Epstein–Barr virus gene polymorphisms in Chinese Hodgkin’s disease cases and healthy donors: identification of three distinct virus variants

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Epstein–Barr virus (EBV) is associated with several malignancies. Specific EBV gene variants, e.g. the BamHI f configuration, a C-terminal region 30 bp deletion in the latent membrane protein-1 (LMP1) gene (del-LMP1) and the loss of an Xhol site in LMP1 (XhoI-loss), are found in Chinese cases of nasopharyngeal carcinoma (NPC), suggesting that EBV sequence variation may be involved in oncogenesis. In order to understand better the epidemiology of these EBV variants, they were studied in virus isolates from EBV-positive Chinese cases of Hodgkin’s disease (HD; n = 71) and donor throat washings from healthy Chinese. Sequencing was performed of 15 representative EBV isolates, including the first analysis of the LMP1 promoter in Asian wild-type EBV isolates. The following observations were made. (i) Three EBV LMP1 variants were identified, designated Chinese groups (CG) 1–3. In both EBV-associated HD and in healthy Chinese, CG1-like viruses showing del-LMP1 and XhoI-loss were predominant. (ii) CG1 viruses were distinct from European and African variants, suggesting that this profile is useful for epidemiological studies. (iii) Specific patterns of mutations were present in the LMP1 promoter in both CG1 and CG2. (iv) The BamHI f variant was not found in Chinese HD, in contrast to Chinese NPC and European HD. This study confirms that EBV isolates in Chinese HD and other tumours differ from those reported in Western cases. However, this reflects the predominant virus strain present in the healthy Chinese population, suggesting that these are geographically restricted polymorphisms rather than tumour-specific strains.

Introduction

Epstein–Barr virus (EBV), the aetiological agent of infectious mononucleosis, is also an important human oncogetic virus, being implicated in the pathogenesis of several neoplasms including Burkitt’s lymphoma, nasopharyngeal carcinoma (NPC), immunodeficiency-associated lymphomas, peripheral T-cell lymphomas (PTLs) and Hodgkin’s disease (HD) (Anagnostopoulos & Hummel, 1996).

Although EBV is ubiquitous in healthy populations throughout the world, the incidence of different EBV-associated tumours shows considerable geographical variation. One possible explanation for this may be the existence of oncogenic EBV strains that cause specific tumours in genetically susceptible populations. However, attempts to identify such viruses have met with little success, several studies suggesting that virus strains are geographically, not disease, restricted (Lin et al., 1995; Khanim et al., 1996; Sandvej et al., 1997; Hayashi et al., 1997). Some viruses appear particularly prevalent in Asia, including the type C strain, which lacks a BamHI site between BamHI W1 and I1, and a proposed substrain (f variant) carrying a deletion in BamHI F (Lung et al., 1990, 1991). The f variant appears to be more frequent in NPC patients in southern China than in healthy Chinese individuals (Lung et al., 1990, 1991), suggesting that this variant may be tumour-associated.

Recently, it has been proposed that sequence variations in the key EBV latent membrane protein-1 (LMP1) gene may be associated with disease. LMP1 is thought to play a central role
sequence variations have been reported in another NPC EBV
an additional 33 bp repeats in the C terminus and (iii) the loss of
tiation (Dawson Lundgren, 1993) and inhibits human epithelial cell differen-
tions in the LMP1 gene compared with
30 bp ( \( \text{del-LMP1} \) ) and 15 bp deletions and the insertion of three
additional 33 bp repeats in the C terminus and (iii) the loss of
an XhoI restriction site (XhoI-loss) resulting from a G → T
mutation at position 169425 (Hu et al., 1991). Similar LMP1
sequence variations have been reported in another NPC EBV
strain, C1510 (Chen et al., 1992). Both CAO and C1510 are
more tumorigenic in SCID and nude mice than B95.8 (Hu et al.,
1993; Chen et al., 1992). Furthermore, transfection studies in
BALB/3T3 cells showed that B95.8 was rendered tumorigenic
following deletion of the 30 bp sequence from the LMP1 gene,
whilst insertion of this sequence into the LMP1 gene of C1510
abolished tumorigenicity (Li et al., 1996). Variants showing
XhoI-loss are found in 97–100% of Chinese NPC and in throat
washings (TWs) from 30–40% of healthy Chinese, a significant
difference (Hu et al., 1991; Chen et al., 1992; Jeng et al., 1994).

In analyses of EBV-associated tumours, del-LMP1 has been
found in about 10–30% of European HD cases, 80% of
Mexican HD cases and 83–100% of human immunodeficiency
virus-related HD cases, in 100% of Malaysian PTLs, 60% of
Danish PTLs and 86% of Chinese PTLs (Knecht et al.,
1993; Sandvej et al., 1994; Santon et al., 1995; Dolcetti et al.,
1997; Dirnhofer et al., 1999; Chang et al., 1995), in 20% of
Burkitt’s lymphoma cases and in 71% of aggressive non-
Hodgkin’s lymphomas (Kingma et al., 1996). In contrast, del-
LMP1 variants have also been found in reactive conditions
(Sandvej et al., 1994; Kingma et al., 1996; Chen et al., 1996 b;
Leung et al., 1997; Dirnhofer et al., 1999) and healthy donors
(Chen et al., 1992; Khanim et al., 1996; Dolcetti et al., 1997;
Sandvej et al., 1997; Chiang et al., 1999). Recently, we
described four main groups of wild-type LMP1 isolates in a
European population, including the del-LMP1 variant (Sandvej
et al., 1997). Khanim et al. (1996) found no increased incidence of
del-LMP1 virus isolates in HD, Burkitt’s lymphomas or
virus-associated carcinomas compared with appropriate normal
populations from the same geographical regions. However,
Chiang et al. (1999) reported a marked predominance of del-
LMP1 compared with wild-type LMP1 (wt-LMP1) variants in
nasal T/natural killer (NK)-cell lymphoma (TNKL) and found
wt-LMP1 to be significantly more frequent in normal tissue
than in tumour tissues. Sung et al. (1998) isolated three LMP1
variants from Chinese NPC. Two of these (China1 and China2)
were specific to Chinese NPC. China1 resembles the CAO
variant, the predominant strain in Chinese NPC. China2 is
characterized by five nucleotide changes in the LMP1 N
terminus in addition to those seen in China1, and by a different
pattern of mutation in the C terminus, with retention of the
30 bp region (168290–168261). The third variant resembles
prototype B95.8. China1 was associated with EBV subtype 1.
The deleted (DV) and retention (RV) variants resembled China1
and China2, respectively. RV was correlated with EBV subtype
2.

The rather contradictory results from these reports indicate
the need for epidemiological studies that map geographical
variation in the frequency of del-LMP1 and other virus
polymorphisms in isolates from EBV-associated tumours.
While EBV isolates have been studied from Chinese NPCs and
PTLs, both of which are relatively common malignancies in
this region, little information is available concerning Chinese
HD. The latter is a rather rare tumour in China, and this may
make it easier to identify an eventual tumour-specific virus
strain compared with the background population. In the
present study, we looked for del-LMP1, XhoI-loss and BamHI f
variants in isolates from 71 EBV-positive HD cases from
mainland Chinese patients and from control TWs from healthy
Chinese. The sequences of the LMP1 promoter region and the
N and C termini were analysed in selected cases.

Methods

Cases. Paraffin blocks were available from 71 EBV-positive Chinese
HD cases with amplifiable DNA. The presence of EBV was demonstrated
by LMP1 immunohistochemistry and EBER-1 in situ hybridization (data
not shown). For comparison, we studied 21 TWs from healthy Chinese,
six Chinese NPCs, two Chinese TNKLs and one Chinese HD case with
EBV-negative Hodgkin and Reed–Stemberg (HRS) cells and EBV-
positive reactive lymphocytes.

DNA extraction. DNA was extracted from paraffin blocks as
described previously (Sandvej et al., 1994). Briefly, tissue sections were
deparaffinized and digested at 55 °C for 48 h with 0.28 mg/ml
proteinase K in 250 ml digestion buffer (50 mM Tris–HCl, 1 mM EDTA
and 0.5% Tween 20, pH 8.5). Proteinase K was inactivated at 95 °C
for 20 min. The supernatant was used as the template for PCR amplification.

In order to obtain TWs, 10 ml Tris buffer was gargled. TW samples
were centrifuged at 2000 r.p.m. for 20 min in graduated conical tubes
(Falcon, Becton Dickinson) and the precipitate was digested with
proteinase K, as described above.

PCR procedure. Amplification of EBNA-2 and EBNA-3C was done
as described previously (Sandvej et al., 1994). The region spanning the
30 bp del-LMP1 was amplified with primers LMP30bp3 (5’ CGTCA-
TCATCTCCACCGAACCAGAAG 3’) and LMP30bp5 (5’ CCGAA-
GAGTTGCAAAACAGAGGTTG 3’). The reaction mixture contained
0.2 µM of each dNTP, 5 µl template, 25 pmol of each primer, 1 U
Tag polymerase (Perkin-Elmer), 2.5 mM MgCl2, 5 µl 10× PCR buffer II
(Perkin-Elmer) and distilled water to a total volume of 50 µl. Hot-start
PCR was performed, comprising 12 cycles of 93 °C for 1 min, 63 °C for
1 min and 72 °C for 2 min and 25 cycles of 93 °C for 1 min, 60 °C for 30 s and 72 °C for 90 s, with a final additional extension at 72 °C for 10 min.

PCR analysis of XhoI restriction site polymorphism (Sandvej et al., 1997), BamHI F region configuration (Khanim et al., 1996) and EBV subtypes 1 and 2 was performed as described previously (Sandvej et al., 1994, 1997). PCR products were electrophoresed in Visigel separation matrix (Stratagene) and visualized with ethidium bromide.

Digestion with XhoI and BamHI. Amplification of DNA fragments covering the XhoI site (113 bp) and BamHI F region (222 bp) was confirmed by electrophoresis in 6% Visigel. Aliquots of 10 µl PCR product were digested with 20 U XhoI (Pharmacia Biotech) or 20 U BamHI (Boehringer Mannheim) at 37 °C for 9 h. The presence of the restriction sites resulted in two bands of 46 and 67 bp (XhoI) or 97 bp and 125 bp (BamHI).

Sequencing of the LMP1 gene. Bidirectional solid-phase sequencing of the promoter region and the N and C termini of the LMP1 gene was performed by using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). The following primer pairs were used: lmp9718 (5′ GGACTCTGGTTTCTAAACACGACCGC 3′)/lmp9529 (5′ CGGATGAGGAGAAGGAGGAGGACAGACACAGGAGG 3′), lmp9602 (5′ CAAAATCCCCCGG-GCCTACATC 3′)/lmp9377 (5′ AGGAGGAGAAGGAGGAGGACAGACACAGGAGGAGG 3′), lmp9442 (5′ CCCCGCGGGCACGGGCTCTCGAG 3′)/lmp9230 (5′ CTCGAGGAGAAGGAGGAGGACAGACACAGGAGGAGG 3′) and lmp9 (5′ AGCGACTCTGGAGAAAGTATGCTGAGGAGGAGGAGGAGG 3′)/lmp30bp3 (5′ CGTCT-ATCCTTCCTACGGAGGAGGAGGAGGAGGAGG 3′). Cycle sequencing was performed according to the manufacturer’s instructions. Briefly, PCR products (2 µl) were mixed with either the forward or reverse PCR primer (5 pmol, 1 µl), 2.5 × buffer (4 µl), terminator ready reaction mix (4 µl) and distilled water (9 µl) to analyse the sense and anti-sense strand. The extension procedure comprised 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. To remove excess dye terminators, the extension reaction was mixed with 2 µl 3 M sodium acetate (pH 4.6) and 50 µl 95% ethanol and spun at 14000 r.p.m. for 20 min. The pellet was washed with 250 µl 70% ethanol. After centrifugation and aspiration, the pellet was dried and mixed with 45 µl loading buffer. The samples were then run on a 6% polyacrylamide gel and analysed using an ABI Prism 373A DNA sequencer with the Collection software.

Controls. Cell lines AG876 and B95.8 were used as positive and negative controls, respectively, for del-LMP1 (Sandvej et al., 1994). Lymphoblastoid cell lines investigated previously for the presence or absence of the XhoI restriction site were used as controls for XhoI-loss (Sandvej et al., 1997). Cases of Chinese NPC analysed previously for configuration of the BamHI F region were used as controls for the BamHI F and J variants. Negative PCR controls were prepared as published previously (Sandvej et al., 1994).

Results

Analysis of Chinese HD and TWs from healthy Chinese is summarized in Table 1. Sequencing results are shown in Tables 2–3 and Fig. 5.

EBV subtype

EBV was detected in 14/21 TWs (67%) from healthy Chinese; 13/14 (93%) could be subtyped, with subtype 1 in 11/13 (85%) and subtype 2 in 2/13 (15%) samples. In 71 informative cases of EBV-positive Chinese HD, EBV subtypes 1 and 2 were demonstrated in 65/71 (92%) and 6/71 (8%) isolates, respectively (Table 1; Fig. 1).

LMP1 30 bp deletion

The region covering del-LMP1 was amplified successfully from 64/71 HD cases (90%). del-LMP1 was found in 53/64 cases (83%); 11/64 cases (17%) contained prototypic (B95.8-like) wt-LMP1 EBV sequences (Table 1; Fig. 2). The presence of del-LMP1 or wt-LMP1 was confirmed by sequencing in eight and four cases, respectively (see Table 3). EBV could be amplified from 14/21 TWs (67%); 12/14 (86%) harboured del-LMP1, one (TW13) carried EBV (subtype 2) with a 15 bp LMP1 deletion, which was confirmed by sequencing (Tables 1 and 3), and the final sample contained wt-LMP1 (Fig. 2).

XhoI RFLP

Results of XhoI RFLP analysis are shown in Table 1 and Fig. 3. Amplification of the XhoI region was successful in 66/71 HD isolates (93%). XhoI-loss was shown in 59/66 cases (89%); seven isolates (11%) carried the wild-type XhoI site (wt-XhoI). Thirteen of 21 TWs (62%) could be amplified for XhoI analysis; 12/13 (92%) showed XhoI-loss.

BamHI F RFLP

Results of BamHI F RFLP analysis are shown in Table 1 and Fig. 4. Amplification of the BamHI F region was successful in 13/71 HD cases (18%) and 11/21 (52%) TWs. All showed a B95.8-like wild-type F configuration. A single HD control case, with EBV-negative HRS cells and EBV-positive reactive lymphocytes, harboured the f variant.

Grouping of EBV LMP1 variants

In order to compare NPC-specific strains reported previously with our isolates, we sequenced the LMP1 promoter region and the N and C termini of the LMP1 gene in selected cases that showed either del-LMP1/XhoI-loss, wt-LMP1/XhoI-loss or wt-LMP1/wt-XhoI. Three distinct sequence variations, designated Chinese groups (CG) 1–3, were identified (see Tables 2–3 and Fig. 5).

Sequence variation in the LMP1 N terminus

Isolates from 14 HD cases, two TWs and five NPC (for comparison) were sequenced at the LMP1 N terminus. CG1 sequences were found in most isolates (eight HD, one TW and three NPC). These showed 13 common nucleotide changes compared with B95.8, all identical to those described in Chinese NPC strains such as C1510 (Chen et al., 1992), China1
Table 1. EBV gene polymorphisms in isolates from Chinese HD and TWs from healthy Chinese donors

Abbreviations: del, deleted; F, F variant; f, f variant; HD, Hodgkin’s disease; LD, lymphocytic depletion; MC, mixed cellularity; NS, nodular sclerosis; TW, throat washing; wt, wild-type. NA, Not applicable.

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* One additional TW isolate harboured a 15 bp deletion.

(Sung et al., 1998) and DV (Cheung et al., 1998) (Table 2). All isolates showed XhoI-loss due to a G → T substitution at codon 17. In 3/14 cases (HD#5, HD#8 and HD#9), there were

Fig. 1. PCR analysis for EBV typing at the EBNA-3C locus. EBV subtypes 1 and 2 are identified by specific 153 and 246 bp fragments. Lane 1 contains molecular mass markers (sizes in bp). Lanes 2, 3, 4, 6 and 7 are representative cases of HD (HD#2, 8, 17, 56 and 57, respectively). Lanes 5 and 8 are representative samples of TWs (TW8 and 13, respectively) from healthy donors. Lanes 9, 10 and 11 are positive controls for EBV subtype 2 and subtype 1 and a negative control, respectively.

Fig. 2. PCR analysis for the LMP1 gene 30 bp deletion in isolates from representative cases of HD and TWs. The specific 153 and 123 bp fragments are respectively consistent with the wt-LMP1 and del-LMP1 gene configurations. Lane 1, molecular mass markers (sizes in bp). Lanes 2 and 3 are non-deleted and deleted controls from B95.8 and AG876 cell lines. Lane 4 is the negative control. Lanes 5, 6, 8 and 9 are representative cases of HD (HD#54, 56, 58, 3 and 2, respectively). Lane 7 and 10 are TW samples (TW13 and 6, respectively). Lanes 9 and 10 are controls for XhoI-loss and wt-XhoI, respectively.

Fig. 3. PCR products from amplification of the LMP1 XhoI restriction site region were digested with XhoI and separated by Visigel electrophoresis. The presence of shorter, 67 bp band indicates an intact XhoI site. The 113 bp band indicates that the XhoI site has been lost. Lane 1, molecular mass markers (sizes in bp). Lanes 2, 3, 5, 6 and 7 are representative cases of HD (HD#54, 56, 58, 3 and 2, respectively). Lane 4 and 8 are TW samples (TW13 and 6, respectively). Lanes 9 and 10 are controls for XhoI-loss and wt-XhoI, respectively.

Fig. 4. PCR products from amplification of the LMP1 BamHI region were digested with BamHI and separated by Visigel electrophoresis. The digestion results in two short bands, of 97 and 125 bp, indicating the presence of an extra BamHI site (f variant). The undigested band of 222 bp indicates the absence of this BamHI site (F variant). Lane 1, molecular mass markers (sizes in bp). Lanes 2, 3 and 6 are representative cases of HD (HD#4, 2 and 72, respectively; HD#72 is a HD control case, with EBV-negative HRS cells and EBV-positive reactive lymphocytes). Lane 5 is a TW sample (TW1). Lanes 4 and 7 are respectively controls for the F and f variants.
Table 2. Sequence variation in the LMP1 N terminus of isolates from selected Chinese tumours and from TWs from healthy Chinese

Only codons exhibiting nucleotide changes compared with B95.8 are shown. Dots indicate residues that are conserved relative to the B95.8 sequence. Previously published prototype sequences were obtained from Fennewald et al. (1984) (B95.8), Hu et al. (1991) (CAO; for corrected sequence see GenBank accession no. AF304432), Chen et al. (1992) (C1510), Sung et al. (1998) (China1 and China2) and Cheung et al. (1998) (DV and RV).

| Isolate | Codon | 3 | 12 | 13 | 16 | 17 | 18 | 25 | 27 | 32 | 33 | 36 | 38 | 41 | 43 | 46 | 51 | 57 | 60 | 63 | 64 | 65 | 67 |
|---------|-------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| B95.8   | CAC   | G | C  | G  | A  | C  | T  | G  | T  | G  | G  | A  | T  | G  | A  | T  | G  | A  | G  | G  | T  | A  | G  | G  |
| CG1     |       |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CG2     |       |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CG3     |       |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HD#56   | –     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HD#58   | –     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| TW13    | –     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Ungrouped |     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HD#49   | –     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HD#51   | –     |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

* HD case with EBV-negative HRS cells.
~ Sequence not available.

Additional changes, at codons 64 (T → G), 36 (A → C) and 27 (T → G), respectively.

CG2 sequences were found in 2/14 HD and 2/5 NPC isolates. One (HD#53) had a sequence similar to that of China2 (Sung et al., 1998). The informative changes involved 15 nucleotides, all of which were seen in China2. One isolate (HD#72) showed 18 nucleotide changes compared with B95.8, of which 17 and 16 changes were respectively identical to those found in RV (Cheung et al., 1998) and China2. One additional change, C → G at codon 12, was absent in this isolate, whilst a change at codon 41 in China2 was absent. HD#72 is a control case showing EBV in reactive lymphocytes but not in HRS cells, thus representing EBV in non-malignant cells. NPC#3 showed the same 16 nucleotide changes reported in the RV strain. Of these, 15 were identical to those found in China2. One change, at codon 60, was different in this case (C → A) compared with China2 (C → G). The change C → T at codon 41 seen in China2 was absent. Finally, NPC#6 harboured 17 nucleotide changes, 16 being identical to those found in China2. One additional change was present at codon...
Table 3. Sequence variation in the LMP1 C terminus in isolates from selected Chinese tumours and from TWs from healthy Chinese

Only codons exhibiting nucleotide changes compared with B95.8 are shown. Dots indicate residues that are conserved relative to the B95.8 sequence. Previously published prototype sequences were obtained from Fennewald et al. (1984) (B95.8), Hu et al. (1991) (CAO), Chen et al. (1992) (C1510), Sung et al. (1998) (China1 and China2) and Cheung et al. (1998) (DV and RV).

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* HD case with EBV-negative HRS cells.
–, Sequence not available.

61 (G → C). In addition, G → A at codon 43 seen in China2 was absent.

Isolates from HD#56, HD#58 and TW13 were identical to B95.8 at the N terminus, apart from two changes (A → C at codons 63 and 65) in HD#56. These isolates were grouped separately as CG3.

In addition, two isolates (HD#49 and HD#51) showed XhoI-loss, but this was unexpectedly not due to the usual G → T at codon 17 seen in CG1 and CG2, but rather to changes in codons 16 and/or 18 (C → T and/or G → C, respectively). Only a limited sequence from codons 8 to 24 was available in these isolates, no more material being available to confirm these findings.

Sequence variation in the LMP1 C terminus

Three distinct sequence variants were also seen at the LMP1 C terminus. CG1 isolates from 8/12 cases of HD, 1/2 TW samples (TW1), 2/4 NPCs and 1/2 TNKL had sequences similar to those of the C1510, China1 and DV strains (Table 3). All harboured del-LMP1 at codons 346–355 and all of the informative cases showed six mutations in the sequence between codons 320 and 366. Surprisingly, the frequent change G → A at codon 335 reported in Chinese NPC isolates (Cheung et al., 1998) was seen in only 1/8 HD isolates. Furthermore, this change was absent from TW, NPC and TNKL isolates. In Chinese NPC, the G → A change at codon
EBV gene polymorphisms in Chinese HD

![Fig. 5. Sequence variation in the LMP1 promoter region in isolates from selected Chinese tumours and from the TWs from healthy Chinese compared with prototype EBV sequences. Only nucleotide changes compared with prototype B95.8 EBV are shown; dots indicate conserved residues relative to the B95.8 sequence. HDg72 is an HD control case with EBV-negative HRS cells and EBV-positive reactive lymphocytes. Previously published sequences were taken from Fennewald et al. (1984) (B95.8), Sandvej et al. (1997) (Group D) and Hu et al. (1991) (CAO).](image-url)

335 results in an amino acid alteration of Gly → Asp, designated deletion variant Asp335 (DV-Asp335). This mutation is reported in 94% of DV in southern Chinese NPC (Cheung et al., 1996, 1998). In contrast, our Chinese HD isolates harboured predominantly Gly335 (7/8; 88%).

In CG2, 2/12 HD and 1/4 NPC isolates shared nine nucleotide changes compared with B95.8, but retained the 30 bp region from codons 346 to 355 (Table 3). The NPC isolate showed an additional change, T → A at codon 360. These changes resemble those seen in RV rather than China2. However, the change C → A at codon 362 in HD and NPC isolates was not seen in China2 or RV.

In contrast to the N terminus sequence, the C terminus sequence of CG3 showed clear differences compared with B95.8. Although 2/12 isolates (HD#56 and HD#58) retained the 30 bp region, they harboured nine and eight nucleotide changes, respectively. Of these, only two were shared, T → C at codon 338 and A → T at codon 342. Similarly, although TW13 had an identical sequence at the N terminus compared with B95.8, it showed six nucleotide alterations and a 15 bp deletion at the C terminus. In addition, 1/2 TNKL and 1/4 NPC also showed several variable nucleotide changes in this region.

### Sequence variation in the LMP1 promoter region

Three distinct sequence variations were also found in the LMP1 promoter region. In CG1, six HD and three NPC isolates were sequenced from −174 to +41 (relative to the transcription start) and two HD and one TW isolates were sequenced from −64 to +41 (Fig. 5). These isolates showed...
similar sequence variation. There were 26 common nucleotide alterations (the only exceptions being the absence of mutations A → C and G → C at positions +26 and +40 in TW1 and HD#47, respectively). In addition, HD#4 had an additional mutation, T → G at position −51, and TW1 had one additional mutation, T → G at −50. CG1 had 26 nucleotide changes in this region, of which 24 were shared with CAO. In comparison, wild-type European EBV group D isolates (Sandvej et al., 1997) showed 23 nucleotide alterations in the promoter compared with B95.8, of which 21 changes were shared with CG1. CAO, group D and CG1 showed identical promoter compared with B95.8, of which 21 changes were shared with CG1. CAO, group D and CG1 showed identical promoter alterations (the only exceptions being the absence of mutations similar sequence variation. There were 26 common nucleotide changes, of which 24 were shared with CAO and European group D. There were four nucleotide changes in the CREB site within the sequence −174 to +41. Of these, 19 and 20, respectively, were shared with CAO and European group D. There were also four nucleotide changes in the CREB site (Fig. 5).

Sequence data were available from +41 to −60 for the single CG2 HD isolate (HD#72; HRS cell EBV-negative). In this region, 18 nucleotide changes were identified, of which 13 and 12, respectively, were shared with CAO and European group D. There were four nucleotide changes in the CREB site (Fig. 5). Isolates from the two CG2 NPCs (NPC#3 and NPC#6) showed 27 and 30 nucleotide changes, respectively, within the sequence −174 to +41. Of these, 19 and 20, respectively, were shared with CAO and European group D. Two CG3 isolates (HD#55 and TW13) had promoter sequences identical to that of B95.8.

Summary of grouping

CG1 isolates show consistent changes, characterized by 26, 13 and six mutations in the promoter region, the N terminus and the C terminus, respectively. A mutation (G → T) at codon 17 in the N terminus causes XhoI-loss. The C terminus contains the 30 bp deletion at codons 346–355. CG2 isolates are characterized by 27, 16 and nine mutations in the promoter region, the N terminus and the C terminus, respectively. XhoI-loss, caused by a G → T mutation at codon 17 in the N terminus, is also seen, but the 30 bp deletion in the C terminus is absent. CG3 is identical to B95.8 in the promoter region and the N terminus, but not at the C terminus.

Based on our sequencing data, we used analysis of the 30 bp deletion and the XhoI restriction site to predict the grouping of the 71 HD isolates. In 58/71 (82%) cases, data were available concerning the configuration at both sites and these could be allocated as follows: 48 sequences were CG1, three were CG2, five were CG3 and two could not be assigned to any of the three groups. For the remaining 13 isolates, data concerning either the 30 bp deletion or the XhoI restriction site were unavailable and these could not be grouped.

Discussion

Deletion of a 30 bp sequence in the C terminus of LMP1 appears to enhance the transforming activity of this key virus oncogene in vitro (Hu et al., 1993; Chen et al., 1992; Li et al., 1996). The detection of del-LMP1 in naturally occurring virus isolates from several EBV-associated malignancies has led to speculation that this variant might be involved in tumour pathogenesis (Knecht et al., 1993; Sandvej et al., 1994; Santon et al., 1995; Chang et al., 1995; Kingma et al., 1996; Dolcetti et al., 1997; Dinhofer et al., 1999). Our study shows that del-LMP1 is common in Chinese HD isolates, being present in 53/64 cases (83%), a frequency similar to that described in Chinese NPC and PTL, in which 80–100% of tumours carry the variant (Miller et al., 1994; Chang et al., 1995; Chen et al., 1996a; Cheung et al., 1996; Khanim et al., 1996). Thus, del-LMP1 is associated with both common and rare EBV-associated tumours in mainland China. However, we also found del-LMP1 in 12/14 (86%) informative TWs from healthy Chinese. This is in agreement with several studies that have described del-LMP1 in up to 92% of the normal Chinese population (Chang et al., 1995; Chen et al., 1996b; Cheung et al., 1996; Khanim et al., 1996). Although the frequency of del-LMP1 in Chinese EBV-associated tumours is considerably higher than in comparable Western tumours, the balance of evidence now suggests that this reflects the level of this variant in the background population.

Whilst it is intriguing that the del-LMP1 genotype appears to correlate with functional changes in vivo that promote tumorigenesis, it is too simplistic to view this molecule as a marker for an oncogenic virus, and we do not believe that it can account for the striking variations in geographical incidence observed for different EBV-associated tumours.

Sequence analysis has previously identified several LMP1 variants in different EBV-related diseases as well as in different geographical populations (Fennewald et al., 1984; Hatfull et al., 1988; Hu et al., 1991; Chen et al., 1992; Miller et al., 1994; Cheung et al., 1998; Sung et al., 1998). Generally, LMP1 variants reported previously in Chinese NPC can be divided into three groups. One is characterized by the loss of an XhoI site at codon 17 and the 30 bp deletion at codons 346–355, together with several other changes in the N and C termini. This group includes CAO (Hu et al., 1991), C1510 (Chen et al., 1992), China1 (Sung et al., 1998) and DV (Cheung et al., 1998). A second group shows the same changes and XhoI-loss but without the 30 bp deletion. Examples include China2 (Sung et al., 1998) and RV (Cheung et al., 1998). The third group resembles B95.8 (Sung et al., 1998).

We found three main groups of isolates in Chinese HD cases and in healthy Chinese, which showed sequence variations in the LMP1 promoter region and the N and C termini. Although these groups (which we have designated CG1, CG2 and CG3) are similar to China1 and DV, China2 and RV and B95.8-like variants, respectively, they show several
notable differences. (i) In DV (derived from NPC from Hong Kong), 94% of variants showed G → A at codon 335, resulting in an amino acid alteration of Gly → Asp (DV-Asp335) (Cheung et al., 1996, 1998). In contrast, the great majority (7/8; 88%) of our CG1 Chinese HD isolates had a non-mutated, DV-Gly335-like configuration. The distribution of DV-Asp335 and DV-Gly335 may reflect geographical variation, since all of the cases in our present study were collected from northern China. (ii) In China1, China2, DV and RV, XhoI-loss was caused consistently by a G → T change at codon 17. XhoI-loss in our CG1 and CG2 variants was also caused by this mutation, but in two HD isolates, it was caused by C → T at codon 16 or G → C at codon 18. Unfortunately, these isolates could not be sequenced further because of lack of material. (iii) In CG2, a C → A change at codon 362 was seen in two isolates. This was found in neither China2 nor RV. (iv) Sung et al. (1998) reported that 5/28 isolates from Chinese NPC were identical to B95.8 at the LMP1 N terminus, but they did not describe the C terminus sequence in these cases. In CG3, we found similar results with respect to the N terminus, with the exception of two nucleotide changes in one isolate. Surprisingly, however, these two isolates respectively showed eight and nine nucleotide alterations in the C terminus. Thus, a B95.8-like N terminus sequence may not necessarily be representative of the entire LMP1 gene sequence. This finding also suggests that the LMP1 C terminus is a hot-spot region for mutations, as we have proposed previously (Sandvej et al., 1997). (v) The promoter region of LMP1 also showed three patterns of sequence variation.

Our study is the first to report sequence variation in the promoters of Asian wild-type LMP1 variants. Examination of this region revealed a number of nucleotide mutations in CG1 and CG2 but only occasional changes in CG3. The significance of these mutations is unclear. However, mutations G → C in CG1 and A → T in CG2 in the CREB recognition sequence (−45 to −38) could reduce LMP1 promoter activity by between three- and nine-fold (Chen et al., 1995; Li et al., 1996). Notably, all of the mutations seen in CG1 and CG2 were also present in TW isolates from healthy Chinese.

Our group has previously identified four wild-type LMP1 variants in a European population (Sandvej et al., 1997). Chinese HD CG3 is similar to European groups A and B. However, Chinese HD CG1 and CG2, which we found in the majority of both tumour and healthy isolates from China, are distinct from all four European groups. These variants also differ from reported African and Alaskan EBV strains (C15 and Par 1; Miller et al., 1994), suggesting that these genotypes could be useful as molecular markers in epidemiological studies.

We have proposed that del-LMP1 arises by misalignment of direct repeats (Sandvej et al., 1994). The present study provides further support for this hypothesis. CG2 variants, which have retained the 30 bp sequence, show mutations that abolish the two 9 bp repeat regions involved in the misalignment (codon 344–346 and 354–356; Table 3).

XhoI-loss is associated significantly with Chinese NPC compared with healthy controls, and it has been considered to be a specific tumour marker (Hu et al., 1991; Chen et al., 1992; Jeng et al., 1994). Similarly, it has been suggested that del-LMP1 is a specific change in several EBV-related tumours (Knecht et al., 1993; Santon et al., 1995; Chang et al., 1995; Kingma et al., 1996; Dolcetti et al., 1997; Dirnhofer et al., 1999). However, our LMP1 sequence analysis shows that XhoI-loss cannot be used alone to determine whether del-LMP1 is present in a particular isolate. Similarly, although the 30 bp deletion is always associated with XhoI-loss, retention of the 30 bp sequence is not specifically associated with either XhoI-loss or del-XhoI (Sung et al., 1998; Cheung et al., 1998). Therefore, examination for only XhoI configuration or the 30 bp region cannot assess the virus strain definitively in all isolates. However, with this proviso, these remain useful markers for screening cases. Sequence analysis of selected cases confirmed the provisional grouping of CG1 isolates based on the presence of del-LMP1 and XhoI-loss. Thus, some 48/58 Chinese HD isolates (83%) and 12/13 informative TW samples (92%) would appear to be CG1 viruses, making this by far the most common EBV variant in our Chinese population.

Previously, the BamHI f variant has been reported predominately in Chinese populations and only rarely outside Asia. This variant has been detected much more often in Chinese NPC (86%) than in healthy Chinese controls (8%) (Lung et al., 1990, 1992, 1994) and it too has been proposed as a specific marker for Chinese NPC. Recently, however, BamHI f variants were detected in 10/21 (48%) cases of European HD but not in European lymphoblastoid cell line variants (Khanim et al., 1996). In our study, we had some problems in amplifying the BamHI F region in all cases, presumably because of the relatively poor quality of DNA available from the paraffin blocks that we examined. We detected the f variant in only one control HD case, in which EBV was present in reactive lymphocytes but not in HRS cells. All informative EBV-positive HD (n = 13) and TWs (n = 11) showed the wild-type BamHI F configuration. These results, together with the findings of LMP1 gene polymorphisms, suggest that European and Chinese HD cases harbour different EBV variants. The different frequencies of the BamHI f variant detected by us in Chinese HD and that reported previously in Chinese NPC is difficult to explain, but may reflect geographical variation in the study populations. Some support for this comes from our analysis of NPC from northern China, in which we found BamHI f variants in 4/17 (24%) NPC isolates (data not shown), a frequency much lower than that reported for carcinomas from southern Chinese patients.

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References


of Epstein–Barr virus genotypes present in direct specimens and lymphoblastoid cell lines established from nasopharyngeal carcinoma patients and healthy carriers in Hong Kong. *International Journal of Cancer* **52**, 174–177.


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