Widespread sequence variation in the Epstein–Barr virus latent membrane protein 2A gene among northern Chinese isolates

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INTRODUCTION

Epstein–Barr virus (EBV) is a herpesvirus infecting >90% of the global adult human population. Besides infectious mononucleosis, a usually self-limiting lymphoproliferation, EBV is also associated aetologically with malignancies of lymphoid and epithelial origins, including Burkitt’s lymphoma, nasopharyngeal carcinoma (NPC), T/NK-cell lymphomas, Hodgkin’s disease (HD) and gastric carcinoma (GC) (Chang et al., 1990; Epstein et al., 1964; Leoncini et al., 1993; Shibata & Weiss, 1992; Suzushima et al., 1995; Weiss et al., 1987). The fact that the incidence distribution of EBV-associated malignancies differs in geographical regions, whilst EBV strains from different regions or populations differ in their gene sequences and/or biological activities, hampers study of the association between EBV variants and the development of tumours. Although EBV is ubiquitous in the human population, it is possible that one contributing factor to the endemic incidence of NPC in southern China could be the presence of a particular virus variant predominating in the endemic region (Lung et al., 1990).

EBV gene expression switches to a nearly quiescent state to enable EBV to persist as a lifelong infection in the host, allowing latently infected cells to avoid immune surveillance (Babcock et al., 2000; Chen et al., 1995; Chen et al., 1999; Hochberg et al., 2004; Tierney et al., 1994). Latent membrane protein 2A (LMP2A), expressed in most EBV-associated malignancies, has been demonstrated to be...
EBV LMP2A variations in northern Chinese isolates

Sequence variations in LMP2A have been explored in a few reports. The functional ITAMs in the amino terminus and the CLG426 epitope were conserved in American EBV-associated reactive and malignant diseases (Berger et al., 1999). According to Tanaka et al. (1999), LMP2A showed several amino acid substitutions in six Japanese EBVaGC cases, one of which was identified at codon 348 (serine to threonine) within a CTL epitope. Two reports on southern Chinese NPC gave inconsistent results, one suggesting conserved CTL epitopes whereas the other showed several mutations among NPC patients and local donors (Lee et al., 1997; Zhang et al., 2006). Few data are available on Chinese EBVaGC, despite China being one of the countries with a high incidence of GC (Chen et al., 2008). We therefore analysed sequence variations in LMP2A in northern Chinese EBVaGC and NPC patients and healthy donors, where NPC is not endemic, to explore the association between sequence variations and EBV-associated diseases, as well as the possibility of a CTL-based therapy.

RESULTS

Sequence variation in LMP2A exon 1

The amplification of LMP2A exon 1, encoding the amino terminus of the protein, was successful in 40 EBVaGC samples, 36 NPC samples and 40 throat-washing (TW) samples from healthy donors.

All nucleotide variations were identified by comparison with the B95-8 prototype sequence (GenBank accession no. V01555) (Fig. 1). Fourteen loci were identified with at least one nucleotide variation in a single isolate, 12 of which resulted in amino acid changes. Five loci causing amino acid mutations (codons 23, 63, 64, 79 and 82) were detected in >40% of the isolates. The nucleotide change at nt 166627, which results in a Y→D substitution at aa 23, was detected in all EBV isolates.

The other four loci with consistent mutations, namely aa 63 P→L, aa 64 Y→D, aa 79 T→N and aa 82 Q→P, defined two types of sequence variation in exon 1 (Fig. 1). Pattern A, in which the amino acids were altered at all four loci, was detected in all EBV isolates.

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In addition to its effect on B-cell signal transduction, LMP2A can also act on epithelial cells. LMP2A is reported to transform epithelial cells, to greatly affect cell growth and differentiation pathways in a human keratinocyte cell line (HaCat) and to inhibit transforming growth factor-β1-induced apoptosis in a GC cell line, through activation of the phosphatidylinositol-3-kinase (PI3K)–Akt pathway (Fukuda & Longnecker, 2004; Scholle et al., 2000).

Downstream of the PI3K–Akt pathway, LMP2A is reported to stabilize β-catenin and to activate the Wnt pathway (Morrison et al., 1993, 1994, 1995).

Analysis of EBV-infected lymphoblastoid cell lines with deletion or point mutations within the LMP2A amino-terminal domain revealed loss of the LMP2A-mediated block in BCR signal transduction (Fruehling et al., 1998). Based on these observations, it is conceivable that the gene variations in LMP2A may be related to the development of malignancy.

Besides its important roles in the maintenance of virus latency and cell transformation, LMP2A could also act as the target for a CTL-based therapy for EBV-associated malignancies. The presence of EBV in malignant cells offers a potential target for a CTL-based therapy (Lee et al., 1997). Among the latent proteins, LMP1 is not expressed in EBV-associated GC (EBVaGC) (Imai et al., 1994). EBNA1 (EBV nuclear antigen 1) has been proposed as a strong immunogen for CD4+ T cells in recent approaches, and its ability to maintain and enhance the protective immunity raised by CD8+ T cells immunogen has been explored (Heller et al., 2006).

LMP2A could serve as a primary target for therapeutic responses in EBVaGC and NPC, as several CD8+ CTL epitopes have been identified in the membrane-associated domain of the protein, all restricted through common HLA alleles such as HLA-A2.1, A11, A23, A24 and B60 (Khanna et al., 1999; Lee et al., 1997; Rickinson & Moss, 1997). A broadly applicable CTL-based therapy requires the existence of highly conserved epitopes among EBV isolates.

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A unique three-codon deletion was detected in one GC isolate. In addition, two consistent nucleotide changes were detected in exon 1: nt 166810 c→t (aa 84 L→A) was altered only in isolates with pattern A, whereas nt 166896 c→t (aa 112 Y→A) was altered in most isolates, with no consistent association with the two patterns.

### Table 1. Distribution of subtypes of LMP2A exon 1 in EBVaGC, NPC and TW isolates

<table>
<thead>
<tr>
<th>Pattern</th>
<th>No. isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EBVaGC (n=40) NPC (n=36) TW (n=40)</td>
</tr>
<tr>
<td>A1</td>
<td>15 (37.5) 9 (25.0) 12 (30.0)</td>
</tr>
<tr>
<td>A2</td>
<td>3 (7.5) 3 (8.3) 6 (15.0)</td>
</tr>
<tr>
<td>B1</td>
<td>21 (52.5) 18 (50.0) 19 (47.5)</td>
</tr>
<tr>
<td>B2</td>
<td>1 (2.5) 6 (16.7) 3 (7.5)</td>
</tr>
</tbody>
</table>

### Sequence variations in LMP2A exons 2–8

DNA sequences from EBV coordinates 58–1612 were obtained from 105 specimens, including 30 EBVaGC, 31 NPC and 44 TW isolates. Isolate grouping by identical sequences in exons 2–8 was not consistent with that of exon 1, indicating inconsistent sequence changes between exon 1 and exons 2–8 (Figs 1 and 2).

The results of comparison with the B95-8 prototype sequence are summarized in Fig. 2. Thirty-three loci were identified with one nucleotide variation in at least two isolates, 18 of which resulted in amino acid changes. Three
nucleotide changes at positions 100, 253 and 1438 were detected in all EBV isolates, of which the substitution at position 1438 also results in an amino acid change (aa 444 S→T). Three clusters of loci always showed variations simultaneously, namely clusters SNS, MLV and TRS. Cluster SNS in exons 2 and 3 includes seven nucleotide changes at positions 157, 159, 207, 213, 247, 391 and 410, resulting in four amino acid changes at codons 153 (T→S), 169 (S→N), 171 (Y→S) and 208 (L→I). Cluster MLV in exon 4 includes three amino acid changes at codons 248 (I→M), 254 (V→L) and 255 (L→V). Cluster TRS in exons 6 and 7 includes eight nucleotide changes at positions 1105, 1134, 1138, 1196, 1350, 1374, 1385 and 1392, resulting in three amino acid changes at codons 370 (I→T), 391 (S→R) and 426 (C→S). Seven characteristic sequence patterns were identified according to these clusters (Fig. 2; Table 2).

Pattern 1 was found in 21 EBVaGC, 11 NPC and 26 TW isolates. Besides three changes at nt 100, 253 and 1438 that were shared by all samples, most of these isolates also harboured the substitution at position 1068, resulting in an amino acid change at codon 348 (S→T). Pattern 2, harbouring the changes in cluster TRS, was detected in three NPC and two TW isolates. Pattern 3, harbouring the changes in clusters SNS, MLV and TRS, was detected in two EBVaGC and three NPC isolates. Twenty isolates, including six EBVaGC, seven NPC and seven TW isolates, represented pattern 4, which harbours the changes in all three clusters. Three isolates of pattern 5, including one NPC and two TW isolates, changed in cluster SNS and MLV, but not in cluster TRS, whilst six isolates, including two NPC and four TW isolates, were of pattern 6, which changed in cluster SNS and TRS, but not in cluster MLV. Seven isolates of pattern 7, including one EBVaGC, four NPC and two TW isolates, were affected in cluster SNS only. The TW isolate, T154, was not grouped into any pattern above, as it had five changes that were not detected in other samples (data not shown). The distribution of the seven patterns in the three specimen groups was not statistically significantly different (Table 2; P>0.05; Fisher’s exact test).

Two nucleotide substitutions, 1068 and 1073 in exon 6, were not detected simultaneously in any isolates, and independently, neither were nt 1177 and 1182.

Fig. 2. Sequence variations in exons 2–8 of LMP2A in northern Chinese EBVaGC, NPC and TW isolates. Legend as for Fig. 1. T154 harboured five inconsistent nucleotide changes at positions 663, 1140, 1143, 1153 and 1432 (data not shown).

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Both patterns A and B of exon 1 were heterogeneous in terms of exon 2–8 variants, which suggests the inconsistent nature of sequence changes between exon 1 and exons 2–8 (Table 3).

### Sequence polymorphisms in motifs of the amino terminus

Several functional critical motifs were identified in the amino terminus of LMP2A and their sequence polymorphisms were analysed in the present work (Fig. 3). The two PY motifs, mediating the interaction of LMP2A with several host proteins, thus acting in the maintenance of viral latency (Ikeda et al., 2001; Longnecker et al., 1991), were not affected by mutations in any isolates. The ITAMs, consisting of paired tyrosines and leucines (YXXL) at Y74 and Y85, were also conserved in all isolates, with up to three point mutations identified in sequence between the two binding sites. The two non-silent mutations at nt 166796 (aa 79 T A N) and nt 166805 (aa 82 Q A P) co-existed in most of the affected isolates, including 19 EBVaGC, 12 NPC and 10 TW isolates, namely all isolates of pattern A (Fig. 1). Two TW and three NPC isolates showed a single mutation at T79 or Q82, respectively. The silent mutation at nt 166810 (aa 84) was limited to isolates with both amino acid substitutions N79 and P82 and was found in 19 EBVaGC, 12 NPC and 10 TW isolates. The tyrosine residues Y60 and Y64 were predicted to form two SH2-binding motifs, YEDP and YWGN (Longnecker & Miller, 1996; Songyang et al., 1993). These motifs were both affected by two mutations next to each other (aa 63 P A L, aa 64 Y A D) in all pattern A isolates, except for one TW isolate in pattern B2 harbouring the Y64 mutation only. No prevalence of any mutation in EBVaGC, NPC or healthy individuals was found in any of the analysed functional motifs, as pattern A was distributed across the three sample groups.

### Variation of epitope sequences among EBV isolates

Sixteen CTL epitopes in LMP2A were identified in previous studies, nine of which showed variation in the detected isolates (Khanna et al., 1999; Lee et al., 1997; Meij et al., 2002; Parker et al., 1994; Rickinson & Moss, 1997). An S A T change was observed at position 3 of the VMS (442–451) epitope of all isolates (Table 4). A mutation in the SSC (340–350) epitope, S A T at position 9 or I A V at position 11, was detected in the majority of specimens, including 28 of 30 EBVaGC, 24 of 31 NPC and 31 of 44 TW isolates. Most EBVaGC isolates (63.3 %) showed changes in the EDP (61–75) epitope, whilst the majority of NPC (61.3 %) and TW (56.8 %) isolates were conserved at this site. EBVaGC and TW isolates showed a prevalence of the prototype sequence in the ASC (141–154), IED (200–208), MFL (249–262), TYG (419–427) and CLG (426–434) epitopes, whereas the NPC isolates showed a relatively high mutant prevalence. The RRR (236–244) epitope to B27 HLA restriction was highly conserved except in two of 31 NPC isolates, which harboured changes R A T at position 2 and V A L at position 9 of the epitope.

### DISCUSSION

This is the first report on sequence polymorphism of the LMP2A gene in northern Chinese EBV isolates. No
sequence identical to the B95-8 prototype was found in any case, as four consistent mutations were detected in all isolates. Frequent mutations in the analysed sequences distinguished two and seven types of sequence variation in exon 1 and exons 2–8, respectively, with no consistent association shown between the genotyping of the two gene fragments. The ITAM and PY motif in the amino terminus were strictly conserved, whilst nine of the 16 identified CTL epitopes were affected by at least one point mutation.

The four consistently mutated nucleotides at positions 100, 253, 1438 and 166627 discriminate all northern Chinese isolates.

Table 4. List of amino acid variations in LMP2A epitopes

Sequences listed are epitope sequences of the B95-8 isolate. Only the mutant amino acids of the specimens are shown; – indicates sequence identical to B95-8. The groups with the highest mutant prevalence are shown in bold. ND, Not determined.

<table>
<thead>
<tr>
<th>HLA restriction</th>
<th>LMP2A position</th>
<th>Epitope sequence</th>
<th>No. isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EBVaGC NPC TW</td>
</tr>
<tr>
<td>ND</td>
<td>61–75</td>
<td>EDPYWNGDRHSDYQ</td>
<td>11 (36.7) 19 (61.3) 25 (56.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––LD––––––––––––</td>
<td>19 (63.3) 11 (35.5) 18 (40.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––D––––––––––––</td>
<td>0 (0) 0 (0) 1 (2.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––LD–––––––––––</td>
<td>0 (0) 1 (3.2) 0 (0)</td>
</tr>
<tr>
<td>ND</td>
<td>141–154</td>
<td>ASCFTASVSTV'TA</td>
<td>23 (76.7) 17 (54.8) 30 (68.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––S––––––––––––</td>
<td>7 (23.3) 14 (45.2) 14 (31.8)</td>
</tr>
<tr>
<td>B60</td>
<td>200–208</td>
<td>IEDPFNSL</td>
<td>23 (76.7) 15 (48.4) 29 (65.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––I––––––––––––</td>
<td>7 (23.3) 16 (51.6) 15 (34.1)</td>
</tr>
<tr>
<td>B27</td>
<td>236–244</td>
<td>RRRWRLTV</td>
<td>30 (100) 29 (93.5) 44 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––T––––––––––––</td>
<td>0 (0) 2 (6.5) 0 (0)</td>
</tr>
<tr>
<td>ND</td>
<td>249–262</td>
<td>MFLACVLVLIVDAV</td>
<td>22 (73.3) 20 (64.5) 35 (79.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––LV–––––––––––</td>
<td>8 (26.7) 11 (35.5) 9 (20.5)</td>
</tr>
<tr>
<td>A11</td>
<td>340–350</td>
<td>SSCSCEPLSKI</td>
<td>2 (6.7) 7 (22.6) 13 (29.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––V–––––––––––</td>
<td>8 (26.7) 6 (19.4) 8 (18.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––T––––––––––––</td>
<td>20 (66.6) 18 (58.0) 23 (52.3)</td>
</tr>
<tr>
<td>A24</td>
<td>419–427</td>
<td>TYGPVFMC'L</td>
<td>22 (73.3) 14 (45.2) 32 (72.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––S–––––––––––</td>
<td>8 (26.7) 17 (54.8) 12 (27.3)</td>
</tr>
<tr>
<td>A2</td>
<td>426–434</td>
<td>CLGGLTV'MV</td>
<td>22 (73.3) 14 (45.2) 32 (72.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––S–––––––––––</td>
<td>8 (26.7) 17 (54.8) 12 (27.3)</td>
</tr>
<tr>
<td>A25</td>
<td>442–451</td>
<td>VMSPNLISAW</td>
<td>0 (0) 0 (0) 0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>––T–––––––––––</td>
<td>30 (100) 31 (100) 44 (100)</td>
</tr>
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</table>
isolates from the B95-8 prototype strain. Zeng et al. (2005) completed the genomic sequencing of EBV from one NPC isolate, GD1 (GenBank accession no. AF961628.3), from a southern Chinese patient. The BLAST result between the northern Chinese EBV isolates and GD1 revealed the same changes at the four loci. These four changes were also detected in all of three analysed Japanese EBVaGC isolates (Tanaka et al., 1999). A change at position 166627, which causes the tyrosine at aa 23 of B95-8 to be replaced by aspartic acid (Y→N), was reported in all isolates in previous work (Busson et al., 1995; Tanaka et al., 1999), as indicated by the BLAST search, whilst the other three sites always changed simultaneously in some of the published sequences (GenBank accession nos AJ507799.2, M24212.1 and Y00835.1), with no preferential association of the changes with either disease type or geographical origin.

Comparative sequence analysis in samples from EBVaGC, NPC and healthy TW isolates from northern Chinese individuals showed classifiable variants in LMP2A exons 1 and 2–8. Patterns in both exon 1 and exons 2–8 showed regional association, with no difference in distribution between the EBVaGC, NPC and TW isolates. The NPC isolates were prone to harbouring more variations than the other two groups, which is in accordance with our results from analysis on polymorphism of EBV-encoded small RNA (EBER) (data not shown). It is conceivable that these variations in multiple viral genes are related to the persistence and latency of EBV in NPC, which possibly contribute to the close association between virus and tumour.

Several motifs in the amino terminus of LMP2A, encoded by exon 1, have been identified as essential for the gene functions, which were largely unaffected. ITAMs and PY motifs were reported to be essential for the inhibition of epithelial-cell differentiation and activation of β-catenin signalling (Morrison & Raab-Traub, 2005). The two PY motifs were strictly conserved, as were the paired pivotal tyrosines and leucines (YXXL) comprising the ITAMs, verifying their critical role in the maintenance of gene function. However, two spacing residues, T79 and Q82, were frequently modified, mostly in pattern A isolates, as reported previously (Berger et al., 1999; Busson et al., 1995). The two SH2-binding motifs, YEDP and YWGN, were both affected by two mutations next to each other (aa 63 P→L, aa 64 Y→D) in all pattern A isolates. Although these sequence polymorphisms were suggested to influence signalling cascades through steric alterations or to affect the protein's specificity for cellular ligands and consequently the host-cell range of various EBV strains (Berger et al., 1999; Busson et al., 1995), none of them was found to have a consistent prevalence in EBVaGC, NPC or healthy individuals.

The prerequisite for any CTL-based tumour therapy is comprehensive knowledge of the CTL epitopes in proteins expressed by the tumour cells. In the present study, we detected consistent mutations in nine LMP2A epitopes in northern Chinese EBV isolates. The VMS 442 epitope was affected by an S→T mutation to the third amino acid in all isolates, as reported previously in isolates from southern Chinese NPC and normal tissues (Zhang et al., 2006). All other changed epitopes showed higher mutation rates in tumour isolates than in healthy TW isolates, in accordance with the idea that the virus strains associated with LMP-expressing malignancies can evade immune surveillance by altering amino acids within LMP epitopes (Knecht et al., 1993). The threonine or serine at codon 348 was suggested to have a preferential substitution in different tumours, including Japanese GC, Caucasian HD and southern Chinese NPC, to confer an advantage on EBV for virus persistence in tumour cells (Tanaka et al., 1999). However, our findings showed almost-equal substitution in northern Chinese NPC and GC isolates. In addition to a report on southern Chinese NPC and normal nasopharyngeal tissue, which indicated mutations in four epitopes, CLG 426, TYG 419, SSC 340 and VMS 442 (Zhang et al., 2006), other studies on HD from the UK (Murray et al., 1998) and on NPC, HD and healthy donors from Caucasian, southern Chinese, New Guinean and African populations (Lee et al., 1997) also revealed the high conservatism of Caucasian and African isolates, as well as frequent mutations in Chinese and New Guinean isolates. It was argued that amino acid substitutions at key positions within peptides presented by MHC class I molecules may reduce or abrogate CTL responses completely (de Campos-Lima et al., 1993, 1994), and the epitope mutation could confer an advantage on EBV for immune escape and virus persistence in tumour cells, thus playing a role in tumorigenesis and tumour maintenance (Tanaka et al., 1999). However, Lee et al. (1997) found no experimental evidence that the mutations affected the antigenicity of synthesized LMP2A epitopes in vitro, although the precise antigen processing of these variant sequences from the whole protein in vivo remains to be determined. In any case, the frequent mutations detected in the epitopes justify more consideration of the development of feasible immune therapy of EBV-positive malignancies.

Interestingly, the two variant patterns in exon 1 did not co-segregate with the seven variant patterns in exons 2–8. The inconsistent variation could be attributed to the segregated location of the two fragments. Also known as terminal protein, LMP2A is encoded by sequences located at both ends of the genome, which requires the episomal form of the viral genome to become an integral transcript (Laux et al., 1988; Sample et al., 1989). The remoteness of exon 1 and exons 2–8 in the genome could lead to their asynchronous mutation during viral DNA replication and virus proliferation. Another kind of classification of exon 1 sequence was described based on the four changed nucleotides at positions 166750, 166796, 166805 and 166810 (Busson et al., 1995), resulting in three types of sequence variation. Pattern A4 harboured altered nucleotides at all four loci, whereas pattern P4 retained prototype sequence at all four loci. The other pattern, PPAP,
harboured the only alteration at position 166805. However, up to 10 TW isolates could not be grouped by the classification. In addition, the non-silent change at position 166748, in the vicinity of and always co-segregating with 166750, was not taken into account. To the best of our knowledge, this is the first reported classification of exons 2–8.

This first detailed investigation of sequence variations in the LMP2A gene flanking both ends of the EBV genome reveals widespread sequence polymorphisms in exons 1 and 2–8. The functional impact of these alterations is potentially very interesting. Strict conservation of PY motifs and ITAMs suggests their important role in virus infection, whilst epitope mutations may confer complexity to proposed immunotherapeutic approaches for EBV-associated malignancies.

METHODS

Specimens. One hundred and sixty-one NPC cases and 1678 GC cases were collected as fresh or paraffin-embedded samples from major hospitals covering Shandong Province on the east coast of northern China. The age of patients ranged from 26 to 95 years (mean age, 60 years). A part of the tumour tissues from each surgical specimen was made into tissue sections and screened by ISH for EBER1, whilst part was used for DNA extraction. TW samples were extracted from 268 healthy adults in the same geographical regions by gargling with 15 ml PBS. The work was approved by the Ethics Committee of Qingdao University, and all carcinoma patients, as well as the healthy individuals, gave informed consent for the study.

Detection of EBV infection. Tumour cases were screened for EBV infection by ISH for EBER1 as described previously (Chang et al., 1992). The sequences of primers and probes used in this study are listed in Supplementary Table S1 (available in JGV Online). As positive controls, GC specimens that had been confirmed as EBV-positive were used in every staining batch. TW DNA was screened for EBV infection by PCR–Southern blot assay on the BamHI W fragment, as described by Tokunaga et al. (1993).

Altogether, 124 of 161 (77.0 %) NPC samples and 102 of 1678 (6.1 %) GC samples were found to be positive for EBER1 in tumour cells, and 108 of 268 (40.3 %) TW samples were positive for the EBV BamHI W fragment. Among the EBV-positive samples, 121 randomly selected samples with high-quality DNA, including 36 NPC samples, 41 EBVaGC samples and 44 TW samples from healthy donors, were used in this study.

DNA was extracted from fresh samples by the standard proteinase K/SDS method and purified with phenol/chloroform, whilst a QIAamp DNA FFPE Tissue kit (Qiagen) was used to extract DNA from paraffin-embedded tumour tissues.

Sequence analysis of the LMP2A amino terminus and trans-membrane domain. Semi-nested PCR was exploited for the amplification of LMP2A exon 1, the DNA fragment encoding the amino terminus of the protein, whilst exons 2–8, encoding the transmembrane domain of the protein, were amplified in three overlapping fragments by nested PCR. Exon 9 was not included in the present work, as neither functional domain nor sequence variation was detected in this fragment (Tanaka et al., 1999). The sequences of primers used in this study are listed in Supplementary Table S1.

The PCR products were electrophoresed on 1.5 % agarose gel, excised, purified by using a QIAex II Gel Extraction kit (Qiagen) according to the manufacturer’s instructions, and directly sequenced bidirectionally with primers used in the second-round PCR. Sequences of respective samples were checked for similarity using BLAST. Sequence polymorphisms were identified by comparison with the B95-8 prototype sequence (GenBank accession no. V01555) using the CLUSTAL_V method of DNASTAR software.

Statistical analysis. Fisher’s exact test (two-sided) was performed to determine the difference of the EBV genotypes among the EBVaGC, NPC and healthy TW samples. Significance was set at P<0.05. Statistical analyses were conducted by using the SPSS 15.0 statistical software program (SPSS).

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