UNIVERSIDADE ESTADUAL PAULISTA JÚLIO DE MESQUITA FILHO

FACULDADE DE MEDICINA DE BOTUCATU - CAMPUS BOTUCATU

Produção de vetores recombinantes para análise das propriedades biológicas e cancerígenas da proteína K1 do herpesvírus associado ao sarcoma de Kaposi (KSHV/HHV-8)

ANNIE CRISTHINE MORAES SOUSA SQUIAVINATO

Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP, para obtenção do título de Mestre.

Orientador: Prof. Dr. Deilson Elgui de Oliveira

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Dedicatória

Dedicatória

Dedico esta dissertação, com todo meu amor e gratidão:

Aos meus pais Ana Maria de Moraes Sousa e José Carlos de Sousa pelo apoio fraternal, confiança e suporte concedido em todas as etapas deste trabalho e por terem, tão cedo, compreendido minha necessidade de ausência.

"Os nossos pais amam-nos porque somos seus filhos, é um fato inalterável. Nos momentos de sucesso, isso pode parecer irrelevante, mas nas ocasiões de fracasso, oferecem um consolo e uma segurança que não se encontram em qualquer outro lugar." (Bertrand Russell)

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"Se o primeiro e o último pensamento do seu dia for essa pessoa, se a vontade de ficar juntos chegar a apertar o coração, agradeça: Deus te mandou um presente: O Amor." (Carlos Drummond de Andrade)

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"Ensinar não é transferir conhecimento, mas criar as possibilidades para a sua própria produção ou a sua construção." (Paulo Freire)

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"O Senhor é o meu rochedo, o meu lugar forte, e o meu libertador; o meu Deus a minha fortaleza, em quem confio; o meu escudo, a força da minha salvação, e o meu alto refúgio." (Salmos 18:2)

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"Não sei...

Se a vida é curta ou longa demais pra nós, mas sei que nada do que vivemos tem sentido, se não tocamos o coração das pessoas.

Muitas vezes basta ser: colo que acolhe, braço que envolve, palavra que conforta, silêncio que respeita, alegria que contagia, lágrima que corre, olhar que acaricia, desejo que sacia, amor que promove.

E isso não é coisa de outro mundo, é o que dá sentido à vida.

É o que faz com que ela não seja nem curta, nem longa demais, mas que seja intensa, verdadeira, pura...

Enquanto durar!

Feliz aquele que transfere o que sabe e aprende o que ensina."

Cora Coralina

"Produção de vetores recombinantes para análise das propriedades biológicas e cancerígenas da proteína K1 do herpesvírus associado ao sarcoma de Kaposi (KSHV/HHV-8)"

SUMÁRIO

RESUMO

O herpesvírus associado ao sarcoma de Kaposi (KSHV) é um gammaherpesvírus associado ao sarcoma de Kaposi (SK). A proteína K1 do KSHV é conhecida por aumentar a sobrevivência e proliferação celular em células infectadas. A ORF-K1 viral apresenta elevada variabilidade, de modo a discriminar diferentes genótipos do KSHV. Até o momento não se sabe se diferentes genótipos virais apresentam características biológicas próprias. Assim, vetores recombinantes contendo a ORF-K1 de genótipos virais A e B foram gerados. A ORF-K1 foi obtida a partir do DNA de linhagens de linfoma de efusão primária (PEL) constitutivamente infectadas pelo KSHV. O amplicon da ORF-K1 e vetor comercial foram digeridos, ligados e clonados em bactéria *E. coli* DH5α. Clones selecionados foram então submetidos à sequenciamento automatizado de DNA. As sequências geradas dos vetores recombinantes foram alinhadas e comparadas com sequências-protótipo de ORF-K1 do KSHV. Sequência de aminoácidos dos vetores recombinantes foram geradas e analisadas quanto a região codificadora do ITAM de K1. Um clone validado de cada vetor recombinante foi transfectado estavelmente em células HEK293 e a expressão de K1 foi avaliada por Western blot (WB) O sequenciamento de DNA demonstrou que a ORF-K1 dos vetores recombinantes de genótipo A, B e C corresponde a de sequências-protótipo depositadas de linhagens de PEL. A presença de K1 no lisado de células HEK293 transfectadas foi demonstrada por WB. A análise da sequência de aminoácidos de K1 codificada nos vetores recombinantes revelou que o domínio ITAM de K1 do genótipo B apresenta aminoácidos distintos em relação ao ITAM de K1 de genótipos A e C. Portanto, vetores recombinantes de ORF-K1 foram produzidos e validados, e serão úteis para se estabelecer modelo experimental para análises das propriedades biológicas da proteína K1 do KSHV.

Palavras-chave: KSHV, proteína K1, Biologia Molecular, Carcinogênesis

ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with the development of Kaposi's sarcoma. The K1 protein of KSHV has been shown to induce increases the survival and proliferation of infected cells. The viral ORF-K1 shows high variability, so it is possible to distinguish different KSHV genotypes (genotypes A, B, C, D). So far, it is unclear whether different viral genotypes have their own biological characteristics. To this intent, recombinant vectors were generated containing the ORF-K1 genotypes A and B. ORF-K1 was obtained from the DNA of primary effusion lymphoma (PEL) cell lines. The amplicon generated and the commercial vector were digested, bonded and cloned in *E.coli* DH5α. Selected clones underwent automated DNA sequencing. The generated sequences were compared with prototype sequences of ORF-K1. The amino acid sequences from vectors were generated and analyzed in the K1 ITAM-coding region. Validated clones were stably transfected into HEK293 cells and K1 expression was evaluated using Western blot (WB). DNA sequencing showed that the ORF-K1 recombinant vectors corresponds to the prototype sequences deposited from PEL cell lines. WB demonstrated the presence of K1 in the lysate of HEK293 transfected cells. Analysis of the K1 amino acid sequence encoded in the vectors revealed that ITAM domain of K1 (genotype B) has distinct amino acids from the ones in the ITAM domain of K1 (genotypes A and C). Therefore ORF-K1 recombinant vectors were produced and validated, and will be useful to establish an experimental model for analysis of the biological properties of the K1 protein.

Running head: KSHV, K1 protein, viral carcinogenesis, Carcinogênesis, Molecular biology

Manuscrito

de Revisão

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1. MANUSCRITO DE REVISÃO

Biological and Carcinogenic Properties of KSHV K1 Protein

Annie Cristhine M.S. SQUIAVINATO (BSc)[1], Deilson ELGUI DE OLIVEIRA (PhD) [1].

1. Unidade Botucatu, UNESP - Univ. Estadual Paulista, FMB, Departamento de Patologia, SP, Brasil.

ABSTRACT - Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS). The K1 protein of KSHV has been shown to induce increases the survival and proliferation of infected cells. In terms of protein structure, K1 can be divided into a peptide signal sequence, an extracellular domain, a transmembrane domain, and a short cytoplasmic tail. In its cytoplasmic region, there is an Immunoreceptor Tyrosine-based Activation Motif (ITAM), responsible for intracellular signal transduction. Genetic analysis of K1 hypervariable sequences, V1 and V2, classifies KSHV into four major genotypes, which are called A, B, C and D. Survival of KSHV-infected cells is increased by K1 through at least two mechanisms: interaction with receptors involved in apoptosis (Fas and/or BCR) and ITAM-mediated intracellular signaling interference. ITAM signaling can activate PI3K/Akt, which is involved in proliferation and survival of K1-expressing cells. In inflammatory processes, when K1 is expressed in B-lymphocytes, it cooperates in cell activation and production of pro-inflammatory cytokines. In angiogenesis and KSHV-infected cells proliferation, K1 contributes through increased secretion and expression of VEGF and MMP-9, respectively. In carcinogenesis, K1 contributes to the immortalization of endothelial cells in vitro and transformation of rodent fibroblasts. Finally, K1 cooperates in tumorigenesis in vivo, since mice inoculated with K1-expressing cells develop tumors. Therefore, the data presented demonstrate that the K1 protein has an important role in the pathogenesis of KS and lymphoproliferative disorders, so the more detailed study of its biological and carcinogenic properties may contribute to a better understanding of these diseases.

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Running head: KSHV, K1 protein, viral carcinogenesis

Apresentação do manuscrito com base nas instruções da revista Virus Reviews & Research (ISSN 2357-9323). Por conveniência, figuras e legendas foram apresentadas no corpo do manuscrito.

1. KAPOSI SARCOMA

In 1872, Moritz Kaposi was the first to report a rare cutaneous malignant tumor he described as "idiopathic multiple pigmented sarcoma of the skin", currently known as Kaposi's sarcoma (KS) (Kaposi, M., 1872). KS is a mesenchymal cancer mimicking blood and lymphatic vessels and showing a prominent inflammatory reaction. The etiology of KS was elusive until 1994, when the human herpesvirus type 8 (HHV-8) – also known as Kaposi's sarcoma-associated herpesvirus (KSHV) – was first described (Ganem, 2010). The KSHV genome was originally isolated from KS lesions arising in patients with the acquired immune deficiency syndrome (AIDS) (Chang et al., 1994). However, in the following years de viral DNA was consistently detected in virtually all KS cases evaluated, even in individuals without infection by the human immunodeficiency virus (HIV) (Moore and Chang, 1995).

KSHV infection is also associated with the development of two rare lymphoproliferative diseases of humans: the multicentric Castleman's disease (MCD) and the primary effusion lymphoma (PEL) (Cesarman et al., 1995; Soulier et al., 1995). MCD is a rare lymphoproliferative disorder of B-lymphocytes that compromises the architecture of lymph nodes. PEL is characterized by proliferation of neoplastic B cells in body cavities, such as the pleural, pericardial, and peritoneal spaces. Both diseases occurs predominantly in immunocompromised patients, in which they cause systemic symptoms and are typically associated with poor prognosis (Du et al., 2006).

KS is the most common malignancy associated with KSHV infection. Four main clinico-epidemiological forms of the disease are described so far: classic KS, endemic KS, iatrogenic KS and AIDS-associated KS. Classic KS recalls the disease originally described by Kaposi: it commonly presents as isolated lesions in lower limbs of men over 50 years old, notably residents in the Mediterranean and near Eastern European. The involvement of internal organs and lymph nodes are uncommon (Antman and Chang, 2000). The endemic KS is typically observed in children in certain regions of Central and South Africa. The disease has aggressive evolution, with involvement of lymph nodes and viscera; adults, on the other hand, have a more mild clinical course. Iatrogenic KS follows develops under immunosuppressive therapy due to organ transplantation. Disease remission can be observed once the use of immunosuppressive drugs is abrogated (Hengge et al., 2002). Finally, the AIDS-

associated KS (AIDS-KS) occurs in individuals with HIV, especially among young men who have sex with other mem (MSM). Without treatment, the disease has aggressive behavior, with multiple lesions and fast progression. Besides the cutaneous involvement, lesions affecting mucosas and viscera are common, especially in the gastrointestinal tract and lungs (Hengge et al., 2002).

The four previously mentioned forms of KS share basic morphological features: early skin lesions present as patch and plaques that evolves to nodules (the tumoral stage of the disease). The pigmentation of lesions range from brown to purple, reflecting a high degree of angiogenesis, a hallmark of this neoplasia (Ganem, 2010). The cellularity is typically heterogeneous, but varies according to lesion stage. For instance, tumors are mostly formed by spindle neoplastic cells, typical endothelial cells, extravasated erythrocytes, and mononuclear cells. Spindle neoplastic cells have endothelial features and a peculiar immunophenotype, with expression of CD31, CD34, CD36, factor XIII, LYVE1 and PROX1 (Giffin and Damania, 2014).

The KS spindle cells actively contribute to the inflammatory microenvironment within the tumor, as they secrete a variety of chemical modulators of immune responses, including the cytokines gamma interferon (IFNγ), tumor necrosis factors (TNF), interleukins 6 and 1 (IL-6 and IL-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), pro-angiogenic molecules, such as vascular endothelial growth factor (VEGF), the platelet-derived growth factor (PDGF), and chemokines, such as monocyte chemotactic protein-1 (MCP-1) and interleukin 8 (IL-8) (Gessain and Duprez, 2005). These molecules participate in a complex network of cell signaling processes, promoting an increase in the life-span of the KS spindle cells and their dissemination within the host organism (Ganem, 2010).

In vivo, most of the spindle cells in KS lesions show evidence of KSHV infection, notably the presence of the viral genome and expression of viral products. The association of KSHV in all forms of KS highlights the role of the virus in the etiopathogenesis of this cancer (Ganem, 2010). Furthermore, there is conclusive evidence that KSHV infection is carcinogenic to humans (IARC, 2012).

2. KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS (KSHV)

KSHV infection is required, though not sufficient, for the development of KS (Chang et al., 1994; Moore and Chang, 1995). Some degree of immune dysfunction is required; notably, HIV-1 infection is an important cofactor in the development of AIDS-

KS. The HIV-1 tat and nef proteins induce the expression of KSHV products that contribute to the development of lesions (Yen-Moore et al., 2000; Zhou et al., 2013; Zhu et al., 2013).

KSHV belongs to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*. Another human gammaherpesvírus, the Epstein-Barr virus (EBV), is well known for establishing latency in lymphocytes and to cause proliferative diseases, including cancer, both in natural or experimental hosts (Lawrence S. Young, John R. Arrand, and Paul G. Murray, 2007). The herpesviral virion is spherical; the linear double-stranded DNA of KSHV is surrounded by a capsid with 162 capsomers and immerse in an amorphous tegument, enclosed by an envelope containing glycoproteins required for viral attachment to the cell surface (Richard Longnecker and Frank Neipel., 2007). The viral genome has approximately 160kpb and more than 90 ORFs. Noteworthy, the KSHV genome encodes products with high homology with human proteins, such as viral cyclin D (vCyc; ORF-72), anti-apoptotic bcl-2 (vBcl-2; ORF-16), viral interleukin 6 (vIL-6; ORF-K2), and viral interferon regulatory factors (vIRFs, encoded in ORFs K9, K11, K11.1, K10.5, K10.6, and ORF K10) (Liang et al., 2008).

The KHSV life cycle consists of latent and lytic phases. In the latent phase, a limited number of viral genes is expressed and the genome is kept as an episome in the nucleus of the infected cell. The KSHV latency associated nuclear antigen (LANA; ORF-73), binds to cellular histones, allowing the viral episome to be segregated to daughter cells upon cell division (Ballestas, 1999). LANA also represses the transcriptional activity of p53 (Friborg et al., 1999), which confers oncogenic potential due to its detrimental impact in the cell cycle regulation. Other KSHV latent proteins with a role in cell transformation and carcinogenesis include the viral FLICE-inhibitory protein (v-FLIP; ORF-K7), which exerts anti-apoptotic stimuli by its interaction with Fas (Djerbi et al., 1999), and activation of the NF-kB intracellular signaling pathway (Sun, 2003). Moreover, KSHV vCyc shares sequence and functional homology with cellular D-type cyclin, it stimulates the cell cycle, can bind and active the cyclin-dependent kinase (CDK6) (Li et al., 1997); on the other hand, vCyc is resistant to cellular inhibitors of CDKs, so that constitutive activity is has been favored in KSHV-infected cell (Swanton et al., 1997).

In contrast with latency, the lytic infection results in expression of all KSHV genes.

Viral DNA is extensively replicated in lytic phase, and this occurs in the oropharynx of humans infected with KSHV, probably B lymphocytes from tonsils. In addition, the replication and transcription activator (RTA), encoded by ORF 50 of KSHV, is essential and sufficient to drive the entire viral lytic cycle (Sun et al., 1998). However, the transition between the lytic and latent phases of the infection depends on the circumstances and the microenvironment.

The lytic protein vIL-6 is worth mentioning: subcutaneous inoculation of mice with vIL-6-expressing cells induces hematopoiesis and angiogenesis, as well as local accumulation of VEGF (Aoki et al., 1999). The lytic phase of KSHV life cycle is also characterized by the expression of viral proteins important for immune evasion, such as viral CC chemokines homologous (vCCL-1, vCCL-2 and vCCL-3; encoded by ORFs K6, K4 and K4 respectively) (Liang et al., 2008), and vIRFs, as vIRF-1 (ORF-K9) (Gao et al., 1997).

Several surface proteins expressed during the lytic phase of KSHV life cycle are pivotal for KSHV pathogenesis due their role in intracellular signaling. For instance, the viral G protein coupled receptor (vGPCR; ORF-74), which resembles a constitutively active IL-8 receptor (Couty, 2001), can induce cell transformation and tumorigenesis in nude mice (Bais et al., 1998). Likewise, the KSHV K1 protein has important oncogenic properties, which will be outlined and discussed below.

3. KSHV K1 PROTEIN

3.1. GENERAL FEATURES

KSHV K1 is a transmembrane glycoprotein of 289kDa and 46 amino acids encoded by the viral ORF-K1. It is structurally divided into a peptide sequence signal at the N-terminal region, an extracellular domain, a transmembrane domain, and a short cytoplasmic tail at the C-terminal region (H Lee et al., 1998). Its extracellular portion is similar to the lambda immunoglobulin light chain (Igλ), and it has approximately 79 amino acid glycosylated (Heuiran Lee et al., 1998). K1 is structurally and functionally similar to the B-cell receptor (BCR); as consequence, it impacts the activation of B-lymphocytes, by deflagrating several signaling pathways and intracellular calcium mobilization (Lagunoff et al., 1999; Lee et al., 2002; Heuiran Lee et al., 1998). The general structure of KSHV K1 is shown in Figure 1.

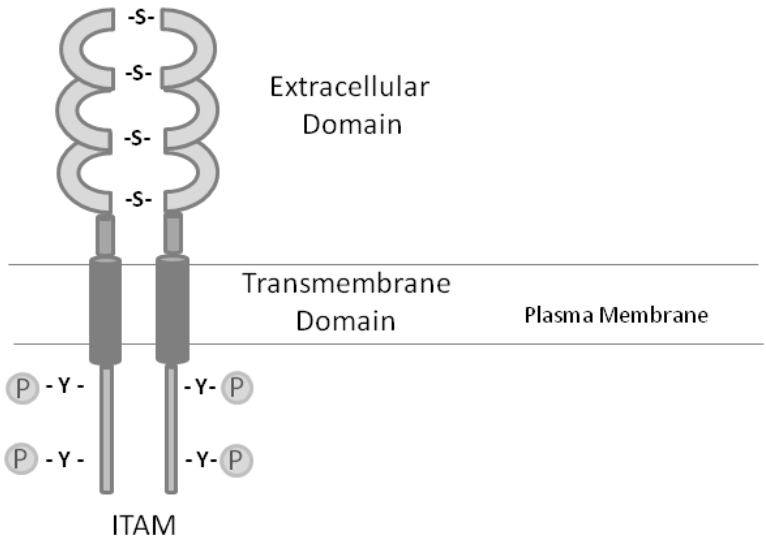

Figure 1 - Schematic structure of K1 protein of KSHV.

The cytoplasmic region of K1 contains a immunoreceptor tyrosine-based activation motif (ITAM), similar to the BCR Igα and Igβ chains (Lagunoff et al., 1999; Heuiran Lee et al., 1998). ITAMs are found in a variety of immune receptors; they are critical for transducing intracellular signals after receptor-ligand binding events, which may culminate in cell differentiation, cell proliferation, or even cell death (Cambier, 1995).

The intact ITAM of K1 is activated upon protein multimerization, leading to phosphorylation its tyrosine residues by src kinases. Two K1 activation motifs enclosing phosphorylated tyrosines (Y1Y2SL-Y3TQP) recruit specific syk kinases (e.g., lyn, syk, p85α, PLCγ2, RasGap, vav SH-PTP1/2, GRB2), relying in their SH2 (Src homology 2) domains to interact with the ITAM. The interaction of syk and PLCγ2 proteins with K1 requires phosphorylation of the ITAM at Y1 and Y3; on the other hand, phosphorylation of Y1 or Y3 is sufficient for recruitment of lyn (Y3 mainly), p85 (particularly Y1), and GRB2 SH2-PTP1/2. The Y2 residue presumably regulates the stability of the phosphorylated ITAM binding sites of SH2 cellular kinases which interact with K1 (Lee et al., 2005).

While tyrosine kinases proteins have a central role at the beginning of signal transduction, the negative regulation of some key receptors in part relies on tyrosine phosphatase proteins. For instance, the negative regulation of BCR activation in B-lymphocytes requires SH2-containing tyrosine phosphatases (SH-PTP). These enzymes catalyze tyrosine dephosphorylation, halting the intracellular signal (Veillette et al., 2002). The interaction between K1 and SH-PTP1/2 suggests that either K1

deregulates their role, or SH-PTP1/2 can modify the signaling that was once initiated by K1, therefore, the signaling becomes either continuous or unstable, respectively (Lee et al., 2005).

The multimerization of cysteine residues in the extracellular domain of K1 possibly makes its ITAM constitutionally active (Lagunoff et al., 1999). This is in contrast with ITAMs within surface molecules of T and B lymphocytes, as they require ligand-receptor interaction for activation (Cambier, 1995). Hence, K1 is prone to trigger uninterrupted intracellular signals, but it may exhibit different properties in cells of the same type, or according to its expression context (e.g., ectopic expression versus KSHV infection). In BJAB cells (KSHV-negative), the expression of CD8/K1 chimera under cell stimulation with anti-CD8 antibody yielded intracellular calcium mobilization, which is associated with activation of the nuclear factor of activated T-cells (NFAT) in cells ectopically expressing K1 (Lagunoff et al., 1999). Moreover, these effects were not significant in BCBL -1 cells (PEL-derived B-cell line constitutionally infected with KSHV) (Lee et al., 2002).

3.2. KSHV K1 VARIABILITY

The ORF-K1 is on the left side of the KSHV genome, within a GC-rich region (Lagunoff and Ganem, 1997). Its genomic position is equivalent to the that encoding the latent membrane protein (LMP1) in EBV (Lagunoff and Ganem, 1997), the saimiri transformation protein (STP) in herpesvirus Saimiri (HSV) (Murthy et al., 1989), and the R1 gene of the rhesus monkey rhadinovirus (Damania et al., 1999). The ORF-K1 and its encoded K1 protein differ among viral genotypes by 87% and about 62%, respectively, in nucleotide and amino acid sequences (Zong et al., 1999). The variations are concentrated in the extracellular portion of K1, within two hypervariable regions called V1 and V2. Nonetheless, 10-12 cysteine residues are well conserved, of which 7 are located predominantly in the activation motifs (Nicholas et al., 1998; Zong et al., 1999). The high variability in the extracellular portion K1 can be partly responsible for protein oligomerization, probably by interfering with disulfide bonds (Zong et al., 1999).

Genetic analysis of the hypervariable sequences of V1 and V2 KSHV K1 classify into four major genotypes, designated A, B , C and D. The V1 and V2 regions of prototype sequences of viral A and C genotypes extends, respectively, between codons 54-92 and 199-227, and between 1-92 and 191-228 for the B genotype. Genotype A differs from B by 39 amino acids (15%), and from C by 85 amino acids (29%) (Zong et

al., 1999). The high variability K1 can be noted in the alignment of amino acid sequences of protein prototypes available at the National Center for Biotechnology Information of the United States (NCBI), as shown in Figure 2.

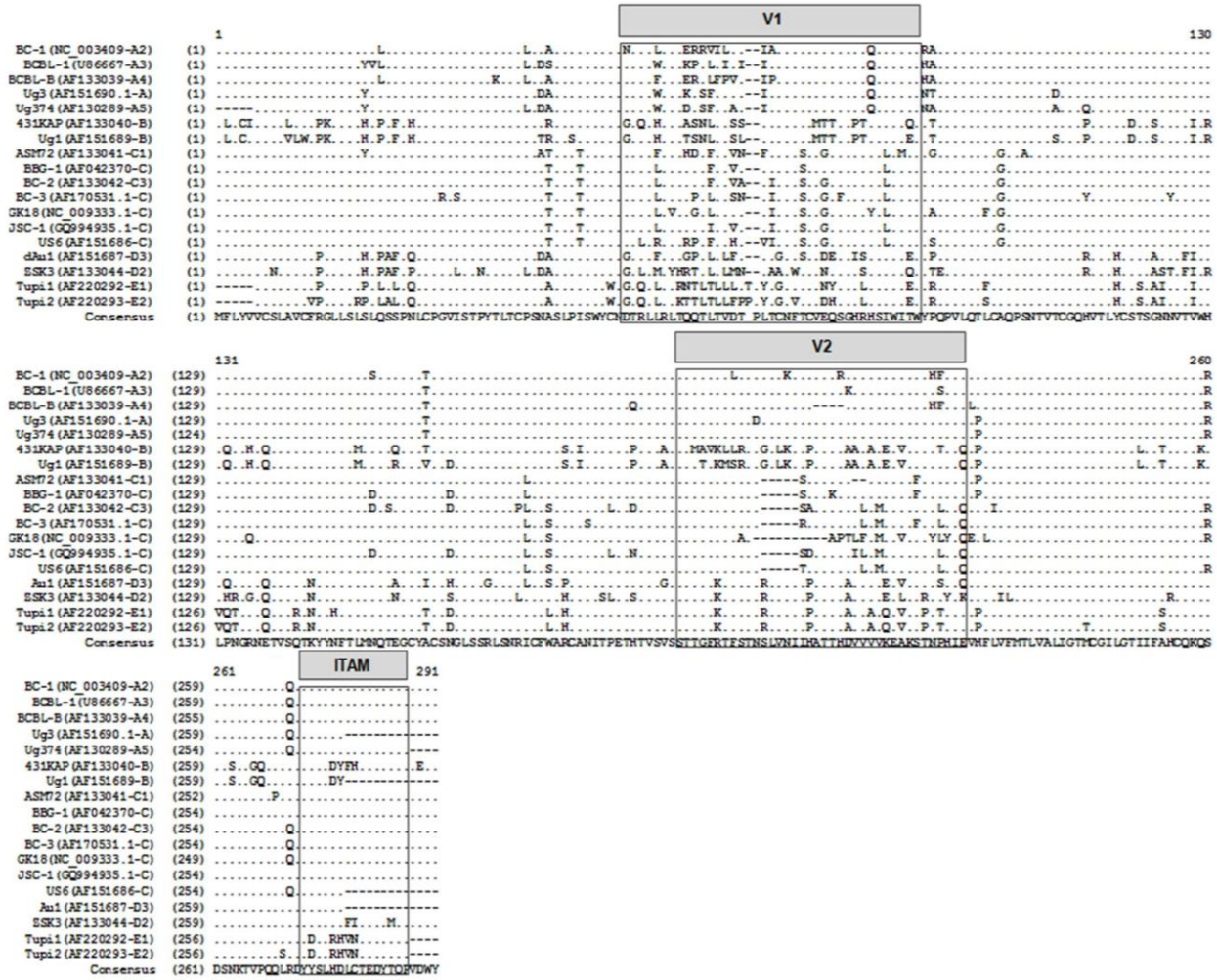

Figure 2. Alignment of amino acid sequences of genotypes A, B, C, D and E from K1 prototype sequences deposited in the NCBI database band. Parenthetically access code (Accession number) of each of the Genbank sequence and the front of the code reported for each genotype cell lines. The variable regions V1/V2 region and the prototype ITAM sequences K1 are indicated.

The distribution of KSHV genotypes in humans depend on geographic region and ethnicity. Overall, isolates of A genotype are found mainly in North America; genotype B in Africa; genotype C in Europe, Asia, and the Mediterranean; and genotype D in Pacific Islands (Zhang et al., 2008; Zong et al., 1999). In addition, there are few more restricted viral genotypes, such as genotype E, identified in South American Amerindians (Biggar et al., 2000), and genotype F, detected in biological samples from individuals in Bantu tribes in Uganda (Kajumbula et al., 2006). In Brazilian natives in the State of Amazonas, KSHV E genotype was found in the totality of samples evaluated (de Souza et al., 2007), while viral genotypes B (2/4), C, (1/4) and E (1/4) were found in natives living in

the State of Pará (Ishak et al., 2007). Except for these regions with endemic KSHV infection, there is no data regarding the distribution of KSHV genotypes in the general population in Brazil. In KS lesions of Brazilian residents, genotypes A and C were reported to be more frequent in HIV-positive (25/50) and HIV negative cases (24/50), respectively (Ramos da Silva et al., 2011). Figure 3 shows the phylogenetic topology based on K1 sequences of different KSHV genotypes.

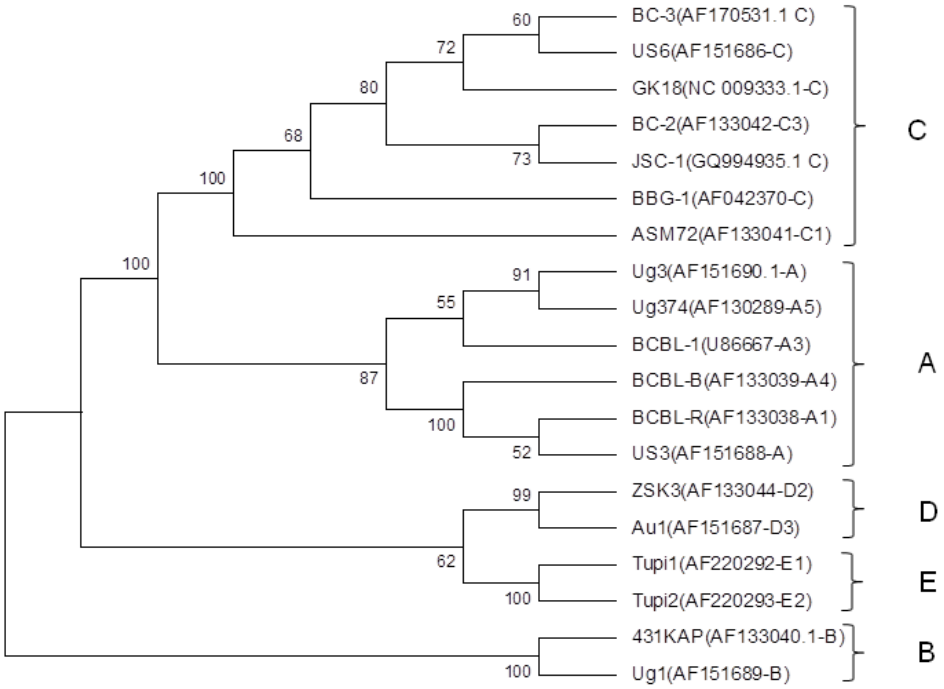

Figure 3 - Phylogenetic analysis based on sequences from prototype KSHV genotypes A, B, C, D and E. Mega 5.2 Software (Tamura et al., 2011) was used, with the maximum likelihood algorithm (Maximum Likelihood) and bootstrap validation of the phylogenetic tree (500 replicates). In parentheses the access code for the sequence (Accession #) in Genbank (NCBI) is provided. Viral genotypes are indicated in the right.

The transmembrane domain of K1 is well conserved among genotypes A, B and C, extending between ORF-K1 positions 229-261 (Zong et al., 1999). The cytoplasmic domain is conserved in genotypes A and C (3% change), but differs 22-30% in the amino acid sequence between viral genotypes A and B (Nicholas et al., 1998; Zong et al., 1999). Although the extracellular portion of K1 is highly variable, the SH2 binding site is usually preserved, ITAM included. Although ITAM is critical for intracellular signal transduction, the extracellular domain is also relevant, since changes in this region may impair calcium mobilization upon stimulation (Lee et al., 2003). On the other hand, the patterns of amino acid substitution in the ITAM of KSHV K1 diverge highly among viral isolates of genotypes A or C compared to genotype B. As consequence of HDLC amino acid sequence at positions 5-8 of the ITAM A/C, compared to DYFH in ITAM within the K1 of B genotypes, proteins from these genotypes might have different properties. In

fact, it was suggested that viral K1 from genotypes A/C versus B evolved distinctly regarding their interference in signaling pathways within infected cells of the host (Hughes and Hughes, 2007).

Analysis of the pattern of nucleotide and amino acid substitutions in different forms of K1 suggests a positive Darwinian selection on V1 (Hughes and Hughes, 2007; Stebbing et al., 2003) and V2 (Hughes and Hughes, 2007) regions of the protein. It was reported that the positive selective pressure in V1 is as high as in immunogenic regions of well-studied viral genes, such as HIV-1 env. The analysis of autologous peptides of K1 (unique to each individual viral isolates), showed epitopes inducing response exclusively from T cytotoxic lymphocytes from the V1 region of K1, with no response to peptides from other individuals. The analyzed epitopes derive only from conserved sequences between isolates of the same viral genotype. The maintenance of antigenicity in isolates of the same KSHV genotype may partially explain their characteristic ethnic and geographical distribution. Consequently, to some extent this selection of epitopes K1 is mediated by antigen presentation in the context of class I MHC. Conversely, to date there is lack of data on putative factors for positive selection in other portions of the K1 protein (Stebbing et al., 2003).

3.3. REGULATION OF K1 IN CELLS INFECTED WITH KSHV

The expression of KSHV K1 has been reported in cells derived from PEL (Lee et al., 2003), MCD (Lee et al., 2003), and KS (Lagunoff and Ganem, 1997; Samaniego et al., 2000). This viral protein is significantly expressed in lytic viral cycle (Lagunoff and Ganem, 1997), and can be induced in PEL cells *in vitro* by treatment with TPA (12-O-Tetradecanoyl Phorbol-13-Acetate) (Samaniego et al., 2000). Contrariwise, low levels of K1 are detected in cells latently infected by KSHV (Chandriani and Ganem, 2010; Wang et al., 2006). The expression of K1 in HEK293 cells peaks at 24h after infection with BCBL-1-derived vírions, declining sharply after 72h, when the majority of infected cells are in the latent phase of the viral cycle (Verma et al., 2006).

The major regulator for the transition between lytic and latent phases is the KSHV Rta protein, which modifies the expression of viral genes by direct binding to Rta-responsive elements (RREs) or indirectly (Liao et al., 2003; Song et al., 2001). Rta activates the K1 promoter B-lymphocytes and epithelial cells (Bowser et al., 2002; Verma et al., 2006); in endothelial cells the effect is less significant, probably due to the particular features of the transcriptional machinery for this cell type (Bowser et al.,

2002). K1 induction by Rta is relies on three binding sites within the ORF-K1 promoter, but only two seem to play a role in protein expression (Bowser et al., 2006).

The K1 expression in B-lymphocytes may increase modestly the Rta-mediated lytic reactivation. Likewise, BCBL-1 cells cotransfected with vectors encoding for KSHV Rta (ORF-50) and a dominant negative version of K1 showed a small reduction of lytic reactivation (75% to 80%) compared to cells expressing Rta only. Furthermore, the suppressive effect of the K1 dominant negative in viral lytic reactivation is abolished when Rta is replaced by TPA treatment (Lagunoff et al., 2001). In contrast, BCBL-1 cells expressing the CD8 surface receptor fused to the cytoplasmic portion of K1 (CD8-K1) efficiently suppresses TPA-mediated viral reactivation, thus suppressing the K8.1 protein and decreasing transcriptional activation mediated by transcription factors such as protein 1 activator factor (AP-1), NF-kB and Oct-1 (Lee et al., 2002). The discrepancy in results between these studies based on the same cell model is possibly due to methodological issues, as TPA treatment exerts various effects on intracellular signaling, activating several transcriptional pathways that culminate in KSHV lytic reactivation. The same does not seem the case for the cytoplasmic region K1, which appear to deflagrate a more restricted network of intracellular pathways.

In KSHV-latently infected cells, the binding of C-terminal region LANA to the terminal repeat promoter of ORF-K1 downregulates K1 expression, which maintains latency by reducing K1-induced signaling (Verma et al., 2006).

Besides regulation of viral products, K1 is also regulated by endogenous proteins of the infected cell. For instance, the heat shock protein 90β (Hsp90β) and ER-associated DNAJ protein 3 (Erdj3) interact with the N-terminal domain of K1 expressed in BJAB, BCBL-1 and HEK293 cells, inhibiting its activity. It is suggested that Hsp90β chaperone associates with K1 during the former *de novo* synthesis, when the peptide moves through the cytoplasm and the endoplasmic reticulum, while Erdj3 possibly acts in the assembly and folding of newly synthesized proteins into the endoplasmic reticulum (Wen and Damania, 2010).

3.4. BIOLOGICAL PROPERTIES OF K1

3.4.1. ACTIVITIES IN MEMBRANE AND CYTOPLASM

KSHV K1 activities predominantly occur at the cell surface, but the protein can also be found in early and late endosomes due to clathrin-mediated K1 internalization. In endosomes, K1 continues to trigger signal transduction, activating the protein kinase

B/AKT (PKB/Akt) pathway. K1 recruit and activate PI3K, promoting its own internalization. Both inhibition of PI3K activation or K1 internalization can suppress the signals triggered by this viral protein as well as its endocytosis. Of note, mutations in the K1 ITAM decrease the rates of protein internalization compared to cells expressing wild-type K1; thus, signaling triggered by ITAM plays a role in K1 endocytosis (Tomlinson and Damania, 2008).

K1 may indirectly contributes to disruption of endothelial cell junctions, allowing an increase in vascular permeability and extravasation of blood components in KS lesions. The increased vascular permeability associated to KSHV lytic infection may also be linked to degradation of the vascular endothelial cadherin (VE-cadherin), a putative effect of KSHV vGPCR (Dwyer et al., 2010) and K15 (Mansouri et al., 2008). Disruption of cell junctions can also be observed in latently infected endothelial cells: KSHV-infected HEK293 cells showed upregulation in the activity of Rac1 GTPase, which is involved in regulation of junctional integrity due to phosphorylation of tyrosine residues of the VE-cadherin and β-catenin. K1 expressing cells activates Rac1 more efficiently than non-expressing cells, indicating that this viral protein can also contribute to increased vascular permeability indirectly (Guilluy et al., 2011).

3.4.2. INCREASED CELL SURVIVAL

Some KSHV products clearly impact apoptosis regulatory proteins. For instance, LANA represses the transcriptional activity of the tumor suppressor protein p53 (Friborg et al., 1999), whereas vFLIP suppresses apoptosis by blocking the assembly of the death inducing signaling complex (DISC) (Thome et al., 1997), as well as stimulating NF-kB activation (Guasparri, 2004). K1 may increase the life-spam of KSHV-infected cells by two mechanisms, at least: interaction with receptors involved in apoptosis (Fas and/or BCR) (Berkova et al., 2009; Lee et al., 2000; Tomlinson and Damania, 2004; Wang et al., 2007), or via ITAM-mediated intracellular signaling (Wang et al., 2006; Wen and Damania, 2010).

The expression of K1 suppresses apoptosis of BJAB, THP-1, U937, and SLK cells treated with a Fas agonist antibody or Fas ligand (FasL) (Wang et al., 2007). In addition, mice treated with a lethal dose of an agonist antibody to Fas (JO2, which induces widespread apoptosis in hepatocytes) survive the challenge when transfected with K1 (Wang et al., 2007). K1 physically interacts with Fas through its immunoglobulin-like domain(Lee et al., 2000), preventing the action of FasL and Fas agonistic antibodies

(Wang et al., 2007). On the other hand, K1 partially inhibits the formation of DISC and significantly blocks the activity of caspase 8 (Wang et al., 2007).

K1 promote survival of B cells via suppression of BCR signaling, which may be due internalization of K1/BCR complexes (Tomlinson and Damania, 2008). Another mechanism involves the accumulation of BCR in the endoplasmic reticulum, by virtue of its interaction with the μ-chain of the N-terminal portion of K1 protein, inhibiting the intracellular transport of BCR and its expression on the cell surface (Tomlinson and Damania, 2004). Of note, in BJAB K1 is found mainly in the endoplasmic reticulum, as assessed by confocal microscopy (Tomlinson and Damania, 2004). K1 downregulates the expression of BCR complex, which blocks B cells activation, promoting the survival of KSHV-infected cells and increasing the repertoire of latently infected cells.

The PI3K/Akt pathway is known for its important role in the negative regulation of cell death by apoptosis (Engelman et al., 2006). Several components of this pathway are deregulated in cancers, including KS (Osaki et al., 2004). KSHV proteins vGPCR, vIL-6, ORF45, and K1 interfere in this pathway (Bhatt and Damania, 2013). Signals triggered by K1 ITAM activate PI3K/Akt in both epithelial cells and B lymphocytes (Tomlinson and Damania, 2008; Wang et al., 2006, 2007).

PI3K is lipid kinase formed by a catalytic p110 and regulatory p85 subunits (Engelman et al., 2006). Once phosphorylated, p85 subunit activates PI3K protein, which catalyzes the phosphorylation of PIP2 to PIP3. PIP3 is essential for Akt translocation to the cell membrane, where it is activated due to phosphorylation by phosphoinositide-dependent kinases (PDK) 1 or 2. The phosphatase and tensin homolog protein (PTEN) inhibits this signaling pathway, since it catalyzes dephosphorylation of PIP3 into PIP2, restraining Akt due to the negative effects on PI3K activation (Osaki et al., 2004). As a result, Akt cannot regulate cell survival by phosphorylation of components of the apoptotic pathway, which usually causes their retention in the cytoplasm and inactivation. This is what happens with FOXO group of proapoptotic transcriptional factors (AFX, FKHR, FKHRL1), as well as the proapoptotic proteins glycogen synthase kinase 3β (GSK3β), caspase-9 and bad. FOXO proteins are known to modulate the expression of cell cycle regulators and proteins that promote apoptosis, such as Fas and bim (Engelman et al., 2006).

K1 expression in endothelial cells and B lymphocytes increase the activation of the p85 subunit of PI3K, Akt, PDK1, and inactivates PTEN, causing PI3K-Akt

hyperactivation (Lee et al., 2005; Wang et al., 2006, 2007). Additionally, cells expressing K1 typically show phosphorylation and consequent inhibition of proteins involved in apoptosis: for instance, FKHR is phosphorylated in BJAB cells, preventing the transcription of FasL; on the other hand, HUVECs exhibited phosphorylation of bad, FKHR, FKHRL1, GSK3β, and mTOR, negatively impacting their proapoptotic properties due to cytoplasmic retention (Wang et al., 2006, 2007).

In summary, K1 induces apoptosis resistance mediated by the signaling triggered by the cytoplasmic portion of the protein, which activates Akt, with consequent inhibition of several pro-apoptotic proteins. Furthermore, the physical interaction of K1 with the Fas receptor prevents Fas-FasL binding. Activation of PI3K/Akt and the simultaneous inactivation of PTEN (its negative regulator), enhance the survival and proliferation of KSHV-infected endothelial cells, promoting viral dissemination, and possibly contributing to the pathogenesis of KSHV-associated diseases.

3.4.3. PROANGIOGENIC AND PROINFLAMMATORY PROPERTIES

The expression of KSHV K1 in B lymphocytes induces the activation of these cells via upregulation of transcription factors such as NFAT and AP-1, as well as production of pro-inflammatory cytokines, such as monocyte-derived chemokine (MCD), IL-1α, IL-1β, IL-8, IL-10, VEGF, and RANTES (Lagunoff et al., 1999; Lee et al., 2005).

In KS lesions, cell migration and angiogenesis are favored by local expression of IL-6, IL-8, VEGF, and matrix metalloproteinases (MMPs) by infection of endothelial cells infected by KSHV (Meade-Tollin et al., 1999; Samaniego et al., 1998). Still, K1 also contributes to this purpose, since increased expression and secretion of MMP-9 and VEGF has been reported in epithelial (HEK293) and endothelial (HUVEC) cells transfected with K1 without mutations in SH2 binding region of ITAM (Wang, 2004). Corroborating this data, K1-expressing HUVEC and BJAB cells exhibited, respectively, overactive VEGF/VEGFR signaling and increased secretion of VEGF (Prakash, 2005; Wang et al., 2006). Thus, under the action of increased expression and secretion of MMP-9 and VEGF, K1 possibly contributes to angiogenesis and has a role in growth and proliferation of infected cells from KS lesions via paracrine mechanisms of cytokine release (Wang, 2004).

In addition to the expression of genes involved in cell survival and proliferation, the NF-kB pathway is responsible for a variety of phenomena in inflammation. Activation of NF-kB is essential for the survival of lymphocytes infected with KSHV. In fact, it is

suggested that this signaling pathway is hijacked by the KSHV to promote viral persistence *in vivo* in the setting of hostile immune responses (de Oliveira et al., 2010).

Although there is some debate on the role of K1 in the activation of NF-kB signaling pathway, some studies report that BC-3 , BJAB and Raji cells transfected with K1 exhibit increased in luciferase activity driven by a NF-kB-responsive promoter (Prakash, 2005; Prakash et al., 2002; Samaniego et al., 2000). In BC-3, this activation seems to be regulated by ITAM and the lyn kinase src, since the use of a lyn-specific inhibitor was able to block cell proliferation and NF-kB activation; on the other hand, NF-kB inhibition was also obtained after ITAM mutation (Prakash, 2005). Splenic B lymphocytes from transgenic mice expressing KSHV K1 showed increased nuclear activity of NF-kB and Oct-2 compared with lymphocytes from non-transgenic mice (Prakash et al., 2002). Likewise, high NF-κB-driven luciferase activity was shown in KVL-1 cells, from a lymphoma in KSHV K1 transgenic mice (Prakash, 2005). Furthermore, as a result of NF-kB activation, K1-expressing COS-1 cells showed increased levels of IL- 6, IL -8 and IL-12 secretion, and co-expression of KSHV K1 and HIV-1 tat in these cells provided an additive effect on luciferase activity (Samaniego et al., 2000).

Conversely, T and B lymphocytes from transgenic mice expressing K1 showed no change in expression of IL-2, IL-4, IL-6, IL-10, GM-CSF, TNF-α and TNF-β compared to cells from control animals (Prakash, 2005). Interestingly, the expression of K1 in transfected HEK293 cells inhibited, in a dose-dependent fashion, the effect of v-FLIP and ORF-75 protein in the luciferase activity regulated by NF-kB-responsive elements (Konrad et al., 2009). Hence, further studies are needed to reconcile these results regarding the effects of KSHV K1 on NF-kB signaling.

3.5. ROLE OF K1 IN CARCINOGENESIS

K1 can activate multiple signaling pathways in KSHV-infected cells, some of them involved in carcinogenesis. The activation of the PI3K/Akt pathway by K1 possibly contributes to the immortalization of initial and sustained endothelial and epithelial cells. KSHV K1-transfected HUVECs showed an increase in the number of passages in cell culture compared to control cells (55 and 32 passages, respectively). After 32th passage, the control cells stopped growing and exhibited morphological features typical of senescence; on the other hand, cells expressing K1 kept growing and had no changes in morphology. However, in this study the KSHV K1-expressing HUVECs did

not induce tumors when injected into nude mice, and their suspected immortalization was not associated with changes in telomerase expression (Wang et al., 2006).

K1 induce cellular transformation *in vitro*. Rodent fibroblasts expressing K1 *in vitro* displayed both morphologic changes and focus formation compared to negative controls (H Lee et al., 1998). K1 may also contribute to tumorigenesis *in vivo*: when the HSV STP oncogene was replaced with the KSHV ORF-K1 in a plasmid that was then inoculated into marmosets, these animals developed lymphomas. Furthermore, immortalized primary T cells can be obtained *in vitro* from these animals (H Lee et al., 1998). Another study reported that 1 out of 4 (25%) BALB/c mice inoculated intranasally with recombinant version of murine herpesvirus 4 encoding KSHV K1 (MHV76-K1) developed salivary glands adenocarcinoma after 120 days and showed increased pulmonary inflammatory response compared to the control animals (Douglas et al., 2004).

Large tumor masses were identified in 2 out of 13 (15.4%) transgenic mice (C57BL/6J) expressing K1 at approximately 14 months of age. One animal developed a submaxilar tumor, supposedly a plasmablastic lymphoma; a second tumor was located in the omentum, and it had features suggestive of sarcomatoid spindle cell neoplasm. The neoplastic cells of plasmablastic lymphoma exhibited higher lyn kinase activity compared to activity in B-lymphocytes expressing K1, suggesting that lyn activation was related to a putative role of K1 in lymphomagenesis (Prakash et al., 2002).

The phenotype of transgenic mice expressing K1 is similar to findings of KSHV-associated lymphomas in humans, including abnormal lymphocyte proliferation in response to antigens and splenomegaly. Furthermore, the KVL -1 cell line derived from murine tumors expressing K1 showed high levels of VEGF (Prakash, 2005). When a lymphoma expressing K1 was subcutaneously injected into nude BALB/c mice, the tumor growth was associated with constitutive activity of NF-kB and VEGF secretion. Additionally, 90% of 12-months old mice expressing K1 showed lymphoid hyperplasia and four times more VEGF produced compared to controls (Prakash, 2005). These results suggest that KSHV K1 may have an impact in the carcinogenic potential of viral infection, notably for lymphomagenesis. However, it must be take into account that rodent cells are more susceptible to cancer and spontaneous transformation compared to human cells (Rangarajan et al., 2004).

Migratory and invasive phenotypes acquisition is a major event for endothelial

KSHV-infected cells, since it is important for both viral contamination and angiogenesis (Qian et al., 2007). Some data indicate that, in established tumors, K1 contributes to an increase in angiogenesis; inoculation of C33A epithelial K1-expressing cells into nude mice displayed tumors that grew significantly faster than the ones in controls, and at the same time, it increased Akt phosphorylation and marker Ki67 expression. Moreover, these K1-expressing tumors were more vascularized than controls, probably due to the activation of VEGF/VEGFR and PI3K pathway caused by K1 (Wang et al., 2006). Finally, microscopic analysis showed an increase in both apoptosis and cell proliferation in endothelial embryonic stem cells expressing K1, derived from subcutaneous murine teratoma. (Mebrahtu et al., 2004).

CONCLUSIONS

Based on the data available so far, the following conclusions can be drawn:

1. In KSHV-infected cells, K1 can activate multiple signaling pathways mediated by ITAM located within its cytoplasmic portion;
2. KSHV K1 increases the life-spam of cells by interfering with receptors involved in apoptosis (e.g., Fas/FasL; BCR), or by activating intracellular pathways that culminate in cell survival programs (e.g.PI3K/Akt).
3. K1 enhances angiogenesis in KSHV-infected cells by promoting cytokines secretion (e.g. VEGF) in the microenvironment.
4. K1 contributes to viral carcinogenesis; it might also be involved in both endothelial cell immortalization and cell transformation.
5. K1 is involved in KSHV-induced tumorigenesis and increases angiogenesis in established tumors.

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herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. J. Virol. 73, 4156–4170.

Manuscrito Experimental

LISTA DE FIGURAS

2. MANUSCRITO EXPERIMENTAL

Produção e validação de vetores recombinantes para expressão de diferentes formas da proteína K1 do herpesvírus associado ao sarcoma de Kaposi (KSHV)

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ABSTRACT: Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with the development of Kaposi's sarcoma. The K1 protein of KSHV has been shown to induce increases the survival and proliferation of infected cells. The viral ORF-K1 shows high variability, so it is possible to distinguish different KSHV genotypes. So far, it is unclear whether different viral genotypes have their own biological characteristics. To this intent, recombinant vectors were generated containing the ORF-K1 genotypes A and B. ORF-K1 was obtained from the DNA of primary effusion lymphoma (PEL) cell lines. The amplicon generated and the commercial vector were digested, bonded and cloned in *E.coli* DH5α. Selected clones underwent automated DNA sequencing. The generated sequences were compared with prototype sequences of ORF-K1. The amino acid sequences from vectors were generated and analyzed in the K1 ITAM-coding region. Validated clones were stably transfected into HEK293 cells and K1 expression was evaluated using Western blot (WB). DNA sequencing showed that the ORF-K1 recombinant vectors corresponds to the prototype sequences deposited from PEL cell lines. WB demonstrated the presence of K1 in the lysate of HEK293 transfected cells. Analysis of the K1 amino acid sequence encoded in the vectors revealed that ITAM domain of K1 (genotype B) has distinct amino acids from the ones in the ITAM domain of K1 (genotypes A and C). Therefore ORF-K1 recombinant vectors were produced and validated, and will be useful to establish an experimental model for analysis of the biological properties of the K1 protein.

Fontes de apoio: Fundação de Amparo a Pesquisa do Estado de São Paulo (Fapesp)
Palavras-chave: KSHV, proteína K1, Biologia Molecular, Carcinogênesis

Apresentação do manuscrito com base nas instruções da revista Virus Reviews & Research (ISSN 2357-9323). Por conveniência, figuras foram apresentadas no corpo do manuscrito.

INTRODUÇÃO

O herpesvírus associado ao sarcoma de Kaposi (*Kaposi's Sarcoma-associated Herpesvirus - KSHV*), também denominado herpesvírus humano tipo 8 (*Human Herpesvirus Type 8 - HHV-8*), é o agente etiológico do sarcoma de Kaposi (SK), uma neoplasia angioproliferativa inflamatória maligna, que acomete principalmente indivíduos imunodeficientes (Chang et al., 1994). Além disso, a infecção viral está associada a dois distúrbios linfoproliferativos de linfócitos B: o linfoma de efusão primário (*Primary Effusion Lymphoma - PEL)* e a doença multicêntrica de Castleman (*Multicentric Castleman Disease - MCD*) (Cesarman et al., 1995; Soulier et al., 1995). De modo semelhante aos demais herpesvírus, o ciclo biológico do KSHV é divido nas fases latente e lítica. Na fase latente poucos produtos gênicos são transcritos; em contraste, na fase lítica o DNA viral é altamente replicado e são expressos vários genes responsáveis pela montagem e propagação de novos vírions, usualmente culminando na morte da célula hospedeira (Ganem, 2010). Diversos genes codificados no genoma do KSHV estão envolvidos na transformação e proliferação celular, sinalização intra e intercelular, imunomodulação e regulação da apoptose – fenômenos tipicamente alterados na carcinogênese (Giffin and Damania, 2014).

K1 é uma glicoproteína transmembrana codificada pela ORF-K1 do KSHV, significativamente expressa na fase lítica do ciclo biológico viral (Lagunoff and Ganem, 1997). Estruturalmente, K1 é dividida em uma sequência peptídeo-sinal na região N-terminal, um domínio extracelular, um domínio transmembrana e uma cauda curta citoplasmática na região C-terminal (H Lee et al., 1998). K1 assemelha-se ao receptor de linfócitos B (*B-cell Receptor - BCR*), podendo mimetizar ou bloquear a ativação dos linfócitos B, iniciando vias de sinalização e mobilização de cálcio intracelulares (Lagunoff et al., 1999; Lee et al., 2002; Heuiran Lee et al., 1998). A região citoplasmática de K1 contém dois motivos de ativação de imunoreceptor de tirosina (*Immunoreceptor Tyrosine-based Activation Motif - ITAM*), que apresenta similaridade com as cadeias α e β do BCR dos linfócitos B (Lagunoff et al., 1999; Heuiran Lee et al., 1998). A ativação dos resíduos de tirosina do ITAM de K1 ocasiona recrutamento de proteínas syk-específicas, que interagem com ITAM por meio de seus domínios SH2, levando a ativação dessas enzimas. Uma vez ativadas, essas enzimas são capazes de ativar diversas vias de sinalização intracelulares (Lee et al., 2005).

Em células endoteliais e linfócitos B, a ativação de vias de sinalização intracelulares mediada por ITAM de K1 é capaz de ativar a via PI3K/Akt (Lee et al., 2005; Tomlinson and Damania, 2004; Wang et al., 2006), que tem importante papel na regulação negativa da morte celular por apoptose (Engelman et al., 2006). K1 também pode aumentar a sobrevivência celular por outros mecanismos: por exemplo, inibindo a transcrição de proteínas pró-apoptóticas (e.g. FasL) (Tomlinson and Damania, 2004; Wang et al., 2006) e por meio de interação física com Fas (mediada por seu domínio semelhante à imunoglobulina), de modo a impedir a ligação do FasL (Berkova et al., 2009). Por outro lado, K1 pode suprimir a expressão do BCR na superfície de linfócitos B (Lee et al., 2000), propriedade que pode ter impacto na sobrevivência e proliferação de linfócitos B infectados pelo KSHV, favorecendo a disseminação viral e possivelmente contribuindo na patogênese tanto do SK quanto de linfomas associados ao KSHV.

K1 também contribui na imortalização de células endoteliais *in vitro*, uma vez que HUVECs transfectadas com K1 apresentam aumento significativo do número de passagens em cultura quando expressam a proteína (Wang et al., 2006). Fibroblastos de roedores que expressavam K1 *in vitro* exibiram mudanças tanto morfológicas como no crescimento celular quando comparadas às células controle (H Lee et al., 1998). Por outro lado, camundongos transgênicos que expressavam K1 desenvolveram tumores e exibiram aumento da atividade nuclear para NF-κB e Oct-2, demonstrando que essa proteína contribui para tumorigênese induzida pelo KSHV (Prakash et al., 2002). Digno de nota, a substituição do oncogene STP do herpesvirus saimiri (*Herpesvirus saimiri* - HSV) em um construto pela ORF-K1 do KSHV e sua inoculação em células de saguis ocasionou linfomas nesses animais, além da geração de células T primárias imortalizadas *in vitro* (H Lee et al., 1998).

K1 apresenta alta variabilidade comparada a outras proteínas do KSHV. A sequência nucleotídica da ORF-K1 possui variação de cerca de 87%. A cadeia polipeptídica de K1 varia em cerca de 62% (Zong et al., 1999), sendo que as variações são concentradas na porção extracelular da proteína, em duas regiões hipervariáveis denominadas V1 e V2. Baseado em análises de V1 e V2, isolados do KSHV foram originalmente classificados em quatro genótipos principais, denominados A, B, C e D (Zong et al., 1999). A distribuição dos genótipos virais em populações humanas infectadas é influenciada por fatores geográficos e étnicos, provavelmente em

decorrência de antigas migrações. Em termos gerais o genótipo A é encontrado predominantemente na América do Norte, B na África, C na Europa, na Ásia e no Mediterrâneo, e o genótipo D em Ilhas do Pacífico (Zong et al., 2002, 1999). Há ainda a descrição de genótipos mais raros, tais como o genótipo E, identificado em Ameríndios sul-americanos (Biggar et al., 2000), e o F, detectado em amostras biológicas de indivíduos na tribo Bantu de Uganda (Kajumbula et al., 2006).

Diante da alta variabilidade da ORF-K1 e das propriedades conhecidas de K1 no ciclo biológico viral e no potencial cancerígeno do KSHV, o objetivo do presente trabalho foi construir e validar vetores recombinantes para expressão de diferentes formas da proteína K1 em células eucariotas. Tais vetores são úteis para investigação dos efeitos *in vitro* de K1, permitindo verificar se os diferentes genótipos de K1 possuem propriedades distintas, de modo a contribuir mais ou menos na patogênese do SK e de linfomas associados ao KSHV.

MATERIAIS E MÉTODOS

Este estudo foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista (UNESP).

Linhagens celulares empregadas

DNA genômico extraído das linhagens celulares BCBL-1, positiva para KSHV e para o vírus Epstein-Barr (*Epstein-Barr virus* - EBV), BC-1 (KSHV+/EBV+), BC-5 (KSHV+/EBV+), VG-1 (KSHV+/EBV-), BC-3 (KSHV+/EBV-), e JSC-1 (KSHV+/EBV+), gentilmente doadas pela Prof[a]. Dra. Ethel Cesarman (*Weill Medical School, Cornell University, NY, USA*). Células HEK 293, doadas pelo Prof. Dr. André Sampaio Pupo (Instituto de Biociências - UNESP), foram cultivadas em meio Eagle modificado por Dulbecco (*Dulbelcco´s Modified Eagle medium - DMEM*) (*LGC Biotecnologia, São Paulo, SP, Brasil*), mantidas com meio suplementado com 10% de soro fetal bovino e 0,4% de gentamicina, e cultivadas à 37ºC em incubadora com 5% de CO_2.

Construção dos vetores recombinantes

Foi empregada reação em cadeia polimerase (*Polymerase Chain Reaction* - PCR) para amplificação da ORF-K1 do KSHV a partir do genoma viral presente nas linhagens celulares mencionadas, a saber: BCBL-1 (genótipo A), BC-1 (genótipo A), BC-5 (genótipo B), VG-1 (genótipo B), BC-3 (genótipo C) e JSC-1 (genótipo C). Os

oligonucleotídeos empregados, KSHVOK1-HindIII.S (GCG CGC AAG CTT TCTT TCA GAC CTT GTT GGA C) e KSHVOK1-BamHI.A (GCG CGC GGA TCC GAA TGT CAG TAC CAA TCC AC), flanqueiam toda a ORF-K1 viral e incorporam no amplicon sítios para enzimas de restrição HindIII e BamHI, respectivamente. O amplicon gerado foi purificado empregando o sistema comercial *GFX™ PCR DNA and gel band Purification* (*GE Healthcare, Little Chalfont, Buckinghamshire, UK*) e clonado no vetor de expressão p3xFLAG-CMV-10 (*Sigma-Aldrich, St Louis, MO, USA*). Recircularização espúria do vetor digerido com HindIII (Promega, Madison, WI, USA) e BamHI (*New England Biolabs, Ipswich, MA, USA*) foi prevenida por meio sua desfosforilação com a enzima fosfatase antártica (*New England Biolabs, Ipswich, MA, USA*).

A ligação do vetor e inserto (ORF-K1 do KSHV) foi realizada com enzima T4 DNA ligase (*New England Biolabs, Ipswich, MA, USA*), empregando tipicamente proporções de massa 1:0 (50 ng do vetor e 0 de inserto) 1:2 (50 ng de vetor para 100 ng de inserto) e 1:3 (50 ng do vetor e 150 ng do inserto). Foi empregada proporção molar 1:3 (1 mol de DNA de vetor para cada 3 moles de DNA do inserto) excepcionalmente nas situações de maior dificuldade de transformação bacteriana. A produção dos vetores recombinantes foi confirmada por amplificação por PCR com os iniciadores CMV-30.S (AAT GTC GTA ATA ACC CCG CCC CGT TAG CGC) e CMV-24.A (TAT TAG GAC AAG GCT GGT GGG CAC), que se ligam aos sítios que flanqueiam a região do cassete de clonagem do vetor. Os vetores produzidos (doravante denominados pKSHVOK1) foram utilizados para transformação de *E. coli DH5α Max Efficiency® Competent Cells* (*Invitrogen, Carlsbad, CA, USA*), que foram selecionadas em placas Lúria-Bertani-Ágar (LB-Ágar) com 100µg/mL de ampicilina.

Validação estrutural dos vetores

Para validação da sequência da ORF-K1 presentes nos vetores, clones arbitrariamente selecionados foram submetidos a sequenciamento automatizado de DNA em equipamento ABI 3730 DNA Analyzer, empregando insumos *Big Dye® terminator v3.1 Cycle sequencing Kit* (*Applied Biosystems, Foster City, CA, USA*) em serviço terceirizado (Centro de Estudos do Genoma Humano, São Paulo, SP, Brasil). As sequências obtidas foram comparadas com sequências-protótipo da ORF-K1 depositadas no banco de dados do Centro Nacional de Informação Biotecnológica dos Estados Unidos (*National Center for Biotechnology Information - NCBI*).

Procedeu-se alinhamento das sequências consenso e de aminoácidos do vetores recombinantes de K1 empregando o software *AlignX* do pacote de software *Vector NTI Advance 11* (*Invitrogen, Carlsbad, CA, USA*). As sequências de DNA e de aminoácidos obtidas foram comparadas às sequências-protótipos depositadas no NCBI utilizando-se a Ferramenta de Pesquisa Básica de Alinhamento Local (*Basic Local Alignment Search Tool – BLAST*), disponível em http://blast.ncbi.nlm.nih.gov/. Subsequentemente foi realizada análise filogenética das sequências da ORF-K1 inseridas nos vetores recombinantes conjuntamente com sequências-protótipo de diferentes genótipos do KSHV selecionados, disponibilizadas no Genbank (NCBI). Para tanto foi utilizado o software *Mega 5.1 Beta 3* (Tamura et al., 2011) com algoritmo de Máxima probabilidade (*Maximum likelihood*) para construção da árvore filogenética, que foi validada por teste de filogenia *Bootstrap (*500 réplicas).

Expressão dos vetores em células transfectadas

Células HEK293 foram cultivadas em placas de 24 poços (densidade inicial de 1,6 x 10^5) até 60-70% de confluência e então transfectadas com 1 µg de DNA dos vetores recombinantes empregando carreador lipídico *FuGENE® HD Transfection Reagent* (*Promega, Madison, WI, USA*), seguindo instruções do fabricante. Os experimentos de transfecção foram realizados em triplicata e empregaram os vetores p3xFLAG-BAP-CMV7 (*Sigma-Aldrich, St Louis, MO, USA*) e p3XFLAG-CMV10 (*Sigma-Aldrich, St Louis, MO, USA*) como controles positivo e negativo, respectivamente. Após 48h de transfecção, as células contendo os vetores recombinantes de ORF-K1 foram selecionadas com 0,8mg/ml de G418 (*Invitrogen, Carlsbad, CA, USA*).

As células HEK293 estavelmente transfectadas com vetores recombinantes de ORF-K1 do KSHV foram submetidas à extração de proteínas totais com reagente *M-PER® Mammalian Protein Extraction Reagent* (*Thermo Scientific, Rockford, IL, USA*) contendo coquetel de inibidores de proteases *Halt™ Protease Inhibitor Cocktail* (*Thermo Scientific, Rockford, IL, USA),* conforme instruções do fabricante. A concentração de proteínas foi estimada por leitura espectrofotométrica à 655nm empregando-se o kit comercial *DC Protein Assay kit* II (*Bio-Rad Laboratories, Hercules, CA, USA*) segundo instruções do fabricante. Lisados proteicos contendo 100µg proteínas totais de cada amostra foram submetidos à eletroforese em gel de poliacrilamida com duodecil sulfato de sódio (*Sodium Duodecyl Sulfate Polyacrylamide*

Gel Electrophoresis - SDS-PAGE) e transferidas para membranas de fluoreto de polivinilideno (*polyvinylidene fluoride* – PVDF) (*Millipore, Billerica, MA, USA)* empregando equipamento *Trans-Blot Semi-Dry Transfer Cell* (*Bio-Rad Laboratories, Hercules, CA, USA).*

A detecção de K1 nas membranas produzidas foi efetuada por meio de incubação com anticorpo primário monoclonal anti-FLAG® M2 (*Sigma-Aldrich, St Louis, MO, USA*) 1:5000 *overnight*, seguida de incubação com o anticorpo secundário antifragmento Fab2 de IgG de camundongo conjugada a peroxidase e produzida em carneiro (*GE Healthcare, Little Chalfont, Buckinghamshire, UK*) a 1:4000 por 4h. Em paralelo foi efetuada detecção da cadeia beta da actina humana por meio de incubação com anticorpo monoclonal anti-β-actina (*Sigma-Aldrich, St Louis, MO, USA)* 1:5000 *overnight,* seguida de incubação com o anticorpo secundário antifragmento Fab2 de IgG de coelho conjugada a peroxidase e produzida em asno (*GE Healthcare, Little Chalfont, Buckinghamshire, UK*) a 1:4000 por 2h. A detecção de sinal no experimento de western blot foi efetuada por quimiluminescência com reagente *Pierce ECL* (*Thermo Scientific, Rockford, IL, USA).*

RESULTADOS

Construção de vetores de expressão para ORF-K1 do KSHV

A construção dos vetores recombinantes para expressão da proteína K1 do KSHV foi efetuada com amplicons da ORF-K1 viral obtida das linhagens de PEL BCBL-1 e BC-1 (genótipo A do KSHV), BC-5 e VG-1 (genótipo B), e BC-3 e JSC-1 (genótipo C). Conforme pode ser observado na Figura 1A, os amplicons obtidos apresentam cerca de 900pb, tamanho estimado para ORF-K1. Subsequentemente os amplicons e o vetor p3xFLAG-CMV10 foram digeridos com as enzimas *BamHI* e *HindIII*. Os insertos produzidos foram denominados KSHVOK1-1, KSHVOK1-2, KSHVOK1-3, KSHVOK1-4, KSHVOK1-5 e KSHVOK1-6, para ORF-K1 de BCBL-1, BC-1, BC-3, JSC-1, BC-5 e VG-1, respectivamente.

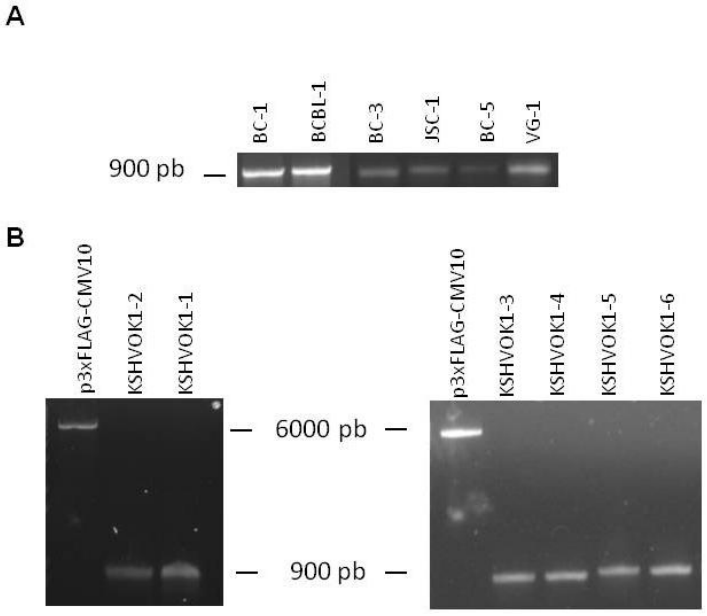

Figura 1 - Eletroforese em gel de agarose 1%, corado com brometo de etídio. (**A**) Amplificação do DNA das linhagens celulares obtidas de PEL KSHV-positivas identificadas como BC-1, BCBL-1, BC-3, JSC-1, BC-5 e VG-1, utilizando os iniciadores KSHVOK1.HindIII.S e KSHVOK1.BamHI.A. A seta indica a banda de tamanho aproximado do amplicon de ORF-K1 (~900pb); (**B**) Resultado de digestão enzimática do vetor p3xFLAG-CMV-10 e insertos de ORF-K1 de BCBL-1 (KSHVOK1-1), de BC-1 (KSHVOK1-2), de BC-3 (KSHVOK1-3), de JSC-1 (KSHVOK1-4), de BC-5 (KSHVOK1-5) e de VG-1 (KSHVOK1-6), com as enzimas *Bam*HI e *Hind*III. Após a digestão, o vetor passa a apresentar 6249pb. Insertos se mantém com aproximadamente 900 pb.

Após a digestão enzimática, o vetor p3xFLAG-CMV10 e os insertos gerados foram avaliados por eletroforese, tendo sido verificado o resultado esperado: perda de cerca de 51pb no vetor, correspondente à sequência intercalada entre os sítios de

restrição utilizados em seu cassete de clonagem (*Multiple Cloning Site – MCS*) (Figura 1B).

Após defosforilação do vetor foi efetuada sua ligação com os insertos, confirmada por PCR convencional com os iniciadores CMV-30S e CMV-24A (Figura 2). O sítio de inserção da ORF K1 distam 317pb no vetor p3XFLAG-CMV10 vazio e sua digestão com as enzimas utilizadas proporciona a remoção de 50pb em seu MCS. Por outro lado, a ligação do vetor com o inserto gera vetor recombinante acrescido de aproximadamente 900pb (ORF-K1, de tamanho variável de acordo com o genótipo). Assim, após sucesso na ligação o tamanho estimado dos construtos é de 1.167pb. Os vetores recombinantes produzidos foram nomeados de pKSHVOK1-1 e pKSHVOK1-2 pKSHVOK1-3, pKSHVOK1-4, pKSHVOK1-5 e pKSHVOK1-6.

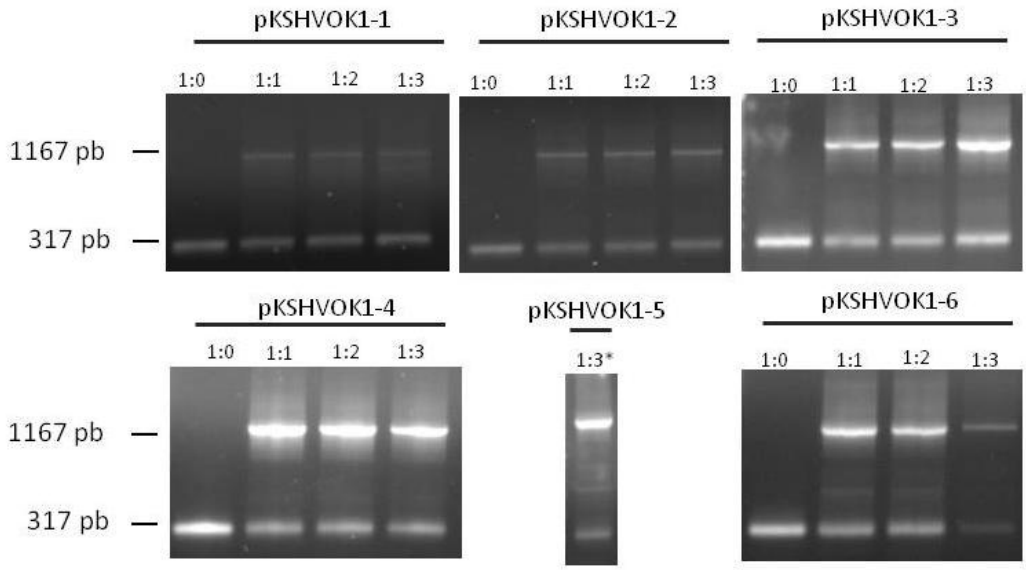

Figura 2 - Eletroforese em gel de agarose 1% corado com brometo de etídio. Avaliação da ligação do vetor p3xFLAG-CMV10 aos insertos. O resultado apresenta produto de PCR amplificado com iniciadores CMV-30.S e CMV-24.A. Amplicon de 1167 pb evidencia que houve ligação adequada entre inserto e vetor; já a banda de 317 pb representa amplificação de resíduo de vetores vazios não digeridos. Foram realizadas ligações com diferentes proporções de massa entre vetor e inserto para KSHVOK1-1, KSHVOK1-2, KSHVOK1-3, KSHVOK1-4 e KSHVOK1-6: 1:0 (sem inserto), 1:1, 1:2 e 1:3. *Para o inserto KSHVOK1-5 utilizou-se proporção molar de 1:3 entre vetor e inserto.

Subsequentemente, bactérias *E. Coli* DH5α foram transformadas com os vetores recombinantes produzidos. A validação da transformação das bactérias foi confirmado por PCR utilizando-se colônias bacterianas selecionadas (Figura 3).

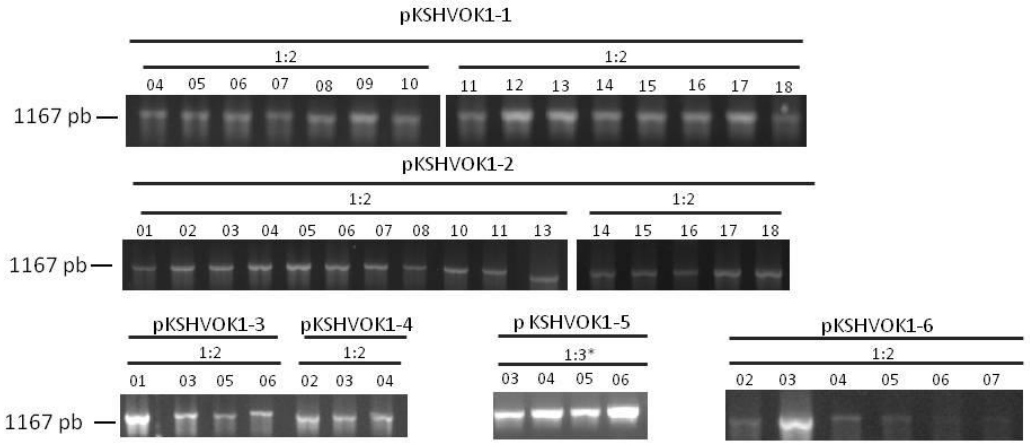

Figura 3 - Eletroforese em gel de agarose 1% corado com brometo de etídio. Produto de *PCR-colony* para os clones dos vetores pKSHVOK1-1, pKSHVOK1-2, pKSHVOK1-3, pKSHVOK1-4, pKSHVOK1-5 e pKSHVOK1-6 após transformação bacteriana. O amplicon gerado (~1167pb) evidencia o vetor recombinante. O número correspondente à colônia selecionada na placa de LB-Ágar está indicado no topo, bem como a proporção de vetor e inserto empregada, sendo 1:2 (massa) para todas as situações, exceto pKSHVOK1-5 (proporção molar 1:3).

Validação estrutural dos vetores recombinantes

Para cada vetor recombinante contendo a ORF-K1 viral, três colônias foram selecionadas e submetidas a sequenciamento automatizado de DNA (colônias 04, 05, 11 para pKSHVOK1-1; 01, 02 e 06 para pKSHVOK1-2; 01, 03 e 06 para pKSHVOK1-3; 02, 03 e 05 para pKSHVOK1-4; 03, 04 e 05 para pKSHVOK1-5; e 02, 03 e 04 para pKSHVOK1-6). As sequências consenso dos construtos foram alinhadas e comparadas com sequências-protótipo. A Figura 4 demonstram a similaridade dos vetores recombinantes em relação a sequências-protótipos conhecidas da ORF-K1 viral.

A sequência da ORF-K1 do vetor pKSHVOK1-1 (genótipo A viral) apresentou alto grau de similaridade com a ORF-K1 de BCBL-1 (*GenBank#* U86667.1), enquanto a do vetor pKSHVOK1-2 foi equivalente a ORF-K1 de BC-1 (*GenBank#* U75698.1). As sequências de ORF-K1 do genótipo B, correspondentes aos vetores pKSHVOK1-5 e pKSHVOK1-6, apresentaram alta similaridade com a ORF-K1 de linhagem celular 431KAP do genótipo B (*GenBank#* AF133040.1). Confirmou-se que as sequências da ORF-K1 viral nos vetores pKSHVOK1-5 e pKSHVOK1-6, provenientes de BC-5 e VG-1, são de genótipo B; entretanto, sequências do genoma viral nessas duas linhagens celulares não estavam depositadas no *Genbank* para comparação. Em tempo, em relação ao genótipo C, a sequência do vetor pKSHVOK1-3 apresentou alta similaridade com ORF-K1 de BC-3 (*GenBank#* AF170531.1), e a de pKSHVOK1-4 foi similar à ORF-K1 de JSC-1 (*GenBank#* GQ994935.1).

A validação estrutural dos vetores foi confirmada por meio de análise filogenética das sequências obtidas (Figura 5). Os resultados obtidos demonstram que os vetores produzidos são representativos dos genótipos virais originalmente definidos da ORF-K1 viral. Assim, foram produzidos e estruturalmente validados dois vetores com ORF-K1 de genótipo A (pKSHVOK1-1 e pKSHVOK1-2), dois de genótipo B (pKSHVOK1-5 e pKSHVOK1-6) e dois de genótipo C (pKSHVOK1-3 e pKSHVOK1-4).

pKSHVOK1-1.04

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ404500.1	Human herpesvirus 8 strain KSHV-BAC36 long unique region, g	1677	1677	100%	0.0	99%
U86667.1	Kaposi's sarcoma-associated herpesvirus ORF K1 gene, comple	1670	1670	100%	0.0	99%
AF178799.1	Human herpesvirus 8 clone K1-32/Bcb K1 protein (K1) gene, c	1602	1602	95%	0.0	99%
AY204658.1	Human herpesvirus 8 isolate K1/E43 K1 glycoprotein gene, con	1596	1596	95%	0.0	99%
AY204662.1	Human herpesvirus 8 isolate K1/E36 K1 glycoprotein gene, par	1594	1594	95%	0.0	99%
FJ884623.1	Human herpesvirus 8 isolate UG-124 K1 glycoprotein gene, cor	1548	1548	99%	0.0	97%
FJ884613.1	Human herpesvirus 8 isolate IT-176 K1 glycoprotein gene, com	1543	1543	99%	0.0	97%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, comp	1543	1543	99%	0.0	97%
FJ884624.1	Human herpesvirus 8 isolate UG-208 K1 glycoprotein gene, cor	1537	1537	99%	0.0	97%
FJ884607.1	Human herpesvirus 8 isolate CM-90 K1 glycoprotein gene, com	1537	1537	99%	0.0	97%

pKSHVOK1-1.05

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ404500.1	Human herpesvirus 8 strain KSHV-BAC36 long unique region, g	1663	1663	100%	0.0	99%
U86667.1	Kaposi's sarcoma-associated herpesvirus ORF K1 gene, comple	1657	1657	100%	0.0	99%
AF178799.1	Human herpesvirus 8 clone K1-32/Bcb K1 protein (K1) gene, c	1596	1596	95%	0.0	99%
AY204662.1	Human herpesvirus 8 isolate K1/E36 K1 glycoprotein gene, par	1591	1591	95%	0.0	99%
AY204658.1	Human herpesvirus 8 isolate K1/E43 K1 glycoprotein gene, con	1591	1591	95%	0.0	99%
FJ884623.1	Human herpesvirus 8 isolate UG-124 K1 glycoprotein gene, cor	1533	1533	99%	0.0	97%
FJ884613.1	Human herpesvirus 8 isolate IT-176 K1 glycoprotein gene, com	1528	1528	99%	0.0	97%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, comp	1528	1528	99%	0.0	97%
FJ884624.1	Human herpesvirus 8 isolate UG-208 K1 glycoprotein gene, cor	1522	1522	99%	0.0	97%
FJ884607.1	Human herpesvirus 8 isolate CM-90 K1 glycoprotein gene, com	1522	1522	99%	0.0	97%

pKSHVOK1-1.11

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ404500.1	Human herpesvirus 8 strain KSHV-BAC36 long unique region, g	1663	1663	100%	0.0	99%
U86667.1	Kaposi's sarcoma-associated herpesvirus ORF K1 gene, comple	1657	1657	100%	0.0	99%
AF178799.1	Human herpesvirus 8 clone K1-32/Bcb K1 protein (K1) gene, c	1596	1596	95%	0.0	99%
AY204662.1	Human herpesvirus 8 isolate K1/E36 K1 glycoprotein gene, par	1591	1591	95%	0.0	99%
AY204658.1	Human herpesvirus 8 isolate K1/E43 K1 glycoprotein gene, con	1591	1591	95%	0.0	99%
FJ884623.1	Human herpesvirus 8 isolate UG-124 K1 glycoprotein gene, cor	1533	1533	99%	0.0	97%
FJ884613.1	Human herpesvirus 8 isolate IT-176 K1 glycoprotein gene, com	1528	1528	99%	0.0	97%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, comp	1528	1528	99%	0.0	97%
FJ884624.1	Human herpesvirus 8 isolate UG-208 K1 glycoprotein gene, cor	1522	1522	99%	0.0	97%
FJ884607.1	Human herpesvirus 8 isolate CM-90 K1 glycoprotein gene, com	1522	1522	99%	0.0	97%

Figura 4 - Alinhamento das sequências consenso de ORF-K1 do KSHV obtidas de clones do vetor recombinante pKSHVOK1-1, pKSHVOK1-2, pKSHVOK1-3, pKSHVOK1-4, pKSHVOK1-5 e pKSHVOK1-6 com sequências-protótipo depositadas no *NCBI*, encontradas através da ferramenta BLAST. Para cada clone foram selecionadas as dez primeiras sequências encontradas no banco de dados que apresentam maior cobertura em relação à sequência consultada (destaque em verde) e maior identidade máxima (destaque em vermelho).

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pKSHVOK1-2.01

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U75698.1	Human herpesvirus 8 type M, partial genome	1683	1683	100%	0.0	100%
FJ884626.1	Human herpesvirus 8 isolate US-216 K1 glycoprotein gene, cor	1609	1609	99%	0.0	99%
FJ884612.1	Human herpesvirus 8 isolate IT-170 K1 glycoprotein gene, com	1604	1604	99%	0.0	99%
FJ884615.1	Human herpesvirus 8 isolate IT-E40 K1 glycoprotein gene, com	1598	1598	99%	0.0	98%
AF133038.1	Kaposi's sarcoma-associated herpesvirus strain BCBL-R transfc	1594	1594	100%	0.0	98%
AF178807.1	Human herpesvirus 8 clone K1-40/Bc1 K1 protein (K1) gene, c	1591	1591	95%	0.0	99%
AY204645.1	Human herpesvirus 8 isolate K1/E5 K1 glycoprotein gene, comp	1568	1568	95%	0.0	99%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, com	1565	1565	99%	0.0	98%
AY204660.1	Human herpesvirus 8 isolate K1/E17 K1 glycoprotein gene, con	1563	1563	95%	0.0	99%
AF130305.1	Kaposi's sarcoma-associated herpesvirus isolate Ema7 K1 glyc	1546	1546	95%	0.0	99%

pKSHVOK1-2.02

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U75698.1	Human herpesvirus 8 type M, partial genome	1672	1672	100%	0.0	99%
FJ884626.1	Human herpesvirus 8 isolate US-216 K1 glycoprotein gene, cor	1598	1598	99%	0.0	98%
FJ884612.1	Human herpesvirus 8 isolate IT-170 K1 glycoprotein gene, com	1592	1592	99%	0.0	98%
FJ884615.1	Human herpesvirus 8 isolate IT-E40 K1 glycoprotein gene, com	1587	1587	99%	0.0	98%
AF133038.1	Kaposi's sarcoma-associated herpesvirus strain BCBL-R transfc	1583	1583	100%	0.0	98%
AF178807.1	Human herpesvirus 8 clone K1-40/Bc1 K1 protein (K1) gene, c	1580	1580	95%	0.0	99%
AY204645.1	Human herpesvirus 8 isolate K1/E5 K1 glycoprotein gene, comp	1557	1557	95%	0.0	99%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, com	1554	1554	99%	0.0	98%
AY204660.1	Human herpesvirus 8 isolate K1/E17 K1 glycoprotein gene, con	1552	1552	95%	0.0	99%
AF130305.1	Kaposi's sarcoma-associated herpesvirus isolate Ema7 K1 glyc	1535	1535	95%	0.0	99%

pKSHVOK1-2.06

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U75698.1	Human herpesvirus 8 type M, partial genome	1683	1683	100%	0.0	100%
FJ884626.1	Human herpesvirus 8 isolate US-216 K1 glycoprotein gene, cor	1609	1609	99%	0.0	99%
FJ884612.1	Human herpesvirus 8 isolate IT-170 K1 glycoprotein gene, com	1604	1604	99%	0.0	99%
FJ884615.1	Human herpesvirus 8 isolate IT-E40 K1 glycoprotein gene, com	1598	1598	99%	0.0	98%
AF133038.1	Kaposi's sarcoma-associated herpesvirus strain BCBL-R transfc	1594	1594	100%	0.0	98%
AF178807.1	Human herpesvirus 8 clone K1-40/Bc1 K1 protein (K1) gene, c	1591	1591	95%	0.0	99%
AY204645.1	Human herpesvirus 8 isolate K1/E5 K1 glycoprotein gene, comp	1568	1568	95%	0.0	99%
FJ884608.1	Human herpesvirus 8 isolate GR-77 K1 glycoprotein gene, com	1565	1565	99%	0.0	98%
AY204660.1	Human herpesvirus 8 isolate K1/E17 K1 glycoprotein gene, con	1563	1563	95%	0.0	99%
AF130305.1	Kaposi's sarcoma-associated herpesvirus isolate Ema7 K1 glyc	1546	1546	95%	0.0	99%

Figura 4 - Continuação

(Continua na próxima página)

pKSHVOK1-3.01

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF170531.1	Kaposi's sarcoma-associated herpesvirus K1 glycoprotein gene	1570	1570	95%	0.0	99%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1528	1528	100%	0.0	97%
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1489	1489	100%	0.0	97%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1489	1489	100%	0.0	97%
AF130300.1	Kaposi's sarcoma-associated herpesvirus isolate UKma1 K1 gly	1471	1471	95%	0.0	98%
AF130274.1	Kaposi's sarcoma-associated herpesvirus isolate Icam1 K1 gly	1454	1454	95%	0.0	97%
AF178774.1	Human herpesvirus 8 clone K1-2/691 K1 protein (K1) gene, co	1448	1448	95%	0.0	97%
AF178816.1	Human herpesvirus 8 clone K1-50/Pbm K1 protein (K1) gene, c	1443	1443	95%	0.0	97%
DQ394060.1	Human herpesvirus 8 isolate I7 K1 glycoprotein (K1) gene, cor	1439	1439	94%	0.0	97%
DQ394051.1	Human herpesvirus 8 isolate D15 K1 glycoprotein (K1) gene, c	1439	1439	94%	0.0	97%

pKSHVOK1-3.03

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF170531.1	Kaposi's sarcoma-associated herpesvirus K1 glycoprotein gene	1574	1574	95%	0.0	99%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1550	1550	100%	0.0	98%
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1511	1511	100%	0.0	97%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1511	1511	100%	0.0	97%
AF130300.1	Kaposi's sarcoma-associated herpesvirus isolate UKma1 K1 gly	1474	1474	95%	0.0	98%
AF130274.1	Kaposi's sarcoma-associated herpesvirus isolate Icam1 K1 gly	1458	1458	95%	0.0	97%
AF178774.1	Human herpesvirus 8 clone K1-2/691 K1 protein (K1) gene, co	1452	1452	95%	0.0	97%
AF278849.1	Human herpesvirus 8 strain J5 K1 glycoprotein (K1) mRNA, par	1448	1448	93%	0.0	98%
AF178816.1	Human herpesvirus 8 clone K1-50/Pbm K1 protein (K1) gene, c	1447	1447	95%	0.0	97%
DQ394060.1	Human herpesvirus 8 isolate I7 K1 glycoprotein (K1) gene, cor	1443	1443	95%	0.0	97%

pKSHVOK1-3.06

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF170531.1	Kaposi's sarcoma-associated herpesvirus K1 glycoprotein gene	1580	1580	95%	0.0	100%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1555	1555	100%	0.0	98%
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1517	1517	100%	0.0	97%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1517	1517	100%	0.0	97%
AF130300.1	Kaposi's sarcoma-associated herpesvirus isolate UKma1 K1 gly	1480	1480	95%	0.0	98%
AF130274.1	Kaposi's sarcoma-associated herpesvirus isolate Icam1 K1 gly	1463	1463	95%	0.0	98%
AF178774.1	Human herpesvirus 8 clone K1-2/691 K1 protein (K1) gene, co	1458	1458	95%	0.0	97%
AF278849.1	Human herpesvirus 8 strain J5 K1 glycoprotein (K1) mRNA, par	1454	1454	93%	0.0	98%
AF178816.1	Human herpesvirus 8 clone K1-50/Pbm K1 protein (K1) gene, c	1452	1452	95%	0.0	97%
DQ394060.1	Human herpesvirus 8 isolate I7 K1 glycoprotein (K1) gene, cor	1448	1448	95%	0.0	97%

pKSHVOK1-4.02

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1633	1633	100%	0.0	99%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1572	1572	100%	0.0	98%
AF130302.1	Kaposi's sarcoma-associated herpesvirus isolate UKma3 K1 gly	1557	1557	95%	0.0	99%
AF178809.1	Human herpesvirus 8 clone K1-42/Tin K1 protein (K1) gene, co	1552	1552	95%	0.0	99%
AF178773.1	Human herpesvirus 8 clone K1-1/663 K1 protein (K1) gene, co	1552	1552	95%	0.0	99%
AF196317.1	Kaposi's sarcoma-associated herpesvirus strain K1-73/Naf K1	1546	1546	95%	0.0	99%
AF178826.1	Human herpesvirus 8 clone K1-61/Fa2 K1 protein (K1) gene, c	1546	1546	95%	0.0	99%
AF178832.1	Human herpesvirus 8 clone K1-69/Fain3 K1 protein (K1) gene,	1546	1546	95%	0.0	99%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1544	1544	100%	0.0	98%
DQ394049.1	Human herpesvirus 8 isolate D14-1 K1 glycoprotein (K1) gene,	1543	1543	95%	0.0	99%

pKSHVOK1-4.03

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1655	1655	100%	0.0	100%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1594	1594	100%	0.0	99%
AF130302.1	Kaposi's sarcoma-associated herpesvirus isolate UKma3 K1 gly	1580	1580	95%	0.0	100%
AF178809.1	Human herpesvirus 8 clone K1-42/Tin K1 protein (K1) gene, co	1574	1574	95%	0.0	99%
AF178773.1	Human herpesvirus 8 clone K1-1/663 K1 protein (K1) gene, co	1574	1574	95%	0.0	99%
AF196317.1	Kaposi's sarcoma-associated herpesvirus strain K1-73/Naf K1	1568	1568	95%	0.0	99%
AF178826.1	Human herpesvirus 8 clone K1-61/Fa2 K1 protein (K1) gene, c	1568	1568	95%	0.0	99%
AF178832.1	Human herpesvirus 8 clone K1-69/Fain3 K1 protein (K1) gene,	1568	1568	95%	0.0	99%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1567	1567	100%	0.0	98%
DQ394049.1	Human herpesvirus 8 isolate D14-1 K1 glycoprotein (K1) gene,	1565	1565	95%	0.0	99%

pKSHVOK1-4.05

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ994935.1	Human herpesvirus 8 strain JSC-1 clone BAC16, complete gen	1655	1655	100%	0.0	100%
AF133042.1	Kaposi's sarcoma-associated herpesvirus strain BC2 transform	1594	1594	100%	0.0	99%
AF130302.1	Kaposi's sarcoma-associated herpesvirus isolate UKma3 K1 gly	1580	1580	95%	0.0	100%
AF178809.1	Human herpesvirus 8 clone K1-42/Tin K1 protein (K1) gene, co	1574	1574	95%	0.0	99%
AF178773.1	Human herpesvirus 8 clone K1-1/663 K1 protein (K1) gene, co	1574	1574	95%	0.0	99%
AF196317.1	Kaposi's sarcoma-associated herpesvirus strain K1-73/Naf K1	1568	1568	95%	0.0	99%
AF178826.1	Human herpesvirus 8 clone K1-61/Fa2 K1 protein (K1) gene, c	1568	1568	95%	0.0	99%
AF178832.1	Human herpesvirus 8 clone K1-69/Fain3 K1 protein (K1) gene,	1568	1568	95%	0.0	99%
U93872.2	Kaposi's sarcoma-associated herpesvirus glycoprotein M, DNA	1567	1567	100%	0.0	98%
DQ394049.1	Human herpesvirus 8 isolate D14-1 K1 glycoprotein (K1) gene,	1565	1565	95%	0.0	99%

Figura 4 – Continuação (Continua na próxima página)

pKSHVOK1-5.03

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transf	1600	1600	100%	0.0	98%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 gl	1557	1557	95%	0.0	99%
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, co	1557	1557	95%	0.0	99%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, com	1550	1550	100%	0.0	97%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, c	1541	1541	95%	0.0	99%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete seq	1528	1528	95%	0.0	98%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, c	1513	1513	95%	0.0	98%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotei	1511	1511	93%	0.0	99%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, c	1507	1507	95%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotei	1489	1489	93%	0.0	98%

pKSHVOK1-5.04

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transformir	1616	1616	100%	0.0	99%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 glycop	1574	1574	95%	0.0	99%
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, comple	1574	1574	95%	0.0	99%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, complete	1567	1567	100%	0.0	98%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, compl	1557	1557	95%	0.0	99%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete sequenc	1544	1544	95%	0.0	99%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, compl	1530	1530	95%	0.0	98%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotein (K	1528	1528	93%	0.0	99%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, compl	1524	1524	95%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotein (K	1506	1506	93%	0.0	98%

pKSHVOK1-5.05

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transformir	1602	1602	100%	0.0	98%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 glycop	1574	1574	95%	0.0	99%
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, comple	1574	1574	95%	0.0	99%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, compl	1557	1557	95%	0.0	99%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, complete	1552	1552	100%	0.0	97%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete sequenc	1544	1544	95%	0.0	99%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, compl	1530	1530	95%	0.0	98%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotein (K	1528	1528	93%	0.0	99%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, compl	1524	1524	95%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotein (K	1506	1506	93%	0.0	98%

pKSHVOK1-6.02

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, co	1567	1567	94%	0.0	99%
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transf	1567	1567	100%	0.0	98%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 gl	1555	1555	94%	0.0	99%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, c	1539	1539	94%	0.0	99%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete seq	1528	1528	94%	0.0	98%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, com	1517	1517	100%	0.0	97%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotei	1515	1515	93%	0.0	99%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, c	1511	1511	94%	0.0	98%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, c	1506	1506	94%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotei	1493	1493	93%	0.0	98%

pKSHVOK1-6.03

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transf	1596	1596	100%	0.0	98%
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, co	1565	1565	95%	0.0	99%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 gl	1554	1554	95%	0.0	99%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, com	1546	1546	100%	0.0	97%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, c	1537	1537	95%	0.0	99%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete seq	1524	1524	95%	0.0	98%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotei	1520	1520	93%	0.0	99%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, c	1509	1509	93%	0.0	99%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, c	1504	1504	95%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotei	1498	1498	93%	0.0	98%

pKSHVOK1-6.04

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF133040.1	Kaposi's sarcoma-associated herpesvirus strain 431KAP transf	1611	1611	100%	0.0	99%
AF178822.1	Human herpesvirus 8 clone K1-57/Eti K1 protein (K1) gene, co	1580	1580	95%	0.0	99%
AF130301.1	Kaposi's sarcoma-associated herpesvirus isolate UKma24 K1 gl	1568	1568	95%	0.0	99%
FJ884618.1	Human herpesvirus 8 isolate UG-65 K1 glycoprotein gene, com	1561	1561	100%	0.0	98%
AF178801.1	Human herpesvirus 8 clone K1-34/E40 K1 protein (K1) gene, c	1552	1552	95%	0.0	99%
AF178783.1	Human herpesvirus 8 clone K1-12/Mou K1 gene, complete seq	1539	1539	95%	0.0	99%
AY042948.1	Human herpesvirus 8 strain Ugd10 from Uganda K1 glycoprotei	1528	1528	93%	0.0	99%
AF178788.1	Human herpesvirus 8 clone K1-19/Edm K1 protein (K1) gene, c	1524	1524	95%	0.0	98%
AF178791.1	Human herpesvirus 8 clone K1-22/Yan K1 protein (K1) gene, c	1519	1519	95%	0.0	98%
AY042949.1	Human herpesvirus 8 strain Ugd29 from Uganda K1 glycoprotei	1506	1506	93%	0.0	98%

Figura 4 - Continuação

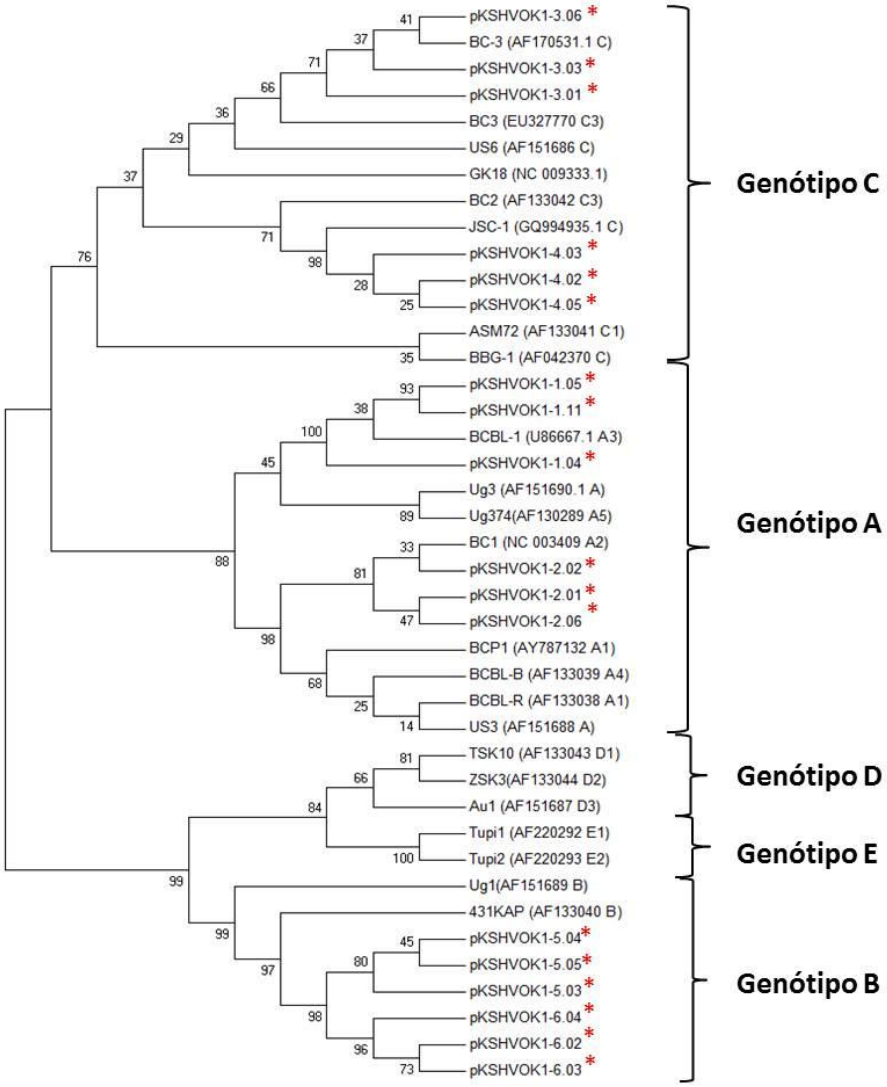
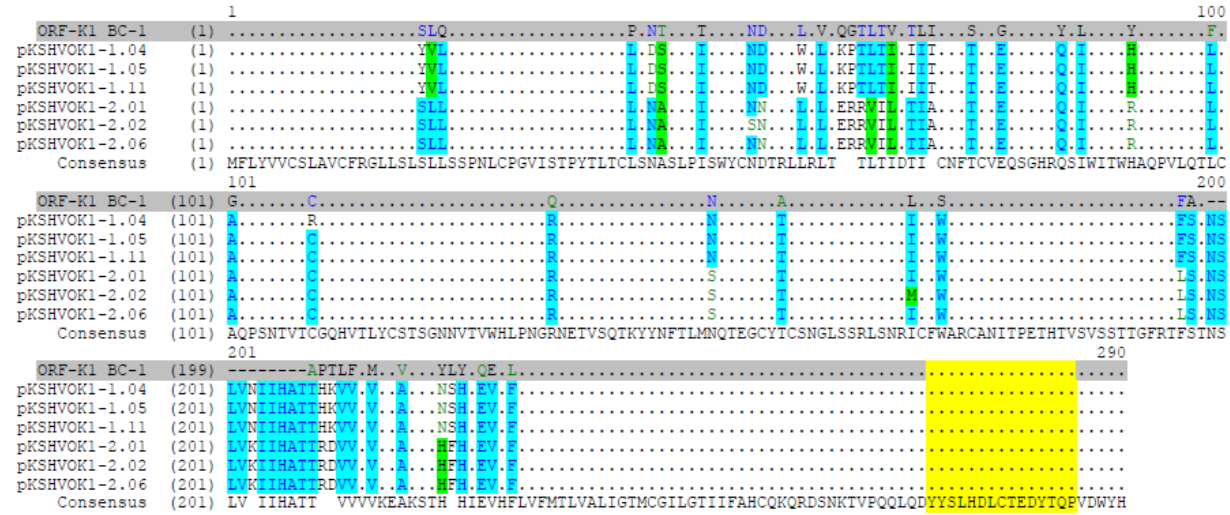


Figura 5 - Análise filogenética das sequências da ORF-K1 do KSHV obtidas dos vetores recombinantes pKSHVOK1-1, pKSHVOK1-2, pKSHVOK1-3, pKSHVOK1-4, pKSHVOK1-5 e pKSHVOK1-6 (destacadas com *). Tais amostras foram comparadas com sequências-protótipo da ORF-K1 viral depositadas no banco de dados *NCBI*. Entre parênteses o código de acesso (*Accession #*) do *Genbank* de cada sequência e, a frente do código, o genótipo a que pertencem cada uma das linhagens celulares. A filogenia confirma que ORF-K1 dos vetores recombinantes pKSHVOK1-1 e pKSHVOK1-2 pertencem ao genótipo A do KSHV, que pKSHVOK1-3 e pKSHVOK1-4 pertencem ao genótipo C do KSHV, e pKSHVOK1-5 e pKSHVOK1-6, ao genótipo B.

As sequências de nucleotídeos dos clones recombinantes foram convertidas em sequências de aminoácidos e alinhadas com as de sequências-protótipo da ORF-K1 de genótipos A, B e C do KSHV para considerações acerca da sequência proteica de K1. Os clones pKSHVOK1 de genótipos A e C apresentaram alta similaridade com suas respectivas sequências-protótipo de K1, revelando ITAM idêntico. Os clones de genótipo B viral revelaram diferença de quatro aminoácidos em relação ao ITAM de K1 dos genótipos A e C (Figura 6).

Genótipo A



Genótipo C

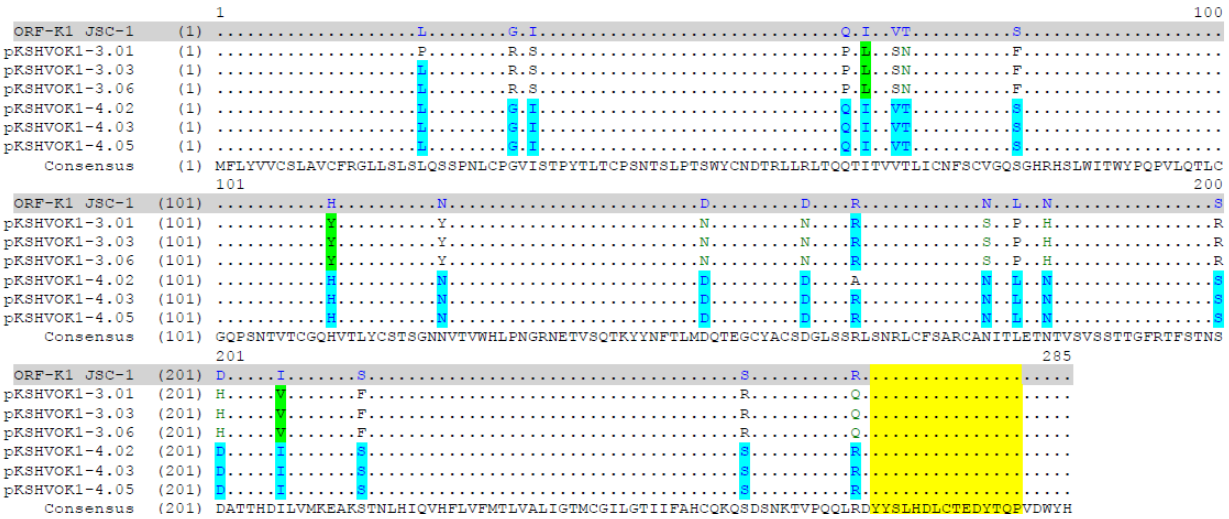


Figura 6 - Alinhamento das sequências de aminoácidos de K1 expressas pelos vetores recombinantes de genótipos B (pKSHVOK1-5 e pKSHVOK1-6), A (pKSHVOK1-1 e pKSHVOK1-2) e C (pKSHVOK1-3 e pKSHVOK1-4), que apresentam alta similaridade com as sequências de K1 dos protótipos 431KAP, BC-1 e JSC-1, respectivamente. Em destaque cinza, sequências-protótipo de K1. A região do domínio ITAM de K1 está destacada em amarelo. Em destaque vermelho, sequência de quatro aminoácidos do domínio ITAM de K1 pertencente ao genótipo B do KSHV, que difere dos demais genótipos.

Genótipo B

Figura 6 - Continuação.

Do mesmo modo, o alinhamento da cadeia polipeptídica de sequências-protótipo de K1 corroborou a equivalência do ITAM de K1 entre os genótipos A e C e sua divergência em quatro aminoácidos em relação ao ITAM de genótipo viral B (Figura 7).

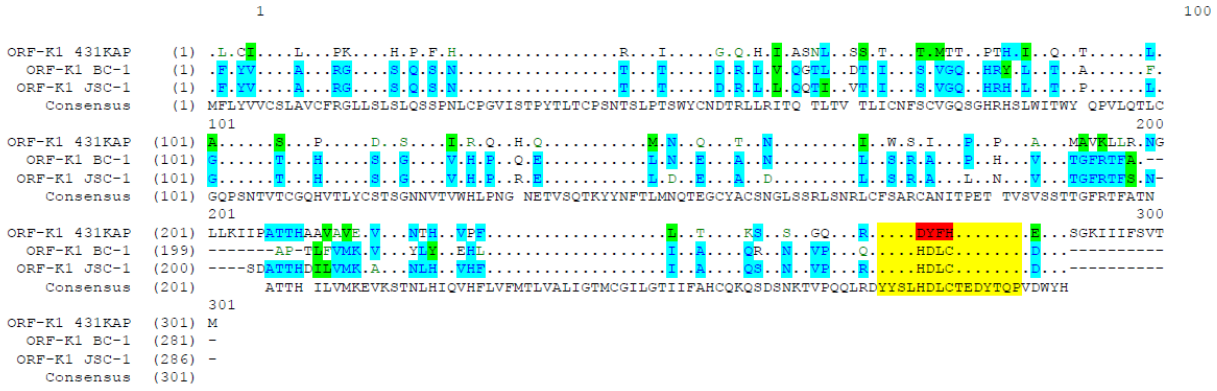


Figura 7 - Alinhamento de sequências de aminoácidos provenientes de sequências-protótipo de K1 de genótipos A (*Genbank Accession* # NC_003409.1, *HHV-8 type M, complete genome,* linhagem celular BC-1), B (*Genbank Accession* # AF133040.1, *KSHV strain 431KAP transforming membrane receptor-like protein (K1-B) gene, complete cds*, linhagem celular 431KAP) e C (*GenBank Accession#* GQ994935.1 - *Human herpesvirus 8 strain JSC-1 clone BAC16, complete genome*, linhagem celular JSC-1). Em destaque amarelo, a sequência do domínio ITAM de K1, idêntica nas sequências-protótipo de genótipo A e C. Em destaque vermelho, região de ITAM de sequência-protótipo de K1 do genótipo B, que se diferencia dos demais genótipos.

Com intuito de avaliar a expressão da proteína K1 a partir dos vetores recombinantes produzidos contendo a ORF-K1 do KSHV de diferentes genótipos, células HEK293 foram transfectadas e a expressão proteica foi avaliada por *Western Blot* (Figura 8). A expressão da proteína K1 fusionada ao epítopo FLAG foi verificada em células HEK 293 estavelmente transfectadas com os vetores pKSHVOK1-1.05 e pKSHVOK1-2.02 (genótipo A), e pKSHVOK1-5.04 e pKSHVOK1-6.02 (genótipo B). Contudo, células HEK 293 transfectadas com os vetores pKSHVOK1-3.06 e pKSHVOK1-4.03 (genótipo C) não apresentaram expressão de K1 em níveis detectáveis nesse experimento (Figura 8 painel C).

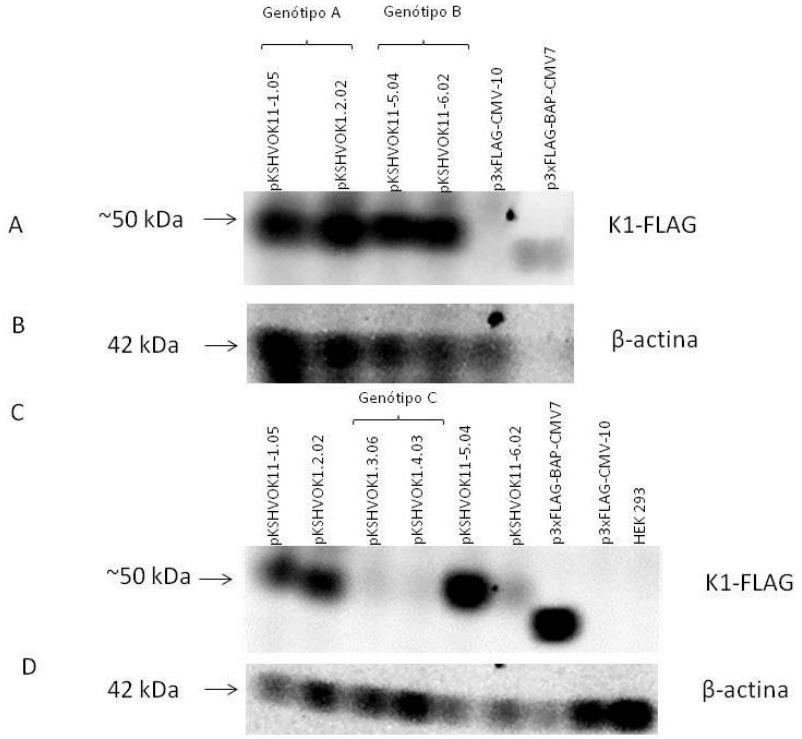


Figura 8 - Expressão de K1 do KSHV em células HEK293 estavelmente transfectadas (**A**) e (**C**) Detecção da expressão de K1 do KSHV acoplada ao epítopo FLAG em membrana de PVDF com 100 µg de proteínas totais (exceto p3xFLAG-BAP-CMV7, com 10 µg). A proteína K1 acoplada a FLAG tem tamanho aproximado de 49,3kDa. Proteína BAP ligada a FLAG é controle positivo para FLAG, com tamanho variável entre 45-55kDa. (**B**) e (**D**) Detecção de β-actina (PVDF com 100µg de proteínas totais, exceto p3xFLAG-BAP-CMV7, com 10µg). A expressão da proteína β-actina (42kDa) foi usado como controle do experimento de western blot. Para esses experimentos as proteínas foram extraídas de células HEK 293 *in vitro* estavelmente transfectadas com vetores pKSHVOK1-1.05, pKSHVOK1-2.02, pKSHVOK1-3.06, pKSHVOK1-4.03 pKSHVOK1-5.04, pKSHVOK1-6.02 e p3xFLAG-CMV-10 (exceto p3xFLAG-BAP-CMV7, para o qual efetuou-se transfecção transiente). Lisado de células HEK 293 não-transfectadas foi empregado como controle negativo de K1.

DISCUSSÃO

A proteína K1 do KSHV pode ativar vias de sinalização importantes por meio de seu ITAM, com efeitos potencialmente relevantes na patogênese do SK (Heuiran Lee et al., 1998). A alta variabilidade das regiões V1 e V2 de K1 e diferenças notadas nas sequências codificadoras do ITAM de diferentes genótipos virais tornam plausível supor eventuais diferenças nas propriedades biológicas de K1. É nessa perspectiva que se insere o presente estudo, no qual foram construídos e validados vetores recombinantes da ORF K1 viral, objetivando seu uso oportuno para investigação das propriedades da proteína codificada por diferentes genótipos do KSHV.

Antigas migrações humanas possivelmente condicionaram a distribuição geográfica dos genótipos do KSHV. Estima-se que primeira onda de migração tenha ocorrido há cerca de 100.000 anos atrás na África subsaariana, possivelmente originando o genótipo B viral. A segunda onda pode ter originado o genótipo D e deve ter ocorrido há 60.000-70.000 anos, seguindo em direção ao sul da Ásia e, por fim, alcançando a Austrália, Taiwan e Ilhas do Pacífico. A terceira onda de migração, com inicio há cerca de 35.000 anos atrás, originou os genótipos A e C, tendo se ramificado para a Europa e norte da Ásia, com rápida expansão para as Américas e norte da Europa no final da Era Glacial (Zong et al., 2002). De fato, dados filogenéticos baseados na ORF-K1 viral (Figura 5) indicam que os genótipos C e A provavelmente são os mais recentes, seguidos dos genótipos D e E e tendo o genótipo viral B como o mais antigo.

K1 é uma proteína significativamente codificada na fase lítica do ciclo biológico do KSHV (Lagunoff and Ganem, 1997). Em sua porção citoplasmática, K1 contém dois motivos de ativação em seu ITAM. A presença do ITAM é uma das características mais relevantes de K1, posto que permite interferência de K1 em vias de sinalização intracelulares. A ativação do ITAM ocorre quando seus dois motivos de ativação (com tirosinas fosforiladas) recrutam syk-quinases específicas (e.g., lyn, syk, p85α, PLCγ2, RasGap, vav SH-PTP1/2, GRB2), as quais interagem com o ITAM por meio de seus domínios SH2 (*Src homology 2*), propiciando fosforilação das mesmas (Lee et al., 2005). Uma vez fosforiladas, essas enzimas ativam outras proteínas envolvidas em muitas vias de sinalização, tais como PI3K/Akt, envolvida no aumento da sobrevivência celular e inibição da apoptose (Tomlinson and Damania, 2004).

As sequências do ITAM dos vetores produzidos de genótipos A e C (Figura 6) e as das respectivas sequências-protótipo depositadas são essencialmente equivalentes, sugerindo compartilhamento de propriedades biológicas. Porém, sequências de K1 de genótipo B viral apresentam variação em quatro aminoácidos em seu ITAM, tanto nos vetores produzidos como em sequências-protótipo depositadas de K1 de genótipo B (Figura 7). Ainda não está definido se essa peculiaridade modifica a sinalização intracelular ITAM-dependente na célula em que a K1 de genótipos B do KSHV é expressa. Assim, os vetores produzidos no presente trabalho se prestam a contribuir na investigação experimental dessa hipótese.

Além de suas atividades dependentes de ITAM, K1 pode interagir com a cadeia μ do BCR por meio de sua a porção N-terminal, o que inibe o transporte intracelular e diminui a expressão do BCR na superfície da célula (Lee et al., 2000). O comprometimento do BCR por K1 ocasiona bloqueio de ativação de linfócitos B, promovendo a sobrevivência de células infectadas pelo vírus e possibilitando um aumento do repertório de células latentemente infectadas. Adicionalmente, é relatado que K1 interage com Fas por meio de seu domínio semelhante à imunoglobulina, tornando-o inacessível para ligação com FasL, de modo a comprometer a deflagração da apoptose pela via extrínseca (Wang et al., 2007).

No presente trabalho, células HEK293 transfectadas com os vetores recombinantes produzidos foram selecionadas e analisadas quanto à expressão de K1 fusionada ao epítopo FLAG (Figura 8). A expressão da proteína viral foi confirmada nos vetores recombinantes de genótipos A e B. Entretanto, vetores produzidos de genótipo C não apresentaram expressão de K1 em níveis detectáveis, embora tenham sido validados estruturalmente. Tal resultado pode decorrer de problemas na construção dos vetores, ou características peculiares a esse genótipo viral.

Em síntese, o presente trabalho apresentou a produção e validação de vetores recombinantes de ORF-K1 do KSHV de genótipos A e B. Vetores do genótipo C foram produzidos, mas não revelaram expressão suficiente de K1 em células HEK293 transfectadas. Os vetores produzidos e completamente validados são um recurso importante para estudos sobre a proteína K1 codificada por diferentes genótipos do KSHV. Assim, serão utilizados em futuros trabalhos do grupo de pesquisa e estão disponíveis aos demais interessados em contribuir na investigação das propriedades biológicas dessa proteína viral.

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Anexos

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Ética Comitê de Ética em Pesquisa

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Ilustríssimo Senhor
Prof. Deilson Elgui de Oliveira
Departamento de Patologia da
Faculdade de Medicina de Botucatu- São Paulo.

Caro Prof. Deilson,

Em relação ao Projeto de Pesquisa **(Protocolo 3529-2010) "Efeitos da proteína K1 de diferentes genótipos do herpesvírus associado ao sarcoma de Kaposi/herpervírus humano tipo 8 na expressão gênica in vitro de células endolteliais humanas"** informo que foi **AUTORIZADA** a inclusão do sub-projeto a seguir:

Sub-Projeto I: "Produção de vetores recombinantes para análise das propriedades biológicas e cancerígenas da proteína K1 do herpesvírus associado ao sarcoma de Kaposi (KSHV/HHV-8)"

Autor: Annie Cristhine Moraes Sousa Squiavinato

Orientador: Prof. Dr. Deilson Elgui de Oliveira

Ao Final da execução dos estudos apresentar ao CEP **"Relatório Final de Atividades"**

Atenciosamente,

Prof. Dr. Trajano Sardenberg
Coordenador do CEP