

Hot-spot variations of Kaposi's sarcoma-associated herpesvirus latent nuclear antigen and application in genotyping by PCR–RFLP

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Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus-8) is aetiologically associated with Kaposi's sarcoma and several other lymphoproliferative disorders. The latent nuclear antigen (LNA) encoded by KSHV ORF73 has important functions in virus latent infection and shows molecular polymorphism. Sequence variations were identified in the internal repeat domain (IRD) of ORF73. DNA sequencing of ORF73 from one KSHV-infected cell line, PK-1, revealed that there were 558 bp (30.2%) deletions and 66 (3.6%) point mutations located mainly in repeat region 2, the glutamine-rich region of ORF73 IRD, compared with ORF73 of BC-1 KSHV. Similar sequence variations of ORF73 were also identified in two other isolates. None of the sequence variations caused any translational frame-shift in these four KSHV isolates examined, suggesting that LNA has a conservative function in virus latent infection. The frequent sequence variations in repeat region 2 of ORF73 IRD were also identified by PCR–RFLP genotyping in 26 KSHV isolates, suggesting that this region is a 'hot-spot' for genetic variations. Each Kaposi's sarcoma lesion sample contained one virus genotype with a unique RFLP pattern, indicating that *in vivo* KSHV infection was established with single predominate genotypes, which was further supported by the presence of invariable genotypes in multifocal lesions from individual KS patients. Four KSHV subtypes were classified based on the RFLP patterns that represent the patterns of DNA sequence variations in the ORF73 IRD. PCR–RFLP genotyping is capable of identifying LNA genetic variations and differentiating individual KSHV isolates, and thus may be useful for KSHV molecular epidemiology studies.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, was first identified in a Kaposi's sarcoma (KS) lesion from a patient with AIDS (Chang *et al.*, 1994). The detection of KSHV DNA sequences and anti-KSHV antibodies in most KS patients strongly supports the aetiological role of KSHV in the development of KS (Boshoff *et al.*, 1995; Chang *et al.*, 1994, 1996; Dupin *et al.*, 1995; Gao *et al.*, 1996*a, b*; Kedes *et al.*, 1996; Lennette *et al.*, 1996; Memar *et al.*, 1995; Miller *et al.*, 1996; Moore & Chang, 1995; Schalling *et al.*, 1995; Simpson *et al.*, 1996; Su *et al.*, 1995; Whitby *et al.*,

1995). KSHV has also been found in several lymphoproliferative disorders such as primary effusion lymphoma (PEL) (Ansari *et al.*, 1996; Cesarman *et al.*, 1995*a*; Gessain *et al.*, 1997; Nador *et al.*, 1996; Otsuki *et al.*, 1996), a subset of multicentric Castleman's disease (Gessain *et al.*, 1996; Soulier *et al.*, 1995) and multiple myeloma (Chauhan *et al.*, 1999; Raje *et al.*, 1999; Rettig *et al.*, 1997; Said *et al.*, 1997), although the latter remains controversial.

Similar to other herpesviruses, KSHV establishes latent infection in the host (Gao *et al.*, 1996*a, b*). KSHV latent nuclear antigen (LNA) encoded by the ORF73 gene is the most immunodominant major latent antigen and the target for several serological assays (Gao *et al.*, 1996*b*; Kedes *et al.*, 1996). In KS lesions, LNA is expressed in > 90% of spindle cells, the hallmark of KS, but not in normal vascular endothelium (Dupin *et al.*, 1999; Rainbow *et al.*, 1997). LNA tethers the KSHV genomic DNA to host chromosomes in KSHV-infected cells

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(Ballestas *et al.*, 1999; Cotter & Robertson, 1999; Szekely *et al.*, 1999), and inhibits p53 to prevent KSHV-infected cells from cell death (Friborg *et al.*, 1999). Thus, similar to Epstein–Barr virus (EBV) latent proteins, particularly EBV nuclear antigen 1 (EBNA 1), KSHV LNA has important roles in virus latent infection. EBNA 1 has genetic variations in the internal repeat domain (IRD) that affect the episomal stability and virus latent infection of EBV (Lee *et al.*, 1999). We have also identified molecular polymorphism in LNA (Gao *et al.*, 1999). The sequence variations of ORF73 IRD were found to correlate with the molecular mass polymorphism of LNA. The IRD of ORF73 is stable in KSHV-infected cell lines after long-term culture and in KSHV-infected subjects (Gao *et al.*, 1999). It is plausible to postulate that LNA genetic variations also affect KSHV episomal stability and latent infection. Identification of genetic variations of LNA and their molecular basis will help to elucidate the biological functions of LNA in KSHV latent infection.

Serological studies have demonstrated that the epidemiology of KSHV mimics that of KS: it is transmissible through male homosexual contact (Dukers *et al.*, 2000; Grulich *et al.*, 1999; Kedes *et al.*, 1996; Martin *et al.*, 1998; Melbye *et al.*, 1998; Simpson *et al.*, 1996; Smith *et al.*, 1999) and iatrogenic organ transplantation (Alkan *et al.*, 1997; Nocera *et al.*, 1998; Parravicini *et al.*, 1997; Regamey *et al.*, 1998, 1999). In Africa, young children have a high prevalence of KSHV infection, indicating that other transmission routes are also present (Mayama *et al.*, 1998; Olsen *et al.*, 1998; Rezza *et al.*, 1998). Despite these serological studies, direct evidence of KSHV transmission has not been demonstrated. Detailed studies to analyse the mode of KSHV transmission and the risk factors are needed but have been impeded by the unavailability of a precise genotyping method that is capable of monitoring the transmission of individual KSHV isolates. Genetic variations have been found among KSHV isolates. Four subtypes (A, B, C and SA) were classified based on the point mutations within ORF26, which encodes a minor capsid protein (Foreman *et al.*, 1998; Zong *et al.*, 1997, 1999). Recently, four subtypes (A–D) were classified based on the highly variable gene ORFK1, which has amino acid substitutions up to 29% (Cook *et al.*, 1999; Kasolo *et al.*, 1998; Meng *et al.*, 1999; Poole *et al.*, 1999; Zong *et al.*, 1999). Two distinct types of ORFK15 alleles at the right-hand end of the KSHV genome could be distinctly defined (Choi *et al.*, 2000; Poole *et al.*, 1999). Nonetheless, the current KSHV genotyping techniques require DNA sequencing, which is time-consuming and not sensitive enough for the identification of individual virus isolates, and thus is not suitable for virus transmission studies. A KSHV genotyping method called KSHV nuclear antigen typing (KVNAtyping) was developed based on the molecular polymorphism of ORF73 (Gao *et al.*, 1999), which is useful for most epidemiological studies. However, it cannot differentiate certain KSHV isolates if they have similar LNA sizes, or provide sufficient information on the molecular basis of LNA genetic

variations and the association of these variations with disease phenotypes.

In this report, we have examined the genetic variations of ORF73 and identified 'hot-spot' variations in the IRD of ORF73. A KSHV genotyping technique, PCR–restriction fragment length polymorphism (RFLP), was also developed and used to identify individual KSHV isolates as well as their genetic variations in the ORF73 gene.

Methods

■ **KSHV DNA source: KS lesions and cell lines.** KS specimens were generously provided by C. Parravicini and M. Corbellino of the Luigi Sacco Hospital in Milan, Italy. Multifocal lesions from African patients with KS were obtained from the Dermatovenereology Clinic, University Teaching Hospital (Lusaka, Zambia). DNA was isolated from KS specimens by phenol–chloroform extraction and ethanol precipitation. KSHV-infected cell lines BC-1, BC-2, BC-3, BCP-1, BCBL-1 and PK-1 were established from PEL patients (Arvanitakis *et al.*, 1996; Cesarman *et al.*, 1995*b*; Gao *et al.*, 1996*b*, 1999; Renne *et al.*, 1996). KSHV-negative cell line P3HR-1 was obtained from ATTC. All cell lines were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum. DNA was isolated from the cell lines with the QIAamp blood kit (Qiagen).

■ **PCR–RFLP.** PCR was performed as previously described with minor modifications (Gao *et al.*, 1999). The IRD of ORF73 was amplified with PCR primers: LNAIF, 5' ATGGGGACAACGAGATTAGC 3'; and LNAIIR, 5' CGACCCGTGCAAGATTATG 3'. Each PCR reaction was carried out in a 25 µl final volume containing 100 ng genomic DNA, 1 unit platinum *Taq* DNA polymerase (GIBCO BRL), 100 µM of each dNTP, 50 pM of each primer, 1.5 mM magnesium chloride, 50 mM potassium chloride, 20 mM Tris–HCl (pH 8.4) and 1 × PCRx enhancer solution (GIBCO BRL). PCR amplification was carried out for 35 cycles at 94 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s and 68 °C for 2 min, and 1 cycle at 68 °C for 5 min. For RFLP, the PCR products were digested with *Ban*II and *Mbo*I restriction enzymes for 2 h at 37 °C before gel analysis. Deionized water and DNA from P3HR-1 were used as negative controls for the PCR amplification. DNA bands were observed under UV illumination after ethidium bromide staining. The gel image was documented with Multi-Analyst software (version 1.1) on a Fluor-S MultiImager gel documentation system (Bio-Rad Laboratories).

■ **DNA sequencing.** The ORF73 IRD as well as its N- and C-terminal fragments from a KSHV-infected PK-1 cell line were amplified as described previously (Gao *et al.*, 1999). The expected band separated in agarose gel electrophoresis was purified by QIAquick gel extraction (Qiagen) and sequenced on an ABI 377 analyser (Applied Bio-systems). The DNA sequences were assembled and analysed with the DNASTAR program.

A segment of KS330 from KSHV-infected cell lines and KS lesions was also PCR-amplified and sequenced as previously reported (Chang *et al.*, 1994). The KSHV subtypes were assigned as previously reported (Poole *et al.*, 1999; Zong *et al.*, 1997).

Results

Identification of 'hot-spot' variations by comparison of ORF73 from KSHV-infected cell line PK-1 with published sequences

We have found LNA molecular mass polymorphism among KSHV isolates that correlates with sequence variations in the

IRD of ORF73 (Gao *et al.*, 1999). To further understand the genetic basis for the variations, we sequenced ORF73 from PK-1 KSHV that had the smallest ORF73 IRD among the six cell lines tested (Gao *et al.*, 1999). Sequence analysis showed that there were seven deletions and 66 point mutations in the IRD of ORF73 from the PK-1 cell line compared with the published BC-1 KSHV DNA sequences (Fig. 1). The total number of nucleotides deleted was 558 (30.2%), ranging from three to 228 bases scattered throughout the IRD of 1845 bp. The 66 point mutations (3.6%) resulted in 13 amino acid changes. The sequence deletions resulted in a change in the PCR-amplified fragment from 1898 bp in BC-1 to 1340 bp in the PK-1 cell line. The endonuclease restriction sites in the IRD were also altered due to the sequence deletions and point mutations. DNA sequencing of the N-terminal and C-terminal segments of ORF73 from PK-1 KSHV revealed two point mutations in the N-terminal and a complete match in the C-terminal segments compared with those of BC-1 KSHV (data not shown).

We further analysed the pattern of repeats and the genetic variations within the ORF73 IRD amino acid sequence. There are three repeat regions in the IRD of ORF73, two acidic regions and one glutamine-rich sequence (Russo *et al.*, 1996). In repeat region 1, there are two types of perfect tandem repeats composed almost exclusively of aspartic and glutamic acid, EEDD (aa 342–385) and EEED (aa 386–397). In repeat region 2, there are six types of perfect tandem repeats, composed of over 50% glutamine, QQQEP (aa 443–472, 479–495, 500–549), QQREP (aa 550–594), QQQDE (aa 595–699), QEQQDE (aa 700–717), QEQQDE (aa 723–752) and QEQQEE (753–764). In repeat region 3, there are three types of perfect tandem repeats containing over 60% glutamic acid, QEQUEEE (aa 765–841), QEVVEEQE (aa 842–848) and VEEQEQEQEEQEE (aa 851–905).

Alignment of PK-1 ORF73 sequences with those from BC-1 and two KS lesions obtained through the GenBank database revealed high sequence variations in the IRD. Compared with ORF73 of BC-1 KSHV, the ORF73 from the two KS lesions (GK18 and KS-F) (Glenn *et al.*, 1999; Zhong *et al.*, 1996) have 105 bp and 222 bp deletions, six and three bp insertions, and 150 and 158 point mutations, respectively (Fig. 1). The deletions are mainly located in two spots of repeat region 2, the left and right side of this region. Most of the point mutations are also scattered in repeat region 2, and the rest are in regions 1 and 3. Our sequencing data of PK-1 ORF73 showed that 516 of 558 bp deletions are located in repeat region 2, 30 bp deletions in repeat region 1 and 12 bp deletions in region 3 (Fig. 1). These sequence variations account for the molecular polymorphism of ORF73. Repeat region 2 appears to be the 'hot-spot' for sequence variations of ORF73.

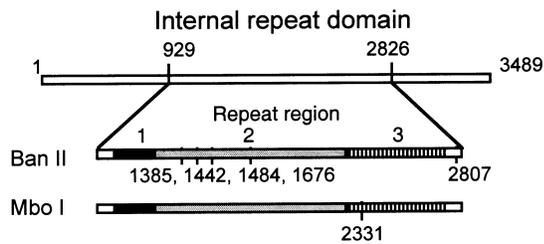
In spite of large sequence deletions in the ORF73 IRD, no frame-shift was observed with each deletion region or the entire ORF73 gene for all the four KSHV isolates examined.

PCR-RFLP genotyping

Sequencing the IRD PCR products is extremely difficult to accomplish due to the high A + G content (75%) and the repetitive sequences. It is therefore not practical to identify the genetic variations in IRD in large numbers of clinical specimens by DNA sequencing. The sequence variations in the ORF73 IRD could be detected by RFLP, which is capable of distinguishing individual KSHV isolates. The size of restriction products will also reflect the location of deletions. We searched the restriction map of the IRD of ORF73 from BC-1 KSHV and found that 64 enzymes had between 1 and 10 cutting sites. Two-thirds of the enzymes would give reasonable fragments visible in agarose gel analysis. After considering the cost and the cutting sites, *BanII* and *MboI* enzymes were selected for RFLP analysis of the ORF73 IRD. *BanII* restriction sites are located in the left half of repeat region 2 and a *MboI* site is at the start of repeat region 3 in the IRD. Thus, one of the restriction fragments represents repeat region 2, the 'hot-spot' for sequence variations. There are five *BanII* restriction sites in the PCR-amplified IRD of ORF73 from BC-1 KSHV (Fig. 2). However, after *BanII* digestion, only three bands (1131, 457 and 192 bp) were expected to be visible, while the other three small fragments (57, 42 and 19 bp) could not be differentiated from PCR primer dimers in regular agarose gel analysis. There is only one *MboI* restriction site in the IRD of BC-1 ORF73 (Fig. 2) and two bands (1403 and 495 bp) are expected after digestion with this single enzyme. There is only one *BanII* restriction site and no *MboI* sites in the IRD of PK-1 ORF73 based on its sequence.

As we expected, unique RFLP patterns were observed for individual KSHV isolates (Fig. 3). After dual digestion with *BanII* and *MboI*, three bands were observed for KSHV from BC-1, BC-3, BCP-1 and BCBL-1 cells, while two bands were observed for KSHV from BC-2 and PK-1 cells. These results indicated that the amplified segments from the first four cell lines had at least two cutting sites, while the segments from BC-2 and PK-1 cell lines had one cutting site. Two bands, 897 and 463 bp, were visible for KSHV from the PK-1 cell line, which was consistent with the presence of one *BanII* site and the absence of a *MboI* site as determined by DNA sequencing. As expected from the sequence analysis of ORF73 of BC-1 KSHV, three bands, 655, 476/457 and 192 bp, were visible for the BC-1 cell line. The *MboI* site was lost in all KSHV isolates except BC-1 as there was no 655 bp band in their corresponding lanes (Fig. 3). A *MboI* site producing bands larger than 655 bp would be impossible as these five isolates have smaller IRD than that of BC-1. The two bands in the BC-2 cell line (Fig. 3) were due to *BanII* digestion, as confirmed by *BanII* enzyme digestion alone (data not shown). The KVNAtypes from BC-2 and BC-3 cell lines as well as those from BCP-1 and BCBL-1 cell lines could not be distinguished (Gao *et al.*, 1999); however, they had different RFLP patterns that can be easily differentiated (Fig. 3). The PCR-RFLP assay is capable of

A. ORF73 gene and restriction sites of *Ban* II and *Mbo* I enzymes



B. Expected Products

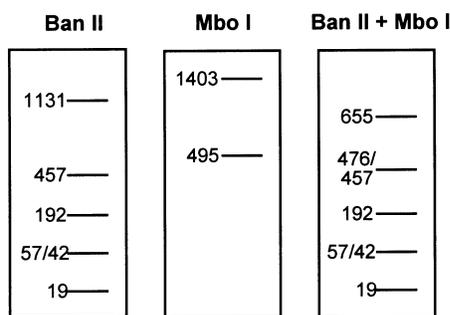


Fig. 2. KSHV ORF73 IRD restriction sites of *Ban*II and *Mbo*I enzymes (A) and expected RFLP profile (B). Locations of repeat regions 1–3 are illustrated above the bar showing restriction sites on the IRD in (A). The sequence numbers are based on the ORF73 sequence from BC-1 KSHV (Russo *et al.*, 1996). The PCR-amplified fragment of the IRD is 1898 bp for BC-1 KSHV.

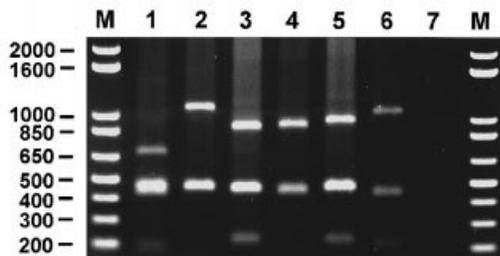


Fig. 3. PCR-RFLP analysis of the KSHV ORF73 IRD from six KSHV-infected cell lines. Lanes 1 to 6 correspond to cell lines BC-1, BC-2, BC-3, PK-1, BCP-1 and BCBL-1. Lane 7 corresponds to the KSHV-negative P3HR-1 cell line used as a negative control. Restriction enzymes *Ban*II and *Mbo*I were used to digest the DNA fragment.

detecting as few as 1–10 copies of KSHV genomes in cellular DNA extract (data not shown).

PCR-RFLP analysis of KSHV from KS lesions

We further analysed KSHV DNA from KS lesions by PCR-RFLP to determine whether the pattern of genetic

Table 1. KSHV subtypes and RFLP patterns

KSHV	RFLP subtypes*	Subtypes based on KS330†
BC-1	1	A
KS-9	1	A
BCP-1	2	A
BCBL-1	2	A3
KS-1	2	A
KS-2	2	A
KS-8	2	C2
BC-3	2	C3
KS-4	2	C2
KS-A1	2	B
KS-A3	2	B
KS-6	3	C2
KS-7	3	A
KS-10	3	A
KS-11	3	A
KS-A2	3	B
BC-2	3	C3
PK-1	3	A
KS-3	3	A
KS-5	4	A

* The RFLP subtypes were assigned based on the number and size of the bands. For details see text.

† KS330 (part of ORF26) of these KSHV isolates was PCR amplified, sequenced and aligned with the BC-1 KSHV sequence. A–C subtype assignment was based on point mutations in certain positions, as described previously (Poole *et al.*, 1999; Zong *et al.*, 1997).

variations of ORF73 IRD that we had identified was consistent in virus isolates from KS tumours, and whether the same genotype or isolate exists between two patients. KSHV from 11 KS lesions from Italy were subjected to PCR-RFLP analysis. Based on the RFLP patterns and DNA sequences of the IRD of BC-1 and PK-1 KSHV, we have grouped the KSHV isolates analysed into four subtypes (Table 1). Subtype 1 had a band pattern similar to that of BC-1 KSHV and had both *Ban*II and *Mbo*I restriction sites. The resulting fragments were 655, 476/457 and 192 bp in size. This subtype could have a complete IRD similar to that of BC-1 KSHV. Subtype 2 had a band pattern that was similar to that of BC-3 KSHV, and had at least two *Ban*II sites and no *Mbo*I sites. The enzyme digestion resulted in three bands with the 655 bp band missing. This subtype could have deletions and point mutations in repeat regions 2 and 3, resulting in the loss of the *Mbo*I site. Subtype 3 had a band pattern similar to that of PK-1 KSHV, and had one *Ban*II and no *Mbo*I sites. There were only two bands visible, while the other two bands (655 and 192 bp) were absent in this pattern. This subtype could have deletions

and 222 bp deletions, six and three bp insertions, and 150 and 158 point mutations, respectively. Most of the deletions and point mutations are located in repeat region 2, the glutamine-rich region of the IRD of ORF73.

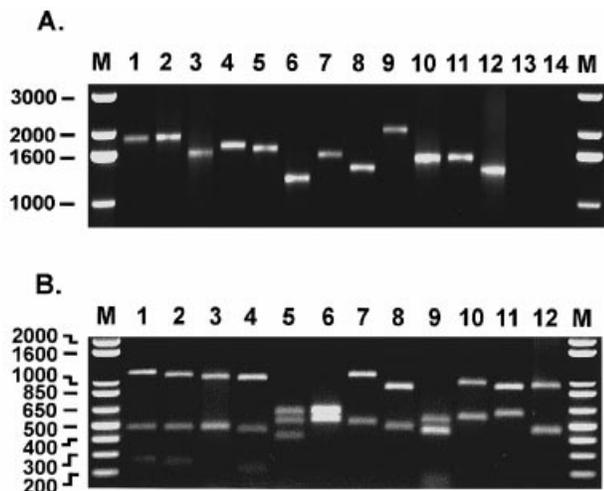


Fig. 4. PCR-RFLP analysis of KSHV from KS lesions. (A) KVNA typing of KS lesions. Lanes 1 to 11 are KS lesion specimens. Lane 12 is the KSHV-infected PK-1 cell line used as a positive control. Lanes 13 and 14 are the KSHV-negative P3HR-1 cell line and water, used as negative controls. (B) RFLP analysis of the PCR-amplified fragments with *Ban*II and *Mbo*I restriction enzymes. Lane designations are the same as in (A), without negative controls.

and point mutations mainly in repeat region 2 and partly in repeat region 3, which reduced the number of *Ban*II sites and eliminated the *Mbo*I site. The largest deletions in the KSHV isolates examined occurred in this group, such as PK-1 KSHV and KS-6 KSHV (Fig. 4). Subtype 4 had a band pattern similar to that of KS-5 KSHV and had one *Ban*II and one *Mbo*I site. There were three bands visible, with the absence of the 192 bp band. This subtype could have deletions in repeat region 2, resulting in the loss of the first three *Ban*II sites. Analysis of the RFLP patterns of KSHV isolates from KS lesions revealed that the majority of the genetic variations in the LNA IRD are in repeat region 2, thus confirming this region to be the 'hot-spot' region for genetic variations.

The results showed that each KS specimen contained a single genotype of KSHV with a unique RFLP pattern (Fig. 4 B). The RFLP patterns had either two or three bands. Similar KVNA types were observed for several KS samples, such as lanes 10 and 11 in Fig. 4(A); however, their RFLP patterns showed that they were different genotypes.

Invariable PCR-RFLP pattern in multifocal KS lesions from the same patient

We have previously demonstrated that there is a single KVNA type in multifocal KS lesions from individual patients (Gao *et al.*, 1999). We further determined whether multifocal lesions from individual patients are due to KSHV infection with multiple KSHV genotypes or isolates. Multifocal KS lesions from three patients were subjected to PCR-RFLP analysis. A unique RFLP pattern was obtained for all the lesions from individual patients, three bands for patients A1 and A3,

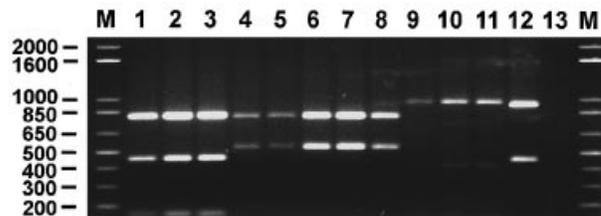


Fig. 5. PCR-RFLP analysis of KSHV from multifocal lesions of individual patients. Lanes 1 to 3 are KS lesion specimens from patient A1, lanes 4 to 8 are KS lesion specimens from patient A2, and lanes 9 to 11 are from patient A3. Lane 12 is the KSHV-infected PK-1 cell line used as a positive control. Lane 13 is the KSHV-negative P3HR-1 cell line used as a negative control.

and two bands for patient A2 (Fig. 5), suggesting the presence of a single KSHV genotype in KS development in individual patients.

KSHV subtypes and the RFLP patterns

To determine the correlation between the RFLP-based subtypes and the KSHV subtypes previously established with KS330 (Poole *et al.*, 1999; Zong *et al.*, 1997), the DNA sequence alignment was analysed and showed that KSHV from the 14 KS samples and six KSHV-infected cell lines fell into the A-C subtypes (Table 1). No apparent correlation was found between the RFLP patterns and the A-C subtypes.

Discussion

We have identified 'hot-spot' variations including sequence deletions and insertions, and point mutations in the IRD of the LNA in KSHV. DNA sequence analysis indicated that the sequence deletions and point mutations were mainly located in repeat region 2 of the IRD of ORF73. These sequence variations account for the LNA molecular polymorphism. The 'hot-spot' variations were further confirmed in KS lesions by PCR-RFLP genotyping techniques, which can differentiate individual KSHV isolates. The distinct RFLP profiles represent the sequence variations in individual KSHV isolates. We found that KSHV from each KS lesion or KSHV-infected cell line had a single KVNA type with a unique RFLP profile, indicating that KSHV infections are established by single predominate isolates. Multifocal lesions from individual KS patients yielded the same RFLP profile, indicating that the development of multifocal KS lesions is also associated with a single KSHV isolate.

The genetic variations of ORF73 focus on certain 'hot-spots' in the IRD. Most of the sequence deletions and point mutations were found in repeat region 2, the glutamine-rich region. The 'hot-spot' variations are located on the left and right side of repeat region 2 among all four KSHV isolates examined. The deletions reduce the number of perfect tandem repeats in region 2. Among the six KSHV-infected cell lines examined, PK-1 KSHV has the largest deletion, 30.2% of the IRD size. It was surprising to find that the sequence deletions in the IRD of ORF73 did not cause a frame-shift in all the

deletion regions and the entire ORF73 gene in all four KSHV isolates analysed, as sequence deletions generally cause coding frame-shift resulting in altered protein structure, function and antigenicity. This result indicates that the in-frame coding of polymorphic LNA maintained in KSHV infection is possibly controlled by functional selection. It would be interesting to determine whether the LNA genetic variations correlate with phenotype expression, such as the types of disease and disease development.

The 'hot-spot' variations were further confirmed in KS lesions by genotyping with PCR-RFLP. Sequence variations cause a change in restriction sites in the IRD. We selected two restriction enzymes that can digest the amplified region into reasonable fragments. From the resulting RFLP pattern, one could identify the location of sequence deletion and/or insertion in the IRD, as we have demonstrated for PK-1 KSHV. Compared with BC-1 KSHV, the RFLP pattern of IRD from PK-1 KSHV had two bands, 897 and 463 bp in size, but did not have the 192 and 655 bp bands. Sequence deletion in repeat region 2 was the reason for the absence of one band at 192 bp in the PK-1 lane. The RFLP profile was consistent with the DNA sequencing result. Based on the ORF73 sequences available, the double enzyme digestion cannot yield fragments over 655 bp in size if a KSHV isolate has a smaller IRD than that of BC-1 KSHV and has restriction sites for both the enzymes. Otherwise, the absence of restriction sites is responsible for a larger band size. Isolates from all the KS lesions examined in Fig. 4 had smaller IRDs, except KS-9, and bands larger than 655 bp in RFLP analysis, except KS-5 and KS-9. Thus, only KSHV from KS-5 and KS-9 in Fig. 4 had both *Ban*II and *Mbo*I restriction sites in the IRD of ORF73. The total size added from the three bands in KSHV from KS-9 was smaller than the undigested fragment size (Fig. 4), suggesting that two fragments were overlapped in one of the bands. A similar situation was also seen in lanes 9, 10 and 11 in Fig. 5. The second band in lane 9 of Fig. 5 is weak, though it was visible in an ethidium bromide-stained gel. Southern blot hybridization can potentially enhance the sensitivity of the assay as demonstrated previously (Gao *et al.*, 1999).

In serological assays, LNA is identified as the major immunodominant latent antigen. It has been found that some samples negative in LNA serological assays were positive in lytic antigen serological assays (Schalling *et al.*, 1995; Simpson *et al.*, 1996; S.-J. Gao, unpublished observation). This could be due to epitope variations in the IRD. However, further studies in this regard are warranted.

LNA plays important roles in maintaining KSHV episomal stability in latent infection (Ballestas *et al.*, 1999; Cotter & Robertson, 1999; Szekely *et al.*, 1999) and inhibits p53 to protect KSHV-infected cells from cell death (Friborg *et al.*, 1999). Expression of LNA in most spindle cells of KS lesions also correlates with the function of LNA in latent infections (Dupin *et al.*, 1999; Rainbow *et al.*, 1997). Similar to EBV latent antigens, genetic variations in the LNA gene might correlate

with KSHV-related pathogenesis. Thus, identification of the genetic variations in LNA is of great importance for understanding KSHV pathogenesis. Because of its important biological function and genetic variations, ORF73 is also a good target for KSHV genotyping.

We classified the KSHV isolates examined in this study into four subtypes based on the RFLP patterns and DNA sequences of the IRD of ORF73 in KSHV isolates with known sequences. Each subtype had a different RFLP pattern, resulting from different sequence variations in the IRD. Through the PCR-RFLP analysis, KSHV isolates could potentially be differentiated in epidemiological studies, for example to track person-to-person transmission. Previous studies have classified KSHV genomes into four subtypes based on DNA sequencing of ORF26 or ORFK1 (Caterino-de-Araujo, 1998; Cook *et al.*, 1999; Diaz-Cano & Wolfe, 1997; Foreman *et al.*, 1998; Luppi *et al.*, 1997; Meng *et al.*, 1999; Zong *et al.*, 1997, 1999). There is no apparent correlation between our genotypes and these four KSHV subtypes, which were all based on the genetic variations of lytic genes. Our PCR-RFLP assay is based on genetic variation of the KSHV latent gene, which might have a different mechanism of genetic variation from that of lytic genes because of different selection pressure encountered during latent and lytic virus infection. Thus it is to be expected that correlation between our genotypes and the KSHV subtype described previously would be low.

Our results show that there is a large repertoire of KSHV genotypes in the KSHV-infected population. Genotyping with PCR-RFLP on LNA is meaningful due to the important roles of LNA in KSHV latent infection. This method is easy to perform and can distinguish individual KSHV isolates without the hassle of sequencing the high A+G and repetitive IRD DNA. We have recently isolated an ostensibly aggressive KSHV variant with large sequence deletions in exclusively lytic cycle genes which is present in some KS lesions (J.-H. Deng, Y.-J. Zhang & S.-J. Gao, unpublished data). This finding suggests that the current lytic cycle gene-based genotyping techniques are useless in certain situations. Genotyping with PCR-RFLP of ORF73 IRD can identify these defective KSHV genomes.

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