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Reflections on the Interpretation of Heterogeneity and Strain Differences Based on Very Limited PCR Sequence Data from Kaposi's Sarcoma Associated Herpesvirus Genomes: A Review

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Background and Purpose

A human gamma class herpesvirus within the rhadinovirus family known as Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8) is now fully accepted as the etiological agent of both Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), as well as of some cases of multicentric Castleman's didease (MCD). The rates of these diseases are greatly increased in certain AIDS subpopulations (epidemic form), less so in organ transplant patients (iatrogenic form), and they also occur in rare non-AIDS associated cases, usually in elderly men (classical form). KSHV DNA can be detected in virtually all KS lesions and PEL cell lines, and in many MCD biopsies, and often also in PBMC or saliva from these same patients, from asymptomatic dual HIV and KSHV seropositive AIDS patients, and in febrile children in Africa undergoing primary KSHV infections. However, except in sub-Saharan Africa (>50%) and parts of the Mediterranean and Middle East (15%), most human populations show only 1 to <3% seropositivity rates and KSHV DNA is rarely detectable in PBMC and saliva samples from healthy individuals.

The purpose of this review is two-fold, 1) to critically evaluate controversial claims linking KSHV to novel disease conditions; and 2) to evaluate claims linking pathological significance to specific sequence variants of KSHV. Our perspective is based on extensive experience with PCR DNA sequencing of both constant and variable gene regions at multiple loci from KSHV (and human cytomegalovirus [HCMV]) in clinical samples obtained from ethnically and geographically diverse human populations. KSHV genomes display significant levels of genetic polymorphism that are both interesting and informative. Variability observed within the most commonly employed ORF26 PCR locus will be evaluated based on the results presented in the accompanying paper, which describes the detection of eight distinct clustered nucleotide polymorphism patterns in an expanded version of this locus (Zong and Hayward, 2007). The significance of KSHV subtypes and DNA sequence polymorphisms will be assessed in the context of global clustering patterns and the overall levels of variability observed

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at multiple loci across the entire KSHV genome. Armed with extensive knowledge of the patterns of DNA sequence variability among KSHV genomes, accurate DNA sequence data becomes a very powerful tool for assessing the validity of the results and interpretations of both past and future epidemiological studies based on PCR analysis. Here we describe typical features of spurious KSHV PCR DNA sequence data, including DNA sequence signatures that are indicative of PEL cell line contamination. We also discuss likely sources of either unexpectedly high levels of sporadic variability or unexpectedly low levels of subtype-specific variability seen within the PCR DNA sequence data reported in some studies.

Use of PCR DNA Data in Clinical and Epidemiological Studies of KSHV Infection

Evidence for KSHV infection is usually based upon detection of antibodies by serological assays or detection of viral DNA by PCR or both. However, there are uncertainties associated with low serological titre, and in some cases (eg., where only tissue or archival paraffin block samples are available) serology is not possible. PCR analysis has usually been based on a single small 233-bp or 330-bp ORF26 DNA fragment of KSHV identified originally by RDA by Chang et al (1994). This has been used successfully to confirm the presence of KSHV DNA in KS, PEL or MCD tissue samples, and in PBMC or saliva from immunosuppressed AIDS or KS patients (Moore and Chang,1995; Cesarman,1995; Whitby,1995; Soulier,1995; Marchioli, 1996; Huang,1995). However, some other clinical or epidemiological studies have claimed detection of KSHV DNA by PCR in other disease conditions, such as multiple myeloma (MM), sarcoid, primary pulmonary hypertension, pemphigus, Bowen's disease, or Kikuchi disease, and in saliva or urogenital specimens from seronegative patients (Luppi,1997; Chauhan, 1999; Hsu,2001; Gao,1999; Said,1997a; Tisdale,1998; Monini,1996; Rettig,1997; Di Alberti, 1997b; Di Alberti,1997a; Cool,2003; Agbalika,1998; Brousset,2000; Boralevi,1998; Said, 1996; Kikuta,1997; Tasaka,1997; Brousset,1997; Huh,1998; Sjak-Shie,1999; Berenson and Vescio,1999; Nishimoto,1997; Memar,1997; Merelli,1997; Said,1997b). For the most part those claims have not been reproducible by other investigators, or by other techniques, and have not gained wide acceptance (Pan,2001; Tarte,1999; Moore and Chang,1998; Moore, 1998; Henke-Gendo,2005; Corbellino,1999).

Many of the disputed reports relied upon nested PCR-amplification using clinical samples that were likely to have extremely low viral DNA copy numbers (if any). Even with rigorous controls, such studies are susceptible to contamination artifacts from either positive control plasmid DNA, other already amplified PCR fragments, or from PEL cell line DNA present within the same laboratory. Unfortunately, some of these studies failed to present the sequence data that they obtained for the ORF26 and other small PCR DNA products involved. This is a serious omission, because useful information can be derived about the likely validity of the claims from the sequence patterns of these PCR products.

As a case in point, two groups (Tisdale, et al.,1998; Brousset, et al.,2000) reported that, although they could not detect any other KSHV DNA, they did detect ORF26 DNA sequences at very low abundance in many samples from patients with MM or monoclonal gammopathy of undetermined significance, and in some control human and primate samples. Tisdale et al (1998) suggested that there may be a second related KSHV-like virus in humans that shares only the ORF26 gene with KSHV. However, presentation of the sequence data could have easily resolved obvious questions about potential contamination with PCR products from other sources. For example, contaminating PCR products or cloned plasmid DNA would likely result in identical sequences matching a known KSHV isolate or subtype in all samples, whereas novel sequence variation would support their hypothesis.

Poor quality DNA sequence data can be equally misleading, such as the original report claiming the presence of KSHV DNA in samples from primary pulmonary hypertension (Cool,et al., 2003). The DNA sequence data presented there for a 774 bp segment of the ORF72 (vCYC) gene contains numerous errors and ambiguities, which led the authors to claim a far higher than expected diversity in this gene. In fact, the human KSHV vCYC gene is highly conserved in KS and PEL samples. We found only seven nucleotide polymorphisms (0.14%) within this region amongst nine different positive samples containing a variety of different VIP (ORF-K1) subtypes.

A different type of problem occurs when attempts are made to correlate nucleotide sequence polymorphisms with biological or pathological features. Ma et al (Ma,2000) argued from short ORF26 and ORF65 DNA sequence analyses that characteristic polymorphisms supposedly found only in KSHV from MM patients represent a distinctive KSHV subset. Similarly, Endo et al (Endo,2003) interpreted that certain nucleotide polymorphisms within a 233 bp region in ORF26 from HIV-infected patients correlate with increased susceptibility to Kaposi's sarcoma. Whilst it is tempting to attribute observed pathological differences with such polymorphisms, this type of data needs to be put into proper perspective. Certainly, in many viruses, including HPV, HIV and adenovirus, genetic sub-grouping can correlate with measurable differences in biological or disease patterns. However, the interpretation of biological correlations from just tiny amounts of PCR DNA sequence data from KSHV genomes (which total 170 kb in size) has to account for the known global patterns and frequencies of heterogeneity in this virus, as well as observations about alternative alleles, partial sub-type linkage patterns and other evidence for recombination and chimerism.

Nature and Extent of KSHV Genome Variability

Two genes at the extreme left-hand-side and right-hand-side of KSHV genomes, which encode the VIP (or ORF-K1) and TMP (or ORF-K15) proteins, respectively, have received considerable attention because of extensive patterns of amino acid hypervariability, some of them associated with divergence that accumulated during the migration of modern humans out of Africa (Hayward,1999; Zong,1999; Poole,1999; Zong,2002; Hayward,2006). In comparison, all other KSHV-encoded proteins are highly conserved, although there is still a significant level of nucleotide polymorphism. We have been sampling up to 7,800-bp of the KSHV genome spread out across eight different PCR-amplified loci from the conserved internal regions (including ORF26). Each locus was sequenced from KSHV-positive DNA samples derived from between 50 and 150 different patients (summarized in Table 1). Using mostly DNA from KS lesions or from PBMC of either KS or PEL patients from all of the major human ethnic groups, we detected a total of 409 of these 7,800 positions with nucleotide polymorphisms (i.e close to 5.5%). Note that the hypervariable VIP protein and the various alternative alleles of the TMP protein are excluded from this analysis. With few exceptions, all KSHV constant region PCR loci had the same eight or nine sets of clustered genotype or sub-type patterns, with either five or six distinct groups among sub-Saharan African samples (B,N,Q,R,F and G), a different one in all aboriginal Pacific rim samples (D/E), and either two or three more in Eurasian samples (A/C, J and K or M). Importantly, the analysis at multiple loci also shows that two genomes that may be identical at several adjacent loci can still differ from each other at other loci because of relatively rare recombination events. The complex patterns of KSHV genome chimerism, including segments containing evolutionarily ancient allelic variants, has been reviewed recently and will not be addressed here (Hayward,2006).

Considering that the unique segment of the KSHV genome is 130,000-bp in size (i.e. 17 times larger than the regions that we have sampled), and allowing for an overall divergence rate of only 35% of that found within the selected regions that we sampled (i.e 2%), yields a minimal estimate of 2,800 total nucleotide polymorphic positions across the entire constant region. Even

if only 25% of these nucleotide polymorphisms lead to amino acid changes, that gives a minimum of 700 amino acid polymorphisms. The greatest variability occurs within sub-Saharanan Africa, but even when the analysis is restricted to just Eurasian, and North African samples, there were 139 total nucleotide polymorphisms within the 7,800-bp block that we sampled (i.e 1.8% variation). A summary of the total number of polymorphisms detected at each of the eight constant region PCR loci sampled is presented in Table 1, which also includes comparisons of the level of variability of pairs of prototype examples from each of the major subtype clusters at each locus. From this data we estimate that an average African B-subtype genome would differ from an average Eurasian A/C- subtype genome at a minimum of 1,373 nucleotide and 343 amino acid positions within the constant region, and at a total of 1440 nucleotide positions and 440 amino acid positions across the entire genome.

There is only a remote chance that the data from Endo et al (2003) or Ma et al (2000) for ORF26 and ORF65 could have serendipitously hit upon key signature polymorphisms that cause KS versus asymptomatic disease, or that differentiated MM viruses versus KS and PEL viruses from the myriad of subtype polymorphisms that occur across the whole genome. Furthermore, to extrapolate from just a single small segment of the genome, one would have to presume that the polymorphisms detected at this locus are fully linked with consistent subtype differences found elsewhere in the genome. Endo et al (2003) stated that they did not need to amplify the more difficult to analyze K1 (VIP) gene, because Zong et al (1999) reported previously that K1 subtypes are tightly linked to the ORF26 subtypes. However, we have pointed out that while there is a general trend for linkage at different loci across the KSHV genome, this is not always so and it cannot be assumed (Poole, et al.,1999; Zong, et al.,2002)).

Levels of Sporadic versus Subtype-Specific ORF26 Variability

We noted previously that the use of either the standard 233-bp or 330-bp fragments of ORF26 (Chang, et al.,1994) is not sufficient to distinguish between all known subtypes. Some African and some Eurasian subtypes have identical patterns in this region, and the A and D subtypes do not differ over this limited stretch of DNA sequence (Poole, et al.,1999). Recently, we examined variability in an expanded 965-bp ORF26E locus by direct PCR DNA sequencing from 136 distinct KSHV-positive samples representing PELs, KS biopsies or PBMC derived from patients with a variety of ethnicies collected from many locations around the world (Zong and Hayward,2007). ,Extension to the larger block permited full resolution of all eight commonly encountered ORF26 subtypes, which have typical characteristic ethnic and geographic associations. Importantly, we found a total of just seven overall sporadic nucleotide changes in addition to the nine diagnostic subtype-specific polymorphic positions out of a total of 38,500 nucleotides sampled over the standard 330-bp ORF26 region (encompassing positions 893 to 1222). One of these was in the BCBL1 PEL cell line and three others were confirmed by multiple PCR reactions. Therefore, only three nucleotides out of the entire 38,500 have some potential to be PCR sequencing artifacts (i.e. 0.007%). In comparison, 25 out of 6,930 nucleotides in data for sarcoid and other samples accumulated by Di Alberti et al (1997a, 1997b) and 27 out of 5,040 nucleotides in the MM data reported by Ma et al (2000) represent changes outside of the six diagnostic polymorphic positions found within their 233-bp region. In other words, the data in both studies contained sporadic variations at a rate of 0.4 to 0.5%, which is 70-fold greater than we observed, and implies artifactual sequencing errors from cloned PCR products (see below).

PEL Cell Line Contamination

In the ORF26 data presented by Endo et al (Endo,2003), more than 85% of their samples have the distinctive A3 subtype pattern characterized by the C to A change at position 1103. Among the 136 samples studied by Zong & Hayward (2007) this particular polymorphism was found

only within the BCBL1 PEL cell line and in one other identical KS sample (BKS14), which raises the strong possibility that most of the samples without KS disease analyzed by Endo et al (Endo,2003) were contaminated with BCBL1 DNA. Similarly, there are a number of studies using the hypervariable regions of VIP (including some from Sub-Saharan Africa), in which the very distinctive amino acid signatures of the commonly encountered PEL cell lines BCBL1, BC1 or BC3/KS1 appear in multiple identical samples. This again raises suspicions about PEL cell DNA contamination of presumably otherwise negative samples.

With regard to MM patients, we analyzed twelve DNA samples provided by another United States laboratory, which had claimed that they were positive by PCR amplification of ORF26 DNA, but we were unable to detect any positive KSHV DNA sequences (including ORF26). Furthermore, ORF26 DNA sequence information provided to us for five MM samples from a second United States group, which claimed to detect KSHV DNA in MM, again proved to have the A-1103 BCBL1 polymorphism in all five cases, but not in any of their positive KS DNA control sequences. In contrast, Ma et al (2000) did not report any examples of the A-1103 polymorphism, despite claiming a distinctive virus subtype for MM. Instead, except for a few sporadic variants, all of their MM samples are essentially identical and match the ORF26 J-subtype pattern DNA pattern found in the BC3 (or KS1) PEL cell line.

Source of Artefactual Sporadic Nucleotide Variations

The results of Ma et al (2000) and Di Alberti et al (1997a,b) illustrate the common problem of unexpectedly high rates of random errors introduced when PCR DNA products are cloned into bacterial plasmids before sequencing. In control experiments, when we carried out direct PCR sequencing of the 500-bp PCR products made with standard Taq DNA polymerase from 20 independent reactions, we obtained exactly the same sequence each time. In contrast, when the products from just one reaction were first sub-cloned into plasmids and then 20 independent clones were sequenced, eight of them had at least one random error and two had either 2 or 4 errors (a 0.14% rate). Many studies in which PCR products were sub-cloned into plasmids prior to sequencing show high levels of random sporadic changes outside the standard ORF26 subtype-specific polymorphic positions described by Zong and Hayward (2007). Therefore, this can lead to incorrect interpretations that differences between clones represent evidence against contamination artifacts or evidence for mixed infections (see below).

Rarity of Multiple KSHV Genotypes in a Single Patient

Another important point involves the question of infection with multiple strains of a single herpesvirus species (Beyari,2003; Cook,2002; Walling,2003). Relevant to this, we have noticed that when two different KSHV subtype DNA samples are admixed together in equal ratios, the direct PCR sequencing procedure shows a simple mixture of the two patterns. However, if the products are first sub-cloned into bacterial plasmids and then sequenced independently, the results yield a complex mixture of randomly re-assorted variants (as well as a minority of the unaltered parent sequences). These novel cloned sequences represent chimeras from apparent copying of parts of one subtype pattern onto the other in addition to the randomly introduced cloned PCR product errors. Much of this presumably results from template switching events during the PCR reactions (Huang and Jeang,1994). Thus, when the PCR products are cloned before sequencing, a simple mixture of two subtypes can be erroneously interpreted as evidence for both multiple infecting strains, and for recombination in vivo between them. While these problems can be reduced by using less error-prone and more processive variants of Taq polymerases, both types of sequencing artifacts can be avoided if the pooled PCR products are sequenced directly.

The presence of a minor second strain of the same virus is not always recognized by pooled direct PCR sequencing, but this procedure does readily detect mixed strains of relatively equal

abundance. This is especially so for hypervariable genes such as KSHV VIP (K1) and the HCMV UL146 beta-chemokine, where certain primers preferentially amplify one subtype over others leading to easily recognized non-matching sequence data. To obtain accurate sequence data from such mixtures, strain-specific primers provide a better solution than plasmid subcloning. However, the issue of resolving mixtures of strains is largely irrelevant for KSHV. Even for the VIP (K1) gene we have never convincingly detected mixed infections (or genotypes) that could be confirmed by duplicate PCR reactions within any single KS tissue or PEL sample, nor among 33 KSHV-positive PBMC samples that we analyzed from KS patients in Uganda (Kajumbula,2006). This result contrasts dramatically with our finding that nearly two-thirds of these same Ugandan PBMC samples contained more than one HCMV strain as detected by mixed sequence data from direct PCR.

Similarly, whereas we occasionally obtained identical VIP (K1) sequences from different patients, we never saw alterations in KSHV DNA sequences occurring in vivo between multiple samples from the same patient. This includes examples of two PEL cell cultures obtained at different times, as well as biopsies from multiple different KS lesions, or when comparing KS and PBMC samples from the same patient. In ten such cases examined, multiple DNA samples were identical, even within the most hypervariable regions of the VIP (K1) gene (Zong et al., 2002; Zong and Hayward,2007). By comparison, direct PCR sequencing of variable HCMV genes in serial bronchoalveolar lavage (BAL) samples collected from immunosuppressed organ transplant patients revealed the simultaneous presence of two different strains in 10% of positive HCMV patients, and in each case the ratio of the two strains changed significantly over time (Alcendor, D.J. et al, unpublished data). Overall, mixed infections are relatively rare even for HCMV, which is evidently horizontally transmitted at much greater frequency than is KSHV, but we have yet to detect an example of multiple infection for KSHV.

Is KSHV Shedding Widespead in Asymptomatic and Seronegative Individuals?

Most investigators agree that KSHV virions and DNA are present at high levels in saliva of KSHV-positive patients, even in the absence of detectable KS lesions, especially in HIV-infected and/or immunosuppressed patients, but not in healthy individuals (Koelle,1997; Blackbourn,1998; Corey,2002). However, several groups (Miller,2006; Duus,2004; Webster-Cyriaque,2006) report KSHV PCR DNA in as high as 24% of saliva or throat-wash samples from control HIV- and KS-negative patients in the USA, suggesting much more asymptomatic infection than is currently appreciated. These reports contradict numerous previous studies (Whitby, et al.,1995; Koelle,1998; LaDuca,1998; Lampinen,2000; Triantos,2004) that failed to detect KSHV DNA in the saliva or PBMC of such controls (<1%), and they also contrast with reliable serological assays that give rates of no more than 1-<3%. Widespread infection would also be hard to reconcile with the observed tight geographically and ethnically restricted patterns of KSHV subtype distribution, which (unlike for HCMV) implies very low rates of horizontal compared to familial transmission for KSHV outside of the AIDS epidemic. We recommend that claims for unusually high frequency detection of KSHV DNA in saliva from normal individuals be treated as only anecdotal as yet in the absence of rigorously controlled and well-documented genotype analyses to rule out contamination.

Conclusions

We have presented evidence and arguments that in our judgment invalidate the interpretations and conclusions of several reports associating KSHV with MM, sarcoid or primary pulmonary hypertension. Most of those data can be explained either by contamination from laboratory sources such as PEL cell DNA, or by DNA sequencing errors introduced using cloned PCR products, or by a combination of both. Similarly, our observations about the extent and patterns

of DNA sequence variability and chimerism in KSHV genomes cast doubt on the claims for pathogenic significance for specific ORF26 variants and other small PCR fragment polymorphisms, which instead correlate closely with viral subtype clustering arising from geographic diasporas and ethnic divergence associated with ancestral human migrations.

On the other hand, we encourage more extensive use of DNA sequence analysis at multiple loci across KSHV genomes in clinical samples, including the hyperviable VIP (K1) and TMP (K15) genes. Despite the trend not to obtain sequence data when using more sensitive and quantitative real-time PCR procedures to measure virus loads, this data is still of great value for potentially revealing real epidemiological connections and for helping to document the various evolutionary lineages of this virus, as well as to provide an important control for artifactual errors and contamination.

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Table 1

Summary and Comparison of Constant Region Polymorphisms Detected Among KSHV Genotypes

						Eurasia			Pacific Rim			Sub-Saharan Africa				
Locus Name	**Map Position**	**Length (bp)**	**Sample No**	**Polymorphisms**		**A/C vs J-A/C**	**A/C vs K-M**	**J-A/C vs K-M**	**A/C vs D**	**A/C vs F**	**A/C vs G**	**A/C vs B**	**A/C vs R**	**A/C vs Q**	**A/C vs N**	**B vs N**
				Total	**Eurasia**											
vIL6	13.3%	1826	69	75	19	11	13	2	5	19	21	22	20	17	20	19
K3	14.4%	920	71	48	12	3	3	2	12	NA	NA	10	9	12	12	5
18/19	23.5%	930	51	28	8	8	8	0	11	NA	NA	13	13	8	13	10
26E	34%	950	73	36	15	5	9	8	4	3	NA	11	8	9	11	12
T0.7	84%	597	149	65	19	3	10	8	11	7	14	11	12	24	18	11
LANA**	90%	980	95	61	15	2	9	9	6	1	6	5	6	3	15	12
75E	96%	850	116	34	16	0	12	12	6	7	9	7	9	12	13	10
UPS75*	96.5%	770	116	66	18	0	16	16	17	7	6	7	NA	8	44	37
Total		7,823	161	409	129	32	80	57	56	44	56	86	77	93	146	116

* Truncated before the hypervariable TMP(K15) C-terminus

** Non-repetitive N-terminus

NA = Not known yet