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AIDS AND ENDEMIC KAPOSI'S SARCOMA DEVELOPMENT: COMPARISON BY HISTOPATHOLOGY, VIROLOGY (HHV-8/KSHV) AND CYTOGENETICS

By

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ABSTRACT

Kaposi's sarcoma (KS) is a highly and abnormally vascularized tumor-like lesion with spindle cells (SC) affecting the skin, lymphnodes and viscera which is found in four different clinico-epidemiological forms as Classic KS (CKS), Iatrogenic KS (IKS), Endemic KS (EKS) and AIDS-associated KS (AKS). All KS forms develop from early stages of patch/plaque to late, nodular tumors and are associated with Kaposi's sarcoma-associated herpesvirus or human herpesvirus-8 (KSHV/ HHV-8). HHV-8 is also associated with primary effusion lymphoma (PEL) and multicentric castleman's disease (MCD). Various studies favour an endothelial origin (CD34+) of the KS SC but whether of vascular (VEC) or lymphatic endothelial cell (LEC) origin has not been settled. The HHV-8 latency-associated nuclear antigen type 1 (LANA-1) protein is the most frequently expressed viral antigen in infected cells. KS is promoted by HIV infection mainly by the angiogenic and pro-inflammatory effects of HIV-Tat. Whether KS represents a predominantly monoclonal neoplastic cell proliferation or a hyperplastic, reactive polyclonal process is still controversial. Reports on cytogenetic and molecular genetic changes in KS are few indicating that initially KS may develop as a reactive polyclonal cell proliferation associated with chromosome instability, followed by clonal chromosome changes in later stages.

In the present KS histopathological studies (paper I & V) by triple antibody immunofluorescence we observed that: (a) the frequency of LANA+/CD34+ cells increased from early patch to late KS nodular lesions; (b) 30-40% of the CD34+ SC were LANA- in both early and late KS, suggesting a continuous recruitment of non-infected endothelial cell into the KS lesion; (c) LANA+/CD34- cells were more frequent in early as compared to late KS and most of them expressed LEC markers such as LYVE-1 and D2-40, suggesting that the resident LECs represent an early target of primary HHV-8 infection; (d) LANA+/CD34-/LYVE-1+ cells decreased from early (25%) to late (4%) KS suggesting a phenotype switch from LEC to VEC; (e) the frequency of proliferating cells ($Ki67^+$) was higher in early as compared to late KS stages and no significant difference in cell proliferation was observed between nodular AKS and EKS, suggesting that the growth of the usually more aggressive AKS tumors may reflect a higher rate of SC progenitor recruitment as compared to the slower growing EKS lesions, consistent with the observed increase in non-proliferative SC during KS (AKS) evolution to nodular stage; (f) infiltrating lymphocytes were LANA negative, whereas some CD68+ monocyte-macrophase appeared to be $LANA^+$.

To validate our results from LANA immunostaining, we established and optimized a semi-quantitative PCR assay for HHV-8 detection in formalin-fixed paraffin-embedded KS biopsies (paper III) and two different protocols for DNA extraction were compared namely the Chelex 100 and Qia-gene kit method. Our result indicate a better performance for Chelex-extracted DNA in paraffin embedded archival biopsies. In late, nodular stage of both AKS and EKS the virus load per unit tissue actin (HHV-8/actin) is higher than in early stages (patch/plaque), which corroborates our findings from double immunostaining for LANA and CD34 of the same cases. Thus these PCR results by serial dilutions of HHV-8 DNA show a correlation between virus load and progressive stages of KS development i.e. the increase in LANA+ SC and does not indicate an increase in viral content of individual tumor cells.

With quantitative real time PCR on sera (paper II), we found higher HHV8 DNA load in AKS compared to EKS, patch compared to nodular KS and males compared to females as well as a significantly higher serum viral DNA load in KS compared to non-KS patients' sera.

AKS patient sera studied by ELISA for HIV-Tat antibodies showed that patients with high HHV8-DNA level had no or low levels of anti-Tat antibodies while patients with very low HHV8-DNA levels had several fold higher anti-Tat IgG titers. Analysis of these KS sera for epitope specificity showed reactivity to various Tat epitopes but not to the transcriptional (functional) epitopes 46-60 (TAR-binding region).

To determine cytogenetic changes during the development of KS as well as possible differences between AKS and EKS we studied 27 KS (10 nodular AKS, 8 patch AKS, 8 nodular EKS and 1 patch EKS) cases by laser microdissection, global amplification of DNA and comparative genomic hybridization (paper IV). Deletion of Chromosome Y was detected in 20 of 23 male KS and was the only chromosomal deletion observed in early (patch) KS biopsies. Late AKS and EKS apart from random aberrations also showed recurrent chromosomal deletions of chromosome 16, 17, Y and a gain of chromosome 21. The deletion of chromosome 16 and Y was confirmed by interphase FISH on paraffin embedded sections. EKS had higher number of chromosomal abnormalities than AKS.

In summary KS SC apparently represents a mixed pool endothelial cells including cells from both VEC and LEC the later being a possible early target for HHV-8 infection. Non-infected CD34+ progenitor cells appears to be continuously recruited to the developing KS lesion and locally infected during the development of KS. Serum HHV-8 DNA load is higher in AKS compared to EKS and HIV-Tat titers were inversely correlated to HHV-8 DNA load in AKS patients. Increased number of recurrent and sporadic chromosomal abnormalities found mostly in a subset of late nodular KS cases may indicate the onset of a clonal cell population (sarcoma).

LIST OF PUBLICATIONS

I. **Pawan Pyakurel**, Charles Massambu, Esmeralda Castaños-Vélez, Susanna Ericsson, Ephata Kaaya, Peter Biberfeld, Thomas Heiden: KSHV/HHV-8 cell association during evolution of Kaposi's sarcoma . J Acquir Immune Defic Syndr. 2004 Jun 1;36(2):678-683

II. Charles Massambu, **Pawan Pyakurel**, Ephata Kaaya, Malin Enbom, Willy Urassa, Ilhan Demirhan, Johannes Loewer, Annika Linde, Angelika Chandra, Thomas Heiden, Hans W. Doerr, Prakash Chandra, Peter Biberfeld: Serum HHV-8 DNA and Tat antibodies in Kaposi's sarcoma patients with or without HIV-1 infection. Anticancer Res. 2003; 23: 2389-2396.

III. Fatemeh Pak, **Pawan Pyakural**, Parviz Kokhaei, Ephata Kaaya, Ali Akbar Pourfathollah, Galina Selivanova, Peter Biberfeld. HHV-8/KSHV during development of Kaposi's sarcoma: evaluation by PCR and immunohistochemistry. J Cutan Pathol. 2005 Jan;32(1):21-7.

IV. **Pawan Pyakurel**, Ulrike Montag, Esmeralda Castaños-Vélez, Ephata Kaaya, Birger Christensson, Peter Biberfeld, Evelin Schröck, Thomas Heiden. Kaposi's sarcoma: CGH cytogenetic analysis of microdissected early and late stage biopsies. Manuscript

V. **Pawan Pyakurel**, Fatemeh Pak, Amos. R. Mwakigonja, Ephata Kaaya, Thomas Heiden, Peter Biberfeld. Recruitment of Kaposi's sarcoma spindle cells during tumor development. Manuscript

LIST OF ABBREVIATIONS

AKS	AIDS associated Kaposi's sarcoma
BCBL	Body cavity based lymphoma (also called PEL)
bFGF	Fibroblast growth factor- β
CDK	Cyclin dependent kinases
CGH	Comparative genomic hybridization
CKS	Classic Kaposi's sarcoma
E2F	DNA binding transcription factor
EBV	Epstein-Barr virus
EKS	Endemic Kaposi's sarcoma
FISH	Fluorescence in situ hybridization
hCG	Human chorionic gonadotropin
HDMEC	Human dermal microvascular endothelial cell
HHV-8	Human herpesvirus type 8 (also called KSHV)
IKS	Iatrogenic Kaposi's sarcoma
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus (also called HHV8)
LANA	Latency associated nuclear antigen (also LNA-1)
LEC	Lymphatic endothelial cell
MCD	Multicentric castleman's disease
MHC	Major histocompatibility complex
MIR	Modulator of immune recognition
NFkB	Nuclear factor kappa B
NK cell	Natural killer cell
ORF	Open reading frame
p53	Tumor suppressor gene
PEL	Primary effusion lymphoma (also called BCBL)
pRb	Retinoblastoma protein or gene
SC	Spindle cell
TAR	Transactivating responsive region (HIV-1 Tat binding region)
Tat	Transactivator of transcription
TNF	Tumor necrosis factor
VEC	Vascular endothelial cell
VEGFR	Vascular endothelial growth factor receptor
v-FLIP	Viral FLICE inhibitory protein (FLICE= FADD-like interleukin converting enzyme; FADD= Fas associated death domain)
v-GCR	Viral G-protein coupled receptor
v-IL-6	Viral interleukin 6
v-IRF	Viral interferon regulatory factor
v-MIP	Viral macrophage inflammatory protein

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1. BACKGROUND

1.1 KAPOSI'S SARCOMA

Kaposi's sarcoma first described by Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin" (Kaposi, 1872) is an angioproliferative, tumour-like lesion most often first developing in the skin (Biberfeld et al., 1998), and if untreated patients develop disseminated disease with multiple cutaneous, visceral and lymph node tumor lesions. Previously a rare disease, it has become an increasing global health care and clinical problem because of its association with the HIV pandemic *(Amir et al., 2001; Ensoli and Cafaro, 1996)* and other immunosuppressed states (Boshoff and Chang, 2001; Lessan-Pezeshki et al., 2001; Schulz, 1999).

Four clinical different KS forms are now recognized (Biberfeld et al., 2002) as:

1) Acquired immunodeficiency syndrome (AIDS)-associated KS (AKS): The most frequent tumor of human immunodeficiency virus type I (HIV-l) infected homo–bisexual men and women and the most aggressive and rapidly growing form of KS, with early dissemination in the skin and viscera.

2) Classical or sporadic KS (CKS): Originally described (Kaposi, 1872) as a slow growing, indolent tumor mostly developing in the extremities of elderly males of eastern and Mediterranean Europe.

3) Endemic KS (EKS): Predominant in eastern and central Africa before the AIDS epidemic (Boshoff and Weiss, 2001) and clinically similar to CKS, but also seen in children in a more fulminant and fatal form. The childhood EKS is mostly lymphoglandular with or without skin involvement.

4) Iatrogenic KS (IKS): seen in immunosuppressed patients, e.g. transplant patients receiving immunosuppressive drugs, emphasizing the importance of immune disturbance as a co-factor in the pathogenesis of IKS and AKS, and possibly also EKS (Biberfeld et al., 2002).

In spite of the clear clinical differences the histopathology of the various KS forms is essentially the same, with characteristic changes related to stage in the development of the KS tumor (Kaaya et al., 1992).

The epidemiology of AKS led to the discovery of a novel herpes virus (Chang et al., 1994), which was shown to be associated with all clinico-epidemiological forms of KS (Schalling

et al., 1995). The virus was first called Kaposi's sarcoma associated herpes virus (KSHV) and further identified as human herpes virus type 8 (HHV-8) (Moore et al., 1996). HHV-8 was found to be also associated with some rare types of lymphomas in AIDS patients, namely primary effusion lymphoma or body-cavity-based-lymphoma (PEL/BCBL) and Castleman's disease (Biberfeld P, 2002; Gaidano et al., 1999).

1.1.1 Histogenesis of KS

The histopathology of KS is characterized by an early infiltration of mononuclear inflammatory cells, formation of small, irregular, endothelial lined slits around new blood vessels (angiogenesis) and extravasation of erythrocytes (Biberfeld et al., 1998) with accumulation of hemosiderin pigments. KS at early stages reflects a predominantly reactive cell proliferation of polyclonal nature that can regress, but may progress to a nodular possibly clonal tumor (Biberfeld et al., 1998; Kondo et al., 2000). Pathognomonic for the KS development from early patch/plaque to late nodular (Fig 1 a & b) tumor lesions is the increased appearance of so-called spindle cells (SC) (Fig 1c) (Enzinger, 2001) expressing CD34 (hematopoietic stem cell and vascular endothelial marker) (paper I). At the nodular KS stage there is less inflammatory cell infiltration, mostly seen at the periphery of the dense nodular accumulation of SC. These nodular KS skin lesions later may ulcerate. Unlike typical metastatic cancers, KS often appears early as a multicentric tumour, with each lesion arising de novo by a localized development and proliferation of SC (Ensoli et al., 2001). These features and our previous findings indicating heterogeneity of the spindle cell compartment and lack of aneuploidy (Kaaya et al., 2000; Kaaya et al., 1996; Kaaya et al., 1995; Kaaya et al., 1992) favour a non clonal growth of the KS lesion.

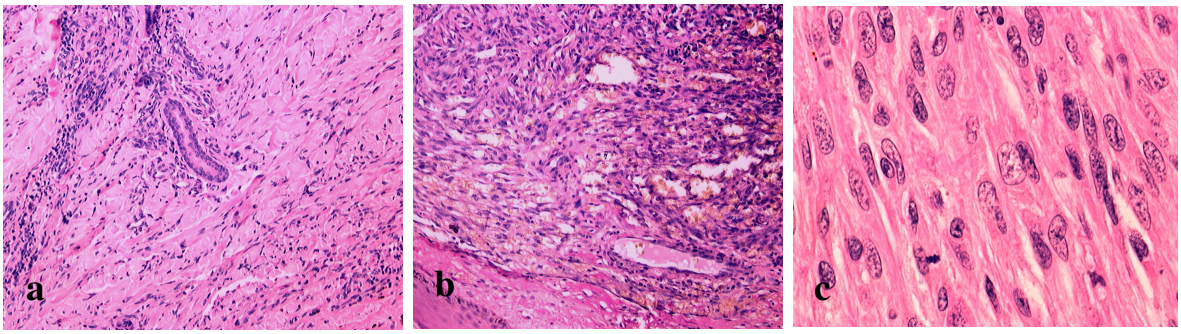

Fig 1: Histology of KS (a) Patch KS (b) nodular KS (c) KS spindle cells

Cytologically characteristic spindle cells (SC) expresses various "mixed" (LEC and VEC) endothelial phenotypic cell markers possibly representing heterogeneous cell populations of endothelial cells at different maturation stages (Biberfeld et al., 2002; Kaaya et al.,

2000; Kaaya et al., 1996; Kaaya et al., 1995). The origin of the SC in KS is controversial (Roth et al., 1992) although an endothelial phenotype has been proposed by several studies including also findings of infected vascular endothelial lining cells as seen in our (paper I & V) and previous studies (Sturzl et al., 1997). However, it has been debated whether SC are vascular (VEC) or lymphatic (LEC) in origin or derive from mesenchymal progenitor cells (Carroll et al., 2004; Jussila et al., 1998; Masood et al., 2004), although most studies by immunohistochemistry have revealed that SC express lymphatic markers, such as D2-40 (Kahn et al., 2002), LYVE-1 (Carroll et al., 2004) and VEGFR-3 (Jussila et al., 1998). Also studies by gene expression microarray show that KS neoplastic cells are closely related to lymphatic endothelial cells (LEC) coexpressing some blood vascular endothelial cells (VEC) markers and that HHV-8 can infect both LEC and VEC in vitro (Wang et al., 2004), and that infected LEC had a higher HHV-8 genome copy number than VEC. In-vitro infection with HHV-8 of human dermal microvascular endothelial cells (HDMEC) (Hong et al., 2004), which also are CD34+ (Unger et al., 2002) resulted in the upregulation of LEC markers such as LYVE-1 expression in the infected HDMEC.

Cell proliferation is relatively low in KS as shown by previous studies on proliferation related protein Ki67 expression and by DNA flow cytometry (Kaaya et al., 2000). No significant difference was observed in these studies between AKS and EKS indicating that the clinical aggressiveness of AKS may reflect a higher rate of tumor growth promoted by various cytokines including HIV-Tat produced during HIV infection and by the compromised state of host immune response in HIV infection. Also a low level of apoptosis in KS lesions, due to strongly expressed Bcl-2 in SC is suggested to contribute to KS tumor growth as well (Kaaya et al., 2000).

KS spindle-like cells have been shown to occur in cultures of peripheral blood of HIV infected patients with KS or at high risk for developing KS (Browning et al., 1994). Furthermore recent studies show that endothelial cells or their precursors residing in donor kidneys may contribute to post-renal transplant KS as demonstrated by the finding that KS SC in the female recipient kidney had a male (donor) karyotype and that KS SC expressed the donor HLA antigen (Barozzi et al., 2003). These findings seem to indicate that KS SC and/or their progenitors can be recruited during development of the KS lesion.

1.1.2 Cytogenesis of KS

It is still a controversy whether KS should be regarded as a monoclonal neoplastic cell proliferation or a polyclonal hyperplastic, reactive process (Kaaya et al., 1995). Reports on cytogenetic and molecular genetic changes in KS are few (Alonso et al., 1987; Casalone et al., 2001; Delli Bovi et al., 1986; Kiuru-Kuhlefelt et al., 2000; Popescu et al., 1996; Saikevych et al., 1988; Scappaticci et al., 1986). Studies from KS cell lines, KS Y-1 (AKS derived) and KS SLK (IKS derived) revealed loss of copies of chromosomes 14 and 21 and non-random translocations and deletions in the short arm of chromosome 3 at region 3p14. These KS cell lines also exhibit loss of heterozygosity of loci at region 3p14-ter. The chromosome 3 alterations observed were suggested to contribute to the neoplastic process in KS (Popescu et al., 1996) but another cytogenetic study with the KS-IMM cell line (IKS) (Casalone et al., 2001) did not reveal any changes in chromosome 3 and showed gains in 1q10→qter, 7p10→pter, 7q22→qter, 8p11→qter, 14pter→q22 (Casalone et al., 2001). These aberrations are compatible with the notion that initially KS may develop as a reactive polyclonal cell proliferation associated with chromosome instability, followed by acquisition of clonal chromosome changes in later stages (Casalone et al., 2001).

Previous CGH studies of formalin fixed paraffin embedded KS biopsies revealed a recurrent gain at 11q13 (Kiuru-Kuhlefelt et al., 2000), which also amplifies two known oncogenes, FGF4 and INT2, residing at 11q13 (Huebner et al., 1988). This was suggested to indicate a possible role of HHV-8 as an integrating oncovirus that causes amplification and activation of genomic oncogenes in humans (Kiuru-Kuhlefelt et al., 2000).

The chromosomal instability suggested by the studies on cell lines may lead to cell apoptotis via p53 pathway (Artandi and Attardi, 2005). However, HHV-8 latency associated nuclear antigen (LANA) binds to p53 repressing its ability to induce apoptosis (Friborg et al., 1999). Furthermore telomerase activity has been found to be upregulated in KS (Chen et al., 2001), which may immortalise the infected cell leading to increased tumor cell survival.

1.2 Human herpesvirus type 8 (HHV-8)

Human herpesvirus 8 or Kaposi's sarcoma associated herpesvirus (HHV-8/KSHV) is a novel gamma-2 herpesvirus of the rhadinovirus genus closely related to the human gamma -1 herpesvirus, Epstein-Barr virus (EBV) (reviewed in (Viejo-Borbolla et al., 2003). HHV-8 is a large virus, with a diameter 140nm and a genome of approximately 170 kb double stranded linear DNA packed into an icosahedral capsid (Renne et al., 1996; Russo et al., 1996), covered by the tegument containing proteins and enclosed during budding from the cell by the cell membrane derived outer lipid membrane envelop containing various host and virus specific glycoproteins (Swaminathan, 2003).

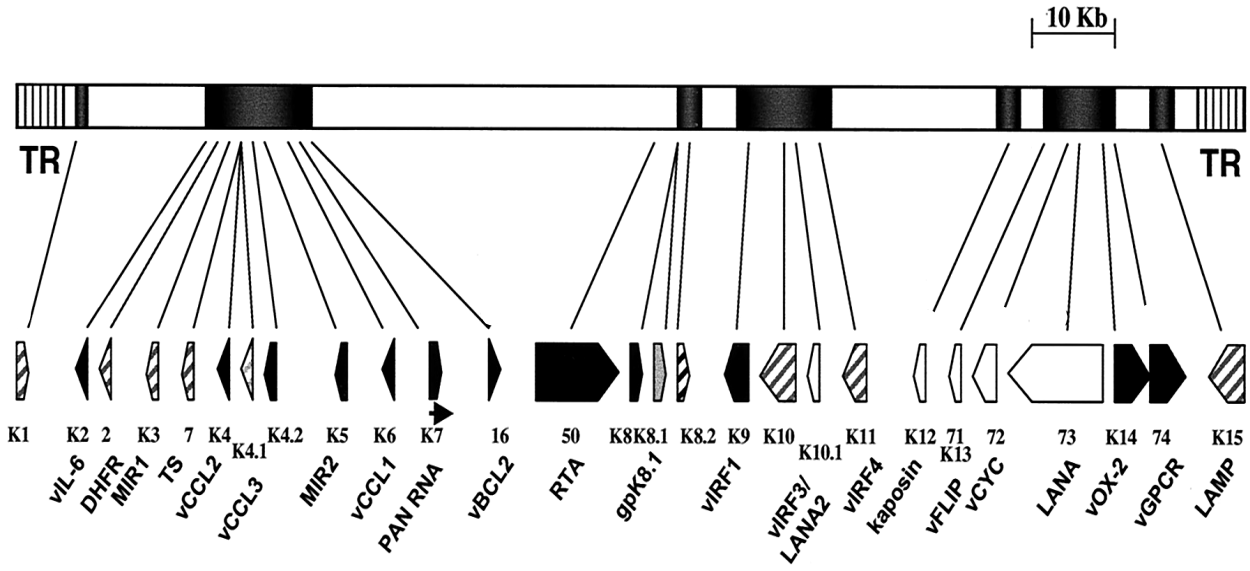

Fig 2: Schematic diagram of HHV-8 genome, adopted from Ablashi et al. (2002), Clin. Microbial Rev

A number of viral glycoproteins have been characterized (Fig 2) including K8.1A and ORF8 (gB) (Zhu et al., 2005) shown to bind to cell surface heparan sulfate (Chandran et al., 1998; Wang et al., 2001) and the cell receptor integrin α3ß1, respectively, thereby mediating virus entry through endocytosis (Akula et al., 2003; Akula et al., 2002). In KS patients HHV-8 is predominantly found in KS SC but was also shown in some lymphocytes, monocytes and keratinocytes (reviewed in (Hengge et al., 2002). The virus replicates in either a lytic or predominantly in the latent form as closed circular episomal DNA (Renne et al., 1996) within the nucleus of KS tumor cells (SC) and B cells of MCD and other infected mononuclear cells (Cathomas, 2000; Schulz, 2000). It has been shown that the episomal viral DNA is tethered to metaphase chromosome and copied in tandem

with host cell DNA during cell division (Szekely et al., 1999). Latent genes shown so far to be expressed in infected KS SC are latent nuclear antigen (LNA-1) encoded by ORF 73, viral cyclin (v-cyclin) (ORF 72), v-FLIP (ORF 71, K13) and a small membrane protein (kaposin, ORF K12), which are all adjacent in the genome (Jenner and Boshoff, 2002). Lytic virus expression is most frequent in MCD, moderate in KS and relatively rare in PEL cells. Common viral genes found during lytic expression include K1 transmembrane protein, v-GCR (ORF 74), v-IRF (ORF K9), v-IL-6 (ORF K2) and v-MIP (ORF K4.1) (Cathomas, 2000; Schulz, 2000).

1.2.1 LANA-1

The latency associated nuclear antigen type 1 (LANA-1) protein is 222-234 kD in size, represents the most frequently expressed immunogenic, latent nuclear antigen of HHV-8 and considered to play an important role in the generation and maintenance of HHV-8 associated malignancies (Komatsu et al., 2002; Rainbow et al., 1997; Russo et al., 1996). Thus it appears important in tethering extra-chromosomal (episomal) viral DNA to host chromosomes and responsible for the segregation of viral episomes with the chromosomes into new daughter cells during mitosis (Cotter and Robertson, 1999). It can also function in cell cycle regulation by competing with E2F for binding of hypophosphorylated pRb thus freeing E2F to activate the transcription of genes involved in cell cycle progression (Radkov et al., 2000). Aberrant E2F activity can also trigger apoptosis via the p53 pathway but LANA-1 binds p53, repressing its transcriptional activity and ability to induce apoptosis. Therefore the inhibition of p53 by LANA-1 allows latent HHV-8 to promote cell cycle progression whilst inhibiting apoptosis (Jenner and Boshoff, 2002).

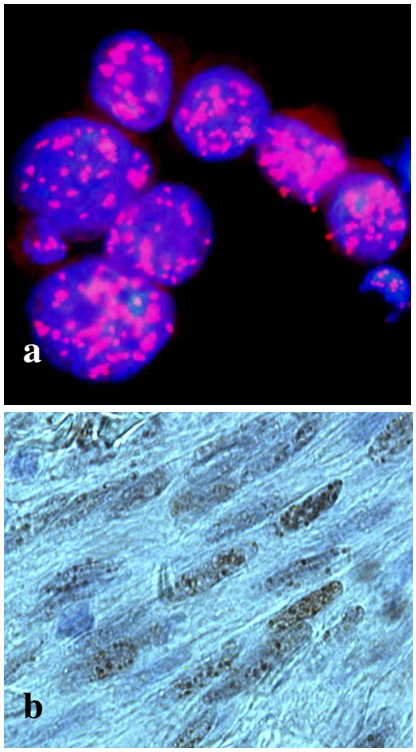

Fig 3: (a) Immunofluorescence assay for LANA on BCBL-1 cells (b) LANA+ KS spindle cells

The LANA-1 antigen can be demonstrated by immunohistochemistry also in formalin fixed paraffin embedded biopsies (Fig 3). It is expressed in most SC of both early and late stage of all the different clinical forms (AKS, EKS, CKS and IKS) (Katano et al., 1999; Schalling et al., 1995) and can serve as a diagnostic marker in suspected HHV-8 related

lesions. It can be used for serology of LANA-1 antibodies by immunocytochemistry or immunofluorescence assay (IFA) on infected BCBL cells that gives a typical speckled nuclear staining (Fig 3). Several studies have shown an increase in the number of LANA-1 positive cells during the progression of KS lesions (Dupin et al., 1999) allowing detection and quantification of HHV-8 infection in KS lesions (paper I & V).

1.2.2 HHV-8 Epidemiology

HHV-8 infection rates in various populations parallels in general the incidence of KS indicating its etiopathogenetic importance (Dukers and Rezza, 2003). A high rate of HHV-8 infection has been documented in sub-Saharan Africa (30-60%), where also the highest prevalence of KS occurs (De-The et al., 1999; Nuvor et al., 2001; Olsen et al., 1998; Serraino et al., 2001; Sitas et al., 1999). Relatively high or intermediate HHV-8 prevalence is noted in southern Italy and other Mediterranean areas (Whitby et al., 1998). In Italy, the prevalence varies between less than 10% in the North to more than 30% in the South (Whitby et al., 1998), with the highest rates observed on the islands of Sicily and Sardinia (Vitale et al., 2001). The lowest HHV-8 prevalence has been reported in northern Europe, Asia and the United States (<10%) (Ablashi et al., 1999; Hudnall et al., 2003), where HHV-8 infection is mostly found among homosexual men at risk of HIV infection (Moore, 2000) and patients with sexually transmitted diseases (STD) (Enbom et al., 2000).

Based on the sequence variation in the open reading frame (ORF) K1, HHV-8 is now classified into subtypes A, B, C, D, E and N (Cook et al., 1999; Zong et al., 1999), of which A and C are found mostly in Europe, whereas subtype B predominates in Africa (Cook et al., 1999; Zong et al., 1999). Subtype D is associated with individuals living in the islands of Pacific ocean and Australia (Meng et al., 1999). Subtype E has been reported in Brazil (Biggar et al., 2000) and subtype N, a novel subtype, has been identified only in South Africa (Alagiozoglou et al., 2000).

1.2.3 HHV-8 transmission

Sero-epidemiological studies of homosexual men and sex workers indicate that HHV-8 can be transmitted by sexual contact as also indicated by the demonstration of the virus in semen, cervical secretions and in saliva (Enbom et al., 2001; Huang et al., 1997; Koelle et al., 1997; Lampinen et al., 2000). Also the presence of infected cells and free virus in blood of healthy individuals has been well documented and it is not so obvious if it represents a risk for blood borne transmission.

In endemic areas like Tanzania where KS is seen in children (Amir et al., 2001; De-The et al., 1999; Manji et al., 2000), oral as well as vertical route of virus transmission (transplacental, during delivery or breast feeding) has been suggested (Plancoulaine et al., 2000). The importance of mother to child horizontal/oral transmission is indicated by the increase frequency of HHV-8 antibodies in infants by age (Bourboulia et al., 1998)and also indicated by high HHV-8 prevalence within families in endemic areas (Pauk et al., 2000). HHV-8 transmission has also been shown after kidney transplantation (Luppi et al., 2000; Parravicini et al., 1997), suggesting viral reservoirs in tissues (kidney) and occasionally by blood products (Renwick et al., 2002).

1.3 Pathogenesis of KS

The pathogenesis of KS is still unclear and appears complex, involving various mechanisms dependent on viral and cellular factors such as, HHV-8, inflammatory cytokines and angiogenesis i.e. endothelial growth factors including HIV-Tat, anti-apoptosis and cell proliferation promoting factors (Biberfeld P, 1998; Dalgleish and O'Byrne, 2002; Hengge et al., 2002). Characteristic for HHV-8 is the high homology of a large number of ORFs to cellular genes suggesting they may have been pirated from host chromosomes during viral evolution. Some of these genes are involved in down modulating the host immune responsiveness to target infected cells, whereas others modulate cell growth, proliferation, cell differentiation and angiogenesis (Hengge et al., 2002). They include the vBcl-2, vIL-8R, and vMIPs, vIL-6, and the D type viral cyclin (Hengge et al., 2002), whose functions are all closely related to that of their cellular homologs.

HHV-8 infected cells escape immune response targeting by down regulation of surface MHC mediated by two transmembrane proteins, MIR1 and MIR2, encoded in ORFs K3

and K5, respectively. MIR1 and MIR2 remove MHC I from the plasma membrane through enhanced endocytosis, and lysosomal degradation which efficiently inhibits MHC I surface expression (Fig 4). Downregulation of MHC I and its accessory immune receptors poses the risk of initiating a natural killer (NK) cell response by initiating receptor-activated apoptosis through Fas (CD95/Apo-1) in cells lacking appropriate MHC I expression. However, HHV-8 can inhibit NK-mediated killing through expression of anti apoptotic v-FLICE-inhibitory proteins (v-FLIPs) (Fig 4). V-FLIP encoded by ORF K13 (ORF71) possesses death effector domains and acts as a dominant-negative inhibitor of receptor-activated apoptosis by binding to Fas-associated death domain protein and caspase 8 (FLICE) (Belanger et al., 2001). This prevents activated caspase recruitment into the death-inducing signaling complex.

HHV-8 v-FLIP shares with c-FLIPs the ability to activate NF-κB through IκB kinase activation (Moore and Chang, 2003) which is essential for the growth and survival of the cell (Fig 4). Our previous studies on KS biopsies have shown that apoptosis clearly decreases during development of early to late nodular KS lesions (Kaaya et al., 2000), and that the expression of anti-apoptotic v-FLIP and cellular Bcl-2 increase from early to late stage KS lesions, (Kaaya et al., 2000; Sturzl et al., 1999). Thus viral exploitation of these two anti-apoptotic pathways contributes to the tumor-like growth and progression of the KS lesion.

HHV-8 encoded v-cyclin, the viral homolog to cellular cyclin, binds with cyclin dependent kinases (CDK6), and this complex phosphorylates pRb releasing the transcription factor E2F, which in turn, activates the transcription of S-phase genes (Fig 4). However, unlike complex of cellular cyclin with CDk6, vCyclin-CDK6 complexes are resistant to CDK inhibitory proteins and can even abolish its inhibitory effect by a concomitant destabilization and degradation, which may lead to unregulated cell cycle progression and cell transformation and thus contribute to tumor development (Verma and Robertson, 2003).

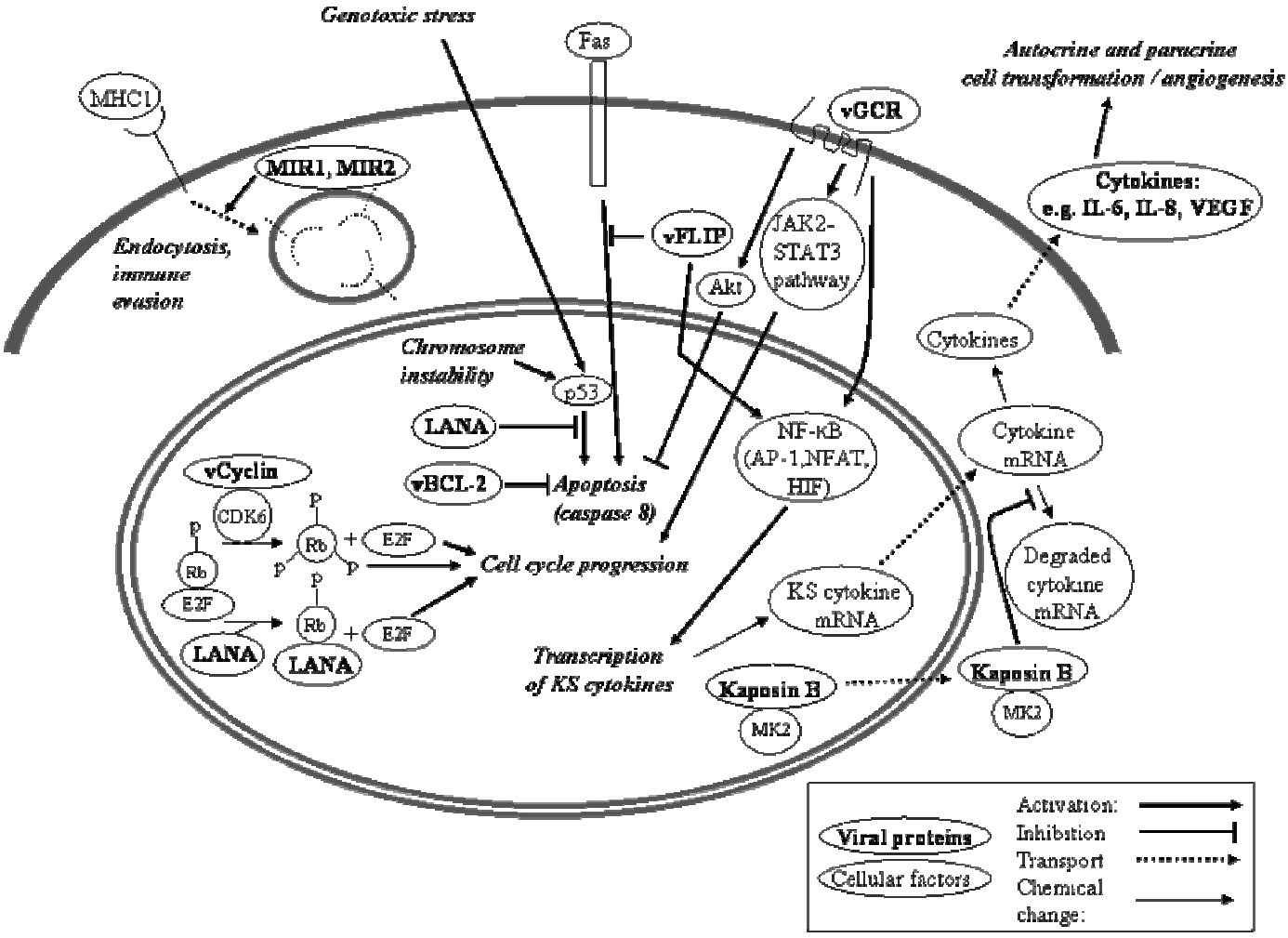

Fig 4: HHV-8 gene expression(pathogenesis) during SC development and tumor growth

Kaposin, a latency gene encoded by ORF K12 represents a potential viral oncogene and is characterized as a transforming gene (Muralidhar et al., 1998), although little is known about its role in deregulating cell signalling (Direkze and Laman, 2004). It is present in three isoforms Kaposin A, Kaposin B and Kaposin C which are all translated from the ORF K12 region (Sadler et al., 1999). Kaposin B is expressed by all HHV-8 infected cell and can activate the p38–MK2 pathway (McCormick and Ganem, 2005). Kaposin B binds to MK2 in the nucleus, which then will be exported to the cytoplasm. The activated cytoplasmic MK2 will then block the degradation of the messenger RNAs transcribing various cytokines necessary for cell survival (normally unstable because they contain AU-rich elements) (Fig 4), hence increasing their translation (McCormick and Ganem, 2005).

Recently MicroRNAs (miRNA), small conserved non-coding RNA molecules which regulate expression of genes by binding to the complementary messenger RNAs have been

identified in HHV-8 (Pfeffer et al., 2005). The HHV-8 miRNAs are confined to a region of the kaposin gene (ORF K12) (Pfeffer et al., 2005), which is expressed by SC at all KS stages (Sturzl et al., 1997) and which can induce tumorigenic transformation of infected cells (Muralidhar et al., 1998). Removal of this miRNA encoded to kaposin may result in abolished protein expression thus hindering the function of kaposin (Pfeffer et al., 2005). The viral and cellular genes are regulated by these HHV-8 miRNAs still remain to be identified.

HHV-8 also encodes a G-protein-coupled receptor (vGCR) homolog to the human angiogenic, chemokine interleukin-8 receptor (IL-8R) (Yen-Moore et al., 2000). Angiogenic responses induced by vGCR are mediated by upregulation of vascular endothelial growth factor (VEGF) (Cannon et al., 2003) (Fig 4). Furthermore, upregulation of VEGF gene expression is mediated through JNK/SAPK and p38MAPK signal transduction pathways, activated by inflammatory cytokines (Cannon et al., 2003) (Fig 4). The constitutive activity of vGCR could therefore have a role for VEGF expression by SC during the development of early stage KS lesions (Choi et al., 2001). Furthermore vGCR dependent expression of autocrine and paracrine growth factors (bFGF, VEGF,) can promote angiogenesis and edema (Biberfeld P, 1998; Samaniego et al., 1998). It was also shown (Zhang et al., 2005) that viral envelop glycoprotein gB (ORF8) can activate the VEGFR-3 receptor and trigger receptor signalling on the surface of microvascular endothelial cells, which can modulate cell migration and proliferation. VEGFR-3 expression and activation may also enhance HHV-8 infection and participate in HHV-8 mediated transformation. Thus VEGFR-3 activation in the endothelium appears to be an important factor in the pathogenesis of Kaposi's sarcoma (Zhang et al., 2005).

The male predominance of adults with KS is as yet not well understood and hormonal factors have been suggested to play a role (Cai et al., 1997). Thus in-vitro studies on AIDS-KS derived cells indicated that glucocorticoid receptors and IL-6 may play a role in the growth of these cells (Gill et al., 1996). Thus IL-6 production in KS-derived spindle cells was shown to be enhanced in vitro by glucocorticoids (Cai et al., 1997) whereas intralesional injections of human chorionic gonadotrophin (hCG) induced regression of KS growth (Gill et al., 1996) which is compatible with the observed male KS predominance.

1.4 AIDS AND KS

KS is one of the AIDS defining conditions (Safai et al., 1992; Thomas, 2001) and the most common malignant tumor associated with HIV infection. Previously a rare indolent tumor with male preponderance, AKS with the HIV epidemic has become a common and aggressive tumor of both sexes with a decrease in male to female ratio from 19:1 to 1.7:1 particularly in East Africa (Thomas, 2001). Epidemiological studies indicate that the risk of developing KS increases from 5-10% in non-HIV infected individuals to 20-70% in HIV infected patients (Cathomas, 2000). Some studies seem to suggest that patients infected first with HIV followed by HHV-8, progress to KS faster than those infected first with HHV-8 followed by HIV (Renwick et al., 1998) suggesting that an established anti-HHV-8 immunity may have some protective effect.

There seems to be a cross talk between HIV-1 and HHV-8 as recent studies have shown that HIV-1 replication stimulates HHV-8 production in PEL cell lines and peripheral blood mononuclear cells from KS patients, possibly due to the activating functions of HIV-Tat (Harrington et al., 1997; Varthakavi et al., 2002). ORF50, the major transactivator of HHV-8 lytic cycle can also induce increased levels of HIV replication by interacting synergistically with HIV-1 Tat leading to increased cell susceptibility to HIV infection and transient permissiveness to HIV replication (Caselli et al., 2003).

1.4.1 HIV-Tat

The increased incidence of KS in patients with AIDS has been shown to be particularly related to the HIV-1 transactivating gene (Tat) protein which is taken up by the infected cells. The HIV-Tat protein stimulates proliferation of spindle cells and inhibits apoptosis. Apparently the Tat protein promotes AIDS KS by at least two distinct mechanisms. First, Tat competes with heparin sulfate binding sites for β-fibroblast growth factor (β-FGF) increasing the concentration of β-FGF, which is a potent angiogenic factor (Albini et al., 1995; Ensoli et al., 1994). Second, HIV-1 Tat has been shown to activate HHV-8 replication in BCBL-1 cells and PBMCs from patients with AIDS and PEL or KS (Harrington et al., 1997) thus increasing viral load and expression of a series of viral genes including vGCR, vBCL2, and vIRF1 with oncogenic potential. Thus, as mentioned earlier Tat promotes tumorigenesis of endothelial cells, e.g. via stimulated synthesis of vascular endothelial growth factor and anti-apoptotic activity. Notably, the functional activity of Tat protein in the pathogenesis of AKS must clearly involve an intercellular signalling cascade

and antibodies to HIV-Tat epitopes were shown to inhibit signalling mediated by Tat (Demirhan et al., 1999). Furthermore, serology studies indicate a deficient anti-Tat response in AKS patients compared to HIV-positive non-AKS subjects (Demirhan et al., 1999; Demirhan et al., 2000), indicating the importance of functional Tat to promote AKS development and aggressiveness (Ensoli et al., 1990; Ensoli and Sturzl, 1998).

2. AIMS

The basic aim of the present studies was: (a) to further elucidate the etiological importance of HHV-8 and possible pathological mechanisms involved in the development of Kaposi's sarcoma and concordantly certain lymphomas in immunosuppressed states like HIV infection; (b) to contribute to the understanding of the possible reactive or clonal growth of KS lesions; (c) to further elucidate the role of certain viral genes in promoting cell proliferation and survival either by cell cycle regulation and/or modulation of apoptosis or by the production of autocrine and paracrine cell growth factors. An improved understanding of the cellular and viral mechanisms and factors involved in the induction and development of tumors in immunodeficiency states could identify new specific targets for prevention and anti tumor therapy.

2.1 Specific aims related to papers I-V:

1. Insitu (on biopsies) characterization of HHV-8 infected Kaposi's sarcoma (KS) cells in early and late developmental tumor stages of the clinical different AIDS related (AKS) and endemic (EKS) KS forms, particularly with regard to:
    - Expression of latency associated nuclear antigen (LANA) (paper I & V).
    - Cell proliferation in infected and uninfected endothelial cells (paper I).
    - Expression of lymphatic and vascular endothelial markers and their relationship to LANA (paper V).
2. Correlation of HHV-8 load in KS biopsies (early and late) (paper III).
3. To compare the HHV-8 DNA load in patient serum at different developmental stages of AKS as compared to EKS (paper II)
4. To investigate the relationship between immune response to HIV-Tat and HHV-8 levels in AKS patients (paper II)
5. Identification of possible cytogenetic abnormalities and characterization of gene expression in micro-dissected KS (AKS, EKS) biopsies at different tumor developmental stages by CGH and interphase FISH (paper IV).

3. MATERIAL AND METHODS

Tissue biopsies (paper I-V): All KS tissues included in this work were routine, diagnostic surgical biopsies, fixed in buffered formalin (10%) and embedded in paraffin at the Department of Histopathology, MUCHS, Dar es Salaam.

Serum: (paper II): Sera were collected from KS patients (paper II) attending Muhimbili University College of Health Sciences (MUCHS), Dar es Salaam, Tanzania. Blood donor and KS patient's sera were taken routinely for HIV diagnostic control and stored at -70^{o}C for further testing.

Histology (paper I-V): KS biopsy sections were stained with hematoxylin and eosin (H&E) and classified as patch, plaque or nodular stage as previously described (Enzinger, 2001). The diagnosis was validated by immunohistochemistry for LANA.

Immunohistochemistry (IHC) (paper I, II, III & V): Triple antibody immunofluorescence staining was performed as described in Paper I & V. Briefly Paraffin sections (4μ) were deparaffinized in xylene and rehydrated in descending grades of alcohol (100% - 70%) to distilled water and heated by microwave for 6 minutes at 100^{o}C (in citrate buffer pH 6) for antigen retrieval as previously described (Shi et al., 1991). Non-specific antibody binding was blocked by incubation with normal serum (species corresponding to that of the detection system). Incubations with primary antibody were done either at 37 ^{0}C for one hour or at 4^{0}C overnight. Detection was performed with anti species Ig conjugated with biotin and detected with Avidin Cy5 and with anti species Ig conjugated with FITC or Cy3 and visualised by a fluorescent microscope (Olympus BX60) equipped with digital camera (Sony DKC-5000). Appropriate filters were used for specific FITC, Cy3, Cy5, and DAPI imaging, which were edited and overlaid using Adobe Photoshop 6.0 (paper I and V). Single antibody IHC was performed by the Avidin Biotin Complex (ABC) method as previously described (Kaaya et al., 2000) and examined by standard light microscope (Nikon Labophot, Japan).

Serology (ELISA) (paper I-V): Antibodies to HIV-1 were screened using Enzygnost anti-HIV-1+2 Plus ELISA (Behring, Marburg, Germany) and reactive samples were confirmed on Wellcozyme recombinant anti-HIV-1 ELISA (Murex Diagnostic, UK) for detection of HIV antibodies as previously described (Urassa et al., 1999) (paper I-V).

Antibodies to HIV-1 Tat (paper II) were detected by using a direct antigen coated ELISA plate method as previously described (Demirhan et al., 1999). The solid phase was coated

with either purified cloned Tat protein or peptides (Neosystem laboratoire) and bound antibodies detected with peroxidase labelled anti IgG and IgM antibodies (Sanofi Diagnostics, Pasteur, Paris, France).

Real-time PCR (paper II): DNA was extraction from sera using the Qiamp Blood Kit (Qiangen Gmbh, Hilden, Germany) according to manufacturer's instruction. Real-time PCR (TaqMan) was performed using primers for HHV-8 ORF26 region. All sera were analysed in duplicates including one inhibition control with the positive control sample added to the test specimen for control of possible inhibitory activity giving false negative results. The serum DNA load was determined as the mean of duplicate values. The number of HHV-8 genomes in each sample was calculated from an EBV DNA standard (Qi Amp Blood kit, Qiagen Gmbh, Hilden, Germany) as previously described (Enbom et al., 2001). The ABI 7700 Sequence Detection System software created the standard curve by plotting the C_t values against each known concentration of EBV standard.

Semi-quantitative PCR (paper III): DNA extraction was performed using the Chelex-100 protocol, briefly three 10 µm sections were deparaffinized in 0.5% Tween 20, heated to 90°C, cooled to 55°C and the wax was separated from the solution. Samples were then digested in proteinase K (10 mg/ml) and heated to 99°C for 10 min with 5% chelex-100 (Bio-Rad, Stockholm) and Tris-EDTA. Samples were heated to 45°C, with chloroform, centrifuged and the top phase with DNA removed and stored at -20°C.

PCR was performed on serial dilution of extracted DNA with HHV-8 primers KS330 Forward-AGCCGAAAGGATTCCACCAT and Reverse-TCCGTGTTGTCTACGTCCAG (Chang et al., 1994) for ORF26, 233-bp (capsid antigen). Amplification of actin sequences as a housekeeping gene was used to evaluate DNA extraction and for controlling efficiency of PCR.

CGH (paper IV): Comparative genomic hybridization (CGH) is a method for molecular cytogenetic analysis, which provides an overview of DNA sequence copy number changes (losses, deletions, gains, amplifications) in a tumor specimen by mapping these changes on normal chromosomes. CGH is based on the in situ hybridization of differentially labeled total genomic tumor DNA and normal reference DNA to normal human metaphase chromosomes. Successful CGH requires high quality of amplified tumor DNA. Non-neoplastic vascular structures, infiltrating lymphocytes and plasma cells in the KS lesion may dilute tumor DNA beyond the detection by CGH (Kallioniemi et al., 1994). Due to these features it is important to separate tumor DNA from the infiltrating and surrounding

normal tissue DNA of the KS lesion which can be done by laser microdissection. However, genetic analysis of paraffin embedded microdissected tissue is made difficult due to the low amount and degradation of DNA recovered from formalin fixed paraffin embedded tussues. Thus we applied the recently developed novel method of whole genome amplification for CHG (Klein et al., 1999) which had been successfully applied on laser microdissected formalin fixed paraffin embedded material (Stoecklein et al., 2002).

Interphase FISH (paper IV): Interphase FISH was performed using DAKO TOP2A FISH kit (paper IV), centromere specific probes (kind gift, Prof. Magnus Nordenskjöld, Clinical Genetics, CMM, Karolinska Institute) for chromosome 11, 16, 22 and Y were hybridized in paraffin sections of two nodular KS that showed deletion of chromosome 16, 22 and Y by CGH.

A centromere specific probe for chromosome 11 was used as an internal control probe and formalin fixed paraffin sections of a human tonsil were used as a normal tissue control. Approximately 150 cells were counted that showed two dots for the internal control (chromosome 11) probe.

Ethical clearance:

The ethical clearance was obtained from Ministry of Health (MoH), and the Muhimbili University College of Health Sciences (MUCHS) in Dar Es Salaam, Tanzania and The ethical committee, Karolinska Hospital (Dnr 01-096)

4. RESULTS AND DISCUSSIONS

4.1 KS Spindle cell (SC) immunophenotypes (paper I and V)

The majority of both AKS and EKS SC expressed the vascular endothelial cell marker CD34 in early (patch/plaque) and late (nodular) stages (paper I), which is also a marker of bone marrow derived stem-precursor cells. They also expressed the lymphatic markers LYVE-1, D2-40 and VEGFR-3 as well as the endothelial precursor markers CD34 and VEGFR-2 (paper V). LYVE-1+ was the most frequent SC phenotype in early as well as late KS and the majority of these cells also expressed VEGFR-3 and D2-40 (paper V). The expression of the lymphatic as well as vascular endothelial cell/endothelial precursor cell markers increases from early to late stage KS. These findings substantiate previous studies suggesting that individual KS SC express a lymphatic (Carroll et al., 2004; Jussila et al., 1998; Kahn et al., 2002; Wang et al., 2004), vascular or "mixed" phenotype (Biberfeld et al., 2002; Kaaya et al., 2000; Kaaya et al., 1996; Kaaya et al., 1995). Tumor growth reflects increase in SC reflects accumulation and survival during development to late stage KS due to; (a) proliferation; (b) increased inhibition of apoptosis by viral genes (Kaaya et al., 2000) and (c) recruitment of precursor cells to the lesion (paper I & V).

4.1.1 LANA expression in KS SC (Paper I and V)

LANA+ cells were significantly more frequent in the late KS stages (46% AKS, 39% EKS) compared to early stages (22% AKS) (Fig 5). This was also validated by PCR showing an increase in tissue viral load during tumor development (paper III). The difference between AKS and EKS in the total frequency of LANA+ cells was not clearly significant (Paper I). A large number of CD34+ cells in early and late KS were also positive for LANA. The double positive (LANA+/CD34+) cells

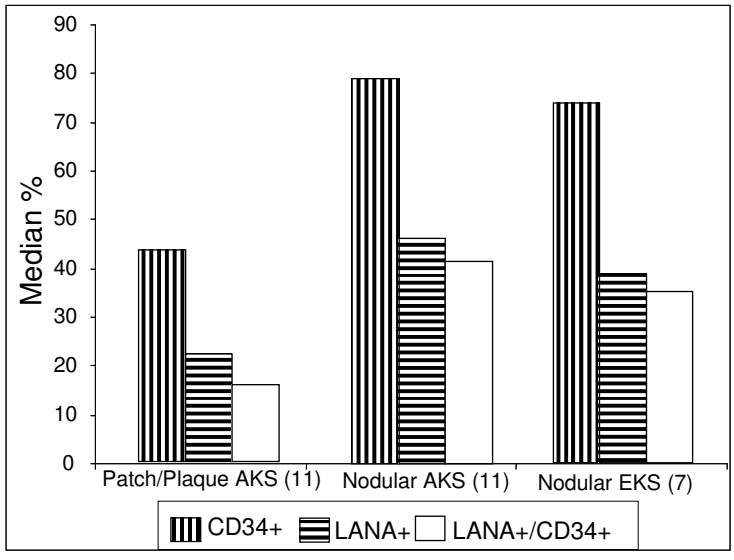

Fig 5: Cells positive for LANA and CD34 in AKS and EKS biopsies

represented a fraction of all CD34+ SC in nodular AKS (41.8%) and EKS (32.6%) as well as early AKS (15.1%). Thus a considerable number of CD34+ SC (36%-38%) were LANA- at all AKS/EKS stages (Paper I & V) (Fig5 & 6). However, all LANA+ cells were

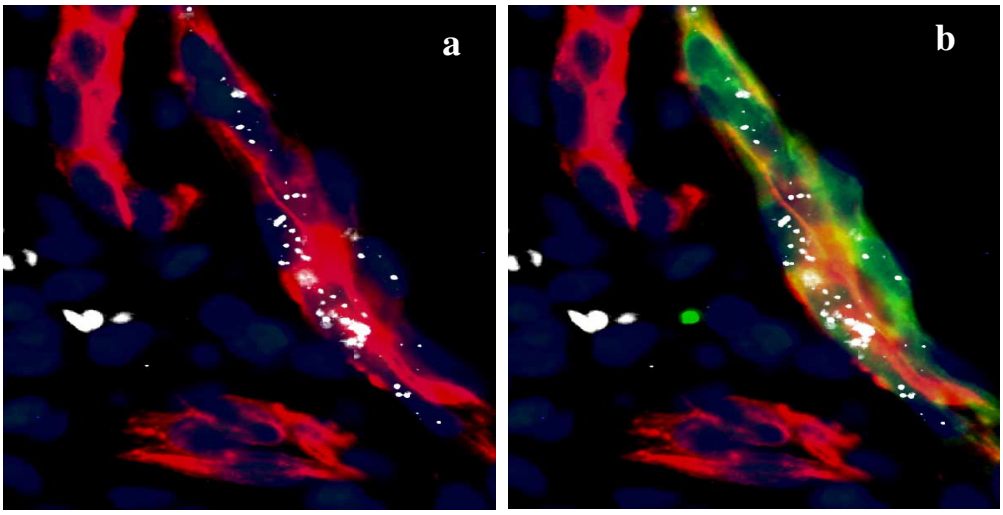

Fig 6: Triple immunofluorescence staining for LANA (nuclear speckling white), CD34 (red membrane) and LYVE-1 (green membrane). (a) note CD34+/LANA- cells. (b) note only cells that were LANA+ are LYVE-1+

LYVE-1+ in early and late KS and LANA appeared better correlated to LYVE-1 than to CD34 expression (Fig 6) (paper V). This apparent heterogeneity in viral permissiveness of CD34+ SC seems less compatible with a clonal CD34+ SC proliferation and virus transfer but appears to indicate that also non-infected $CD34^+$ SC are continuously recruited from progenitor cells and locally triggered to develop permissiveness to HHV-8 infection. Furthermore cells belonging to the non-cycling SC (Ki67-) population showed a clear increase during development from patch/plaque (median 13.5%) to nodular (median 40.3%) (Paper I), also supporting the concept of continuous recruitment of CD34+ cells to the lesion.

LANA+ but CD34- (paper I & V) (Fig 7a) cells were more frequent in early as compared

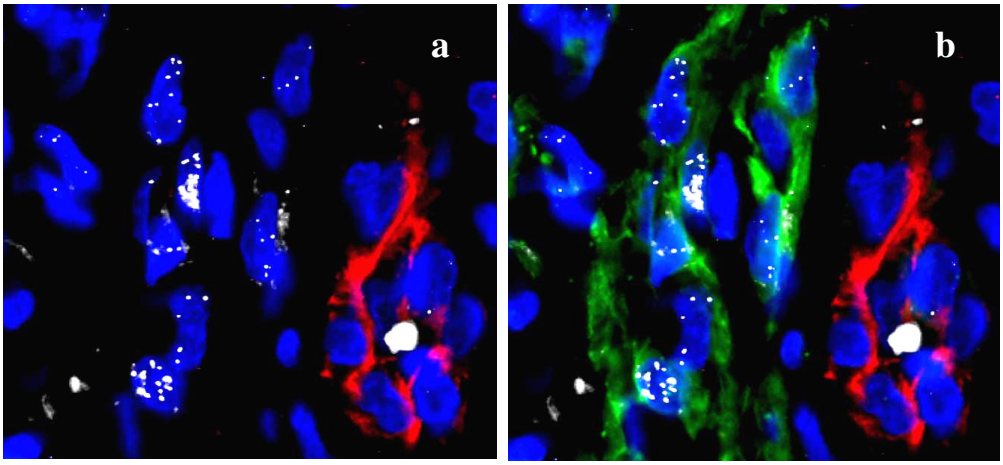

Fig 7: Triple immunofluorescence staining for LANA (nuclear speckling white), CD34 (red membrane) and LYVE-1 (green membrane). (a) note LANA+ but CD34- cells. (b) note all cells that were LANA+/CD34- are LYVE-1+

to late lesions and did not express a leucocytic phenotype (CD3, CD20, CD45, CD68) (Paper I), but most of these cells expressed lymphatic endothelial (LEC) markers such as LYVE-1 (Fig 7b), VEGFR-3 and D2-40 (Paper V), suggesting that the resident LECs represent an early target of primary HHV-8 infection. This is also supported by the findings from Wang and colleagues (Wang et al., 2004) that infected LECs have a higher HHV-8 genome copy number than VECs. Obviously a high viral copy number may result in an efficient maintenance and propagation of episomal HHV-8 DNA in dividing and migrating LECs. Thus in-vitro activation of VEGFR-3 has been shown to increase endothelial cell migration and to enhance cell susceptibility to HHV-8 infection and transformation (Zhang et al., 2005). Hence, the activation of VEGFR-3 (Paper V) in LANA+/VEGFR-3+ SC observed in our study at the early stage of KS will probably favour an increased endothelial cell migration (recruitment) and transformation to tumor SC including formation of pathological vascular slits during KS development.

Most likely recruited endothelial precursor cells become infected in the lesion as evident from the increase in frequency of LANA+/CD34+/VEGFR-2+ cells (Paper V) during KS development. All these LANA+/CD34+/VEGFR-2+ cells also expressed LEC markers (LYVE-1, VEGFR-3 & D2-40), indicating that after infection these cells apparently undergo a phenotype switch including upregulation of the LEC markers (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004). These findings corroborate those by Wang and colleagues (Wang et al., 2004) showing that HHV-8 infects both LECs and VECs in-vitro, driving their gene expression profile closer to that of each other, and also studies on in-vitro infection of CD34+ human dermal microvascular endothelial cells (HDMEC) (Unger et al., 2002) with HHV-8 (Hong et al., 2004), showing upregulation of LEC markers such as LYVE-1 expression in the infected HDMEC. Our finding that during progression to nodular KS, the portion of LANA+/LYVE-1+/CD34+ cells increases significantly (60% to 85%) could thus indicate that HHV-8 infection promotes (a) change of LEC towards a VEC phenotype (CD34+) (b) changes of VEC towards a LEC phenotype, (c) recruitment of CD34+ endothelial progenitor cells or (d) accumulation of a mixed population according to (a), (b) and (c).

4.1.2 SC and proliferation (paper I)

The frequency of proliferating (Ki67+) cells usually decreased during development from early to late KS lesions, consistent with the notion that KS growth in the development from a reactive, early lesion to a nodular tumor depends not only on SC division but also

impaired apoptosis (Kaaya et al., 2000) and progenitor recruitment (paper I & V). No significant difference in cell proliferation was observed between nodular AKS and EKS. These findings could therefore indicate that the usually more aggressive growth of the AKS tumors may reflect a higher rate of SC progenitor recruitment compared to the more indolent EKS lesions.

Most proliferating cells were $LANA^+$ (3.4%) particularly in patch/plaque lesions indicating that $LANA^+$ cells could have a proliferative advantage (Friborg et al., 1999). Proliferating LANA+/CD34- cells (Paper I) appeared to be more frequent in the early patch/plaque stages (median 1.8%) as compared to the nodular stage (median 0.1%). These proliferating LANA+/CD34- cells probably represent resident lymphatic endothelial cells since from our study (paper V) all LANA+/CD34- cells were LYVE-1+. However, the LANA+/CD34-/LYVE-1+ cell population decreased during the development of early (patch) to late (nodular) KS stage (paper V) indicating that in the initiation of the KS lesion, proliferation of resident lymphatic endothelia is necessary, but that later during KS development there is a continuous recruitment of non infected endothelial precursor cells into the KS lesion probably promoted by various inflammatory cytokines (e.g. IL-1, IL-6, TNF) (Biberfeld P, 1998; Kaaya et al., 1996) as well as by HIV related angiogenic growth factors (FGF, VEGF, HIV-1 Tat) (Biberfeld P, 2002; Ensoli and Sturzl, 1998; Samaniego et al., 1998).

4.1.3 Leucocytes in KS lesions (paper I)

Previously it has been shown that some circulating monocytes and macrophages in AKS become HHV-8 infected and it was suggested they function as a route of transmission (Blasig et al., 1997; Monini et al., 1999). Our studies with double and triple immunostaining with either markers for macrophages (CD68+) or leukocytes (CD45+) showed that 5-9% of cells in the lesions were CD68+, and 10% CD45+ of which 3-5% were T-lymphocytes (CD3+) and 2% were B-lymphocytes (CD20+). Most $CD20^+$ leucocytes were located at the borders of tumor areas and around vascular endothelium in both early and late KS lesions. Unexpectedly, less than 0.5% of the CD 68+ macrophages were LANA+ and all T and B lymphocytes were negative, suggesting that LANA-1 is expressed in some macrophages, but not in infiltrating lymphocytes (paper I), and that lymphocytes within KS lesions are therefore less likely to transmit HHV-8.

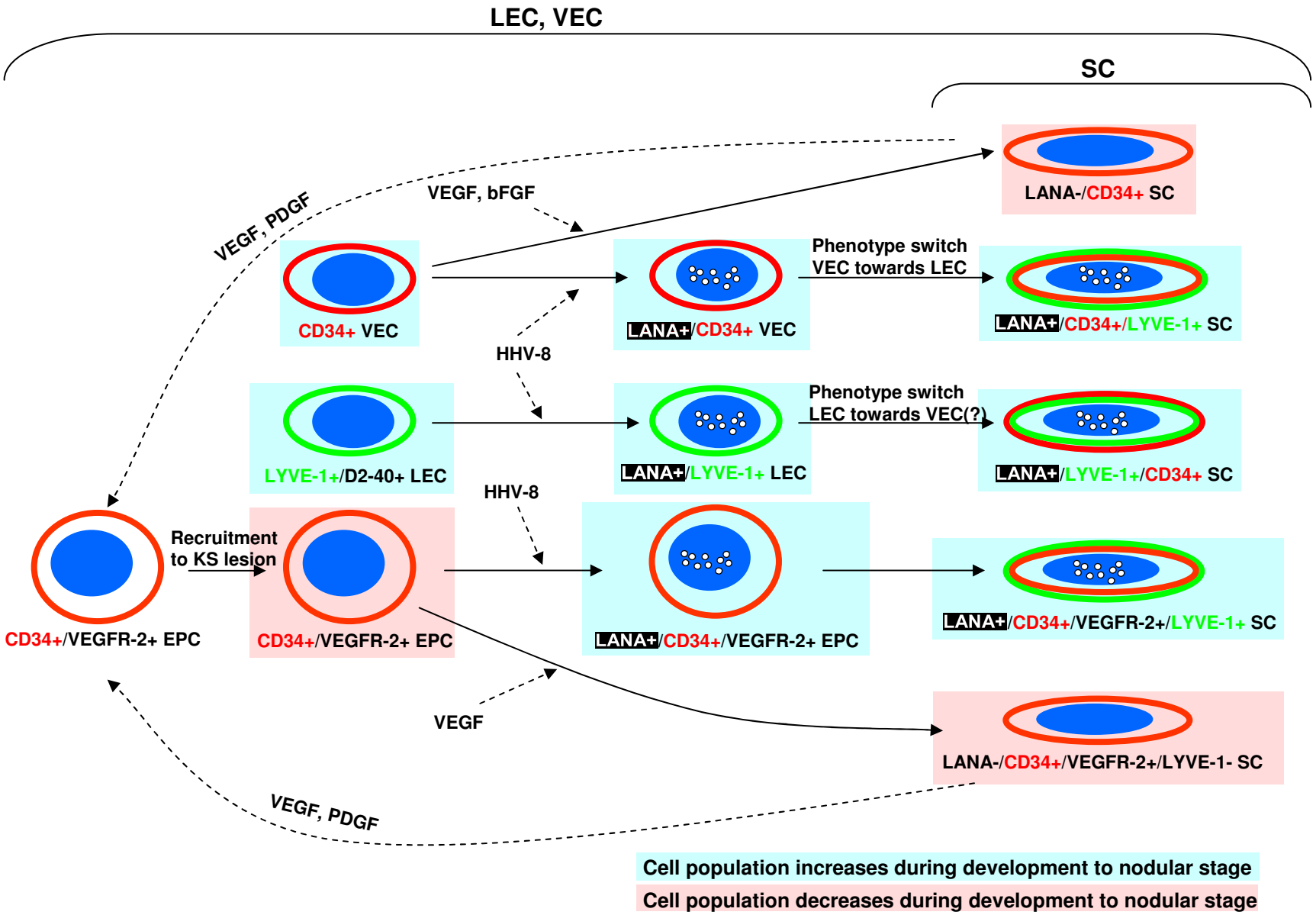

Fig 8: Spindle cell phenotypes observed and possible precursors during tumor development from early to late KS (paper I & V). SC: spindle cell, VEC: vascular endothelial cells, LEC: lymphatic endothelial cells, EPC: endothelial precursor cell, LANA: latency associated nuclear antigen, VEGF: vascular endothelial growth factor, PDGF: platelet derived growth factor, bFGF: basic fibroblast growth factor, CD34: bone marrow derived precursor cell/vascular endothelial cell marker, LYVE-1 & D2-40: Lymphatic endothelial cell marker. Dotted arrows indicate activity of soluble factors /HHV-8 virions.

4.2 Serum HHV-8 DNA in KS patients (Paper II)

HHV-8 DNA was detected by PCR in 17/23 (74%) KS serum more in AKS (12/14 (86%) than EKS 3/5 (60%) and non-KS patients 2/4 (50%). HHV-8 DNA load ranged from 25 to 7000 (median 1400) genomes/ml, values similar to other studies done by real time PCR (Campbell et al., 2000; Lallemand et al., 2000). KS sera had significantly higher HHV-8 viral DNA load compared to non-KS patients, also found in other studies (Kennedy et al., 1998; Monini et al., 1999). Our results also corroborate previous findings that activation of viral replication, as seen by serological assays, indicating an increased risk for KS development (Schulz, 1998) and that HHV-8 viral load correlates with clinical progression (Mendez et al., 1998). The two non-KS patients in our study with detectable HHV-8 DNA reflect the high HHV-8 seroprevalence in healthy Tanzanian blood donors (Enbom et al., 2002).

Our finding showed a higher HHV-8 DNA load in AKS (median 1,400 copies/ml) compared to EKS (median 200 copies/ml), which was suggested to reflect a higher AKS aggressiveness due to stimulation of HHV-8 activation by HIV replication, particularly the HHV-8 activating functions of HIV-Tat (Harrington et al., 1997; Varthakavi et al., 2002).

HHV-8 DNA load was higher in males (median 580 copies/ml) compared to females (median 120 copies/ml), which is consistent with the well recognized male predominance of EKS and CKS found in adults and the suggested influence of female/male hormonal factors on HHV-8 activation (Cai et al., 1997). Thus intralesional injections of human chorionic gonadotrophin (hCG) showed a suppressed effect on SC tumor growth (Gill et al., 1996) but whether it has any effect on HHV-8 viral replication is not clear.

Serum HHV-8 DNA load was also often higher in early (2750 genomes/ml) than in late KS (85 genomes/ml) stages and may reflect higher viremia in early stages followed by segregation of HHV-8 to tissues during KS development. Correlation of serum HHV-8 with the histological stage of KS lesions is poorly documented (Campbell et al., 2000; Quinlivan et al., 2002), but our study indicates a negative correlation in HHV-8 DNA load between KS sera (paper II) and KS lesions (paper III) suggesting that the level of virus in serum does not reflect only the amount of virus in KS tumour tissue, but possibly also of other viral reservoirs. However, the relatively small number of cases in the present study does not provide conclusive evidence on the apparent higher viral load at early KS stage.

It has been shown that antibodies against certain HIV-Tat epitopes can abolish the functional activity of Tat in the pathogenesis of Kaposi's sarcoma (Demirhan et al., 1999; Ensoli et al., 1994). It would therefore be expected that AKS patients without HIV-Tat antibodies would have higher levels of serum HHV-8 DNA. Seven out of 14 AKS patients (50%) had anti-Tat IgG. Sera with the lowest level of HHV-8 DNA (25, 85 copies/ml) showed highest anti-Tat serological index (0.143, 0.138) and sera with the highest HHV-8 DNA levels (6,000-7,000 copies/ml) had lower anti-Tat serological index (0.025-0.044), where none of the non KS sera showed any antibody reactivity to HIV-Tat. These finding thus appears to suggest a correlation between high anti-Tat IgG titers and low HHV-8 serum viral load, although not statistically significant and supports the importance of HIV-Tat in HHV-8 activation and the pathogenesis of AKS.

Interestingly the highest HHV-8 DNA level was observed in the serum of a patch AKS patient (25500 genomes/ml) which case had no anti-Tat reactive antibodies. This particular case is however difficult to evaluate due to lack of detailed clinical information and it could represent a high level of virus activation in advanced clinical stage. Interestingly none of the sera in our study had reactivity to functional anti-Tat epitopes responsible for the transactivation by HIV-Tat. Thus, not only quantitative, but also qualitative aspects of the humoral anti Tat response have to be considered in evaluation of prognosis.

4.3 Semi quantitative PCR for HHV-8 (Paper III)

Diagnostic formalin fixed, paraffin embedded biopsies are usually easily available from archives. However, DNA extractions from such tissues are troublesome due partially degradation of DNA during fixation, paraffin embedding, and the extraction process. In the current study we evaluated the efficacy of DNA extraction by two established methods namely the Chelex and the Qia-gene kit for analyzing for possible changes in HHV-8 viral load, assessed by PCR as a HHV-8/actin ratio in AKS and EKS biopsies taken at different stages of tumor development. A better performance for Chelex-extracted DNA compared to Qia-gene kit was found corroborating a previous study also comparing Chelex and Qia-gene kit (Coombs et al., 1999).

The sensitivity of the HHV-8 PCR was assessed on serial dilutions of DNA extracted from known number of BCBL-1 cells with a known number of Plasmid vector containing the 233bp DNA sequence of ORF26. A borderline reaction was obtained for DNA from 25 BCBL-1 cells.

With this PCR assay the borderline DNA concentration for HHV-8 and actin in different stages of KS (AKS and EKS) was evaluatedin relation to tumor area of early and late KS specimens. Our results indicate similar virus load (HHV-8/actin) in late, nodular stage of both AKS and EKS and higher viral load in late (nodular) than in early stages (patch/plaque). As expected, the tumor area/section increased from the early (patch) to late (nodular) stages. This increase in viral load from the early to late stage KS was comparable to the increase of LANA+ cells in sections corresponding/parallel to those DNA extracted sections.

Thus by an improved DNA extraction it was possible to compare semiquantitative PCR and immunohistochemistry of fixed, paraffinized tissue biopsies, which assay also may be useful for differential diagnosis of HHV-8 associated lesions in standard histological tissue biopsies.

4.4 Cytogenetics of Kaposi's sarcoma (Paper IV)

In twenty of twenty three (87%) male (early and late) KS biopsies loss of chromosome Y was observed and in seven of nine (78%) early KS (all male) no chromosomal aberration apart from the deletion of chromosome Y was observed (Fig 9). Deletion of chromosome Y was also reported by previous studies on short term cultures of primary KS tumor cells (Saikevych et al., 1988; Scappaticci et al., 1986) and established KS cell lines (Popescu et al., 1996). In our study, six cases of nodular AKS and EKS had besides loss of chromosome Y, also recurrent deletions on chromosomes 16, 17, and gain of chromosome 21. The finding that chromosome Y was the only chromosomal aberration observed in early KS and that additional chromosome abnormalities were found in late KS shows that chromosomal instability is related to the development of KS (Fig 9). The deletion of Chromosome 16 and Y was confirmed also by interphase FISH in sections of two nodular KS biopsies, which showed no dots for chromosome Y in 61% of the SC and less then 2 dots in 68% of the SC for chromosome 16. Only SC that had two dots for the control probe (chromosome 11) were counted. Epidermis included in the section KS was evaluated as an internal control, persistently showed two dots in most of the nuclei. The increase in genomic alterations during KS development support the view that KS gradually can progress to a true clonal sarcoma.

Known aberration of 1p, 19 and 22 (Karhu et al., 1997) were also seen in our controls (normal tonsil), and those genomic regions were excluded from the present analysis.

Unexpectedly EKS showed more chromosomal abnormalities than AKS (Fig 9), but the cases examined were too few to reach statistical significance. This might indicate that genomic instability could be a more important factor in the development of EKS than AKS. Most likely AKS development is also promoted by various cytokines and growth factors produced during HIV infection and by the compromised state of host immune response in HIV infection.

One single EKS case showed a gross hypodiploidy which needs to be further validated. The observed chromosomal aberrations were not clearly related to age or gender of the patients.

Published CGH data on chromosomal imbalances in KS are difficult to interpret since they were based on analysis using bulk tumor material including varying amounts of normal DNA (Klein et al., 1999). Our study using microdissected KS lesions therefore minimized

the contributions of normal diploid cell DNA and should be more representative for KS SC. The fact that short-term as well as long term cultures of established tumor cell lines promote selection of in-vitro induced karyotypes also hampers interpretation of such data.

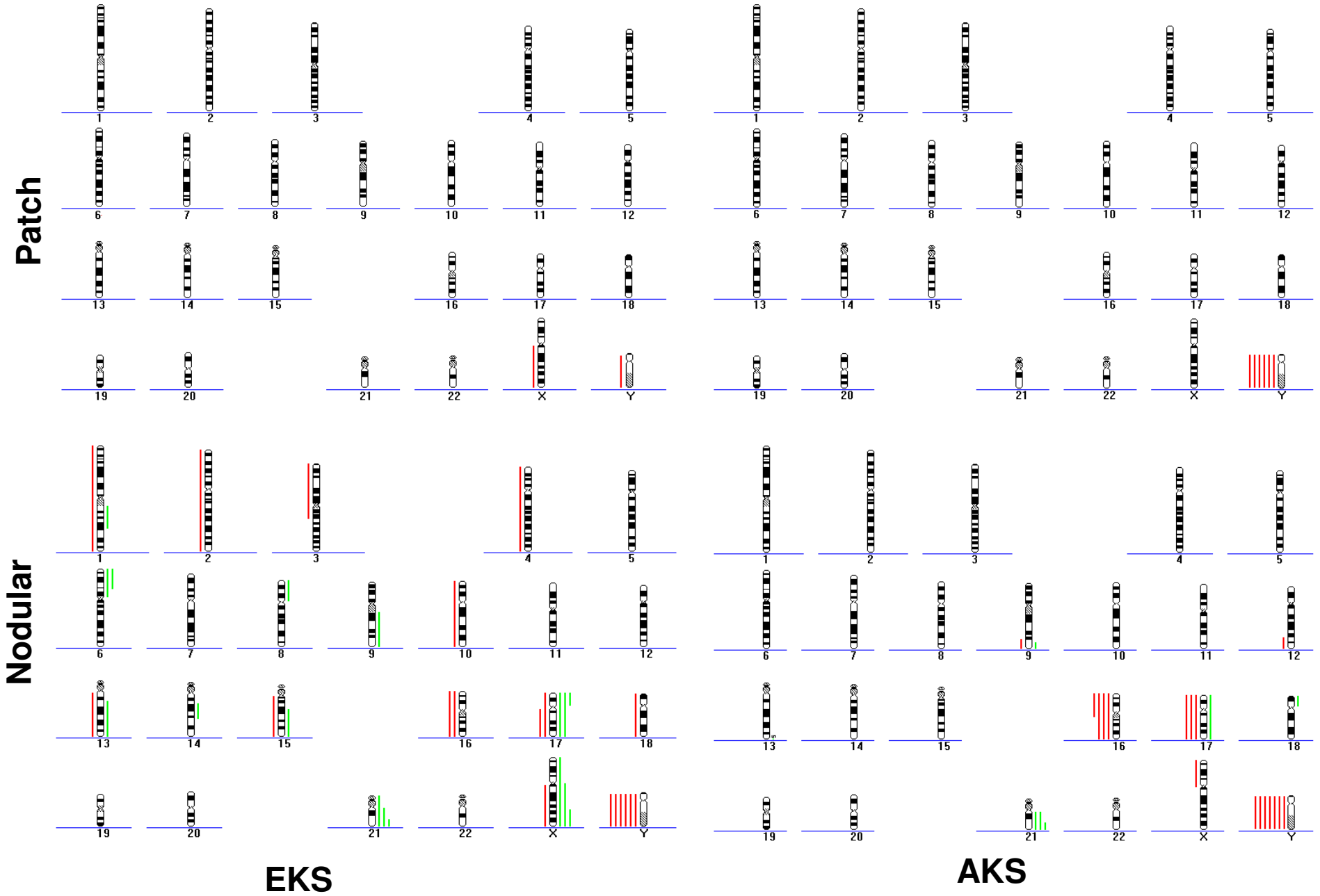

Fig 9: CGH finding on patch (early) and nodular (late) endemic (EKS) and AIDS (AKS) kaposi's sarcoma biopsies

5. CONCLUSIONS

Immunohistochemistry studies (paper I & V)

- The majority of CD34+ SCs are LANA+ suggesting a selective advantage in viability for infected cells (paper I & V).
- The presence of considerable number of LANA-negative CD34+ SC may suggest heterogeneity among SCs for HHV-8 infectivity and a continuous recruitment of non-infected SC precursors during KS development (paper I & V).
- Some LANA+ cells are CD34-, but are all LYVE-1+, indicating that resident lymphatic cells most likely are the early target of HHV8 infection (paper V).
- Cell proliferation is higher in early as compared to the late KS stages, consistent with the notion that KS tumors develop from a predominantly reactive early lesion (paper I).
- The clinical aggressiveness of AKS compared to EKS is apparently not strictly correlated to cell proliferation in the lesions (paper I).
- Few macrophages and no T or B-cells are LANA+ suggesting that also other vectors/pathways maybe of importance for transmission of HHV8 to the KS lesions (paper I).

Serum HHV-8 (paper II)

- Serum HHV8 viral load was higher in AKS, males and early KS stage than in EKS, females and late stage, and was not clearly related to the tumor viral load.
- The observed inverse correlation between HHV8 DNA load and anti Tat IgG titres indicates a relationship between HHV8 and HIV-Tat production.

Semiquantitative PCR (paper III)

- PCR evaluation of HHV-8 DNA indicates a correlation between virus load and a progressive stage of KS development i.e. increase in LANA+ SC and does not suggest an increase in viral content of individual tumor cells (paper III).

Cytogenetics of Kaposi's sarcoma (Paper IV)

- Chromosome Y was the only chromosomal deletion observed by CGH in early (patch) KS. Late (nodular) KS lesions showed together with chromosome Y deletion also recurrent loss of chromosome 16, 17 and gain of chromosome 21. The increase in genomic alterations during KS development supports the view that KS gradually can progress to a true clonal sarcoma (paper IV).

- The more frequent random chromosomal aberrations detected in late EKS appears to indicate that chromosomal instability could be a more important factor for the development of EKS than for AKS, which may also be promoted by the compromised state of host immune response and various cytokines produced during the HIV infection.

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8. APPENDIX (PAPERS I-V)