

Chapter 1: Literature Review and Hypothesis

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1.1 Introduction

Kaposi's sarcoma (KS) is a rare mesenchymal tumour originally described in 1872 as an idiopathic, multipigmented sarcoma of the skin (Kaposi 1872). It attracted little attention until the 1980s when an aggressive form of the disease was diagnosed in homosexual men in the United States heralding the acquired immunodeficiency syndrome (AIDS) epidemic. Viral oncogenesis by human herpes virus 8 (HHV8), also known as KS associated herpes virus, cytokine induced growth, and immunosuppression represent important factors for this tumour development in AIDS-related KS.

The anatomical distribution, rates of progression, and clinical outcomes of KS vary dramatically among the different clinical subtypes. A classical indolent subtype is found in elderly men of Mediterranean origin, usually arising on the lower extremities, and rarely involving visceral organs. A mild form of KS is also found in transplant patients treated with immunosuppressants, particularly patients of Italian or Jewish descent (Penn 1997). An endemic and aggressive form of KS involving visceral and/or lymphatic organs is seen in young adults and children of subequatorial Africa (Slavin, Cameron and Singh 1969; Taylor, Smith, Bull and Pike 1972). The most aggressive form of KS is found in patients with HIV infection (Friedman-Kien 1981; Haverkos and Drotman 1985). KS was one of the first conditions to be recognised as a sequela of infection with HIV. It is the most common malignancy associated with AIDS and develops in 20-30% of patients with AIDS (Levine 1994; Clarke 2001). In this

aggressive subtype, the KS can be generalised with widespread subcutaneous and visceral involvement. Among HIV infected patients, KS is particularly common in homosexual men, regardless of their ethnic origin (Safai, Johnson, Myskowski, Koziner, Yang, Cunningham-Rundles, Godbold and Dupont 1985; Beral, Peterman, Berkelman and Jaffe 1990).

Although these clinical subtypes have a very variable natural history, they share the same histopathological appearance. Pathologically, KS is characterised by infiltration by spindle-shaped cells, microvascular endothelial cells, extravasated erythrocytes and mononuclear cells (Safai 1997). In addition, it has been recently found that virtually all forms of KS are associated with infection with HHV8 (Antman and Chang 2000). These features suggest that the development of the different forms of KS is probably initiated by the same mechanism but the rate of progression is modified by different factors in individual clinical subtypes. With the diverse variation in clinical behaviour, KS serves as a unique tumour model for the studying of tumour progression.

1.2 Clinical subtypes

1.2.1 Classical Kaposi's sarcoma

Classical KS, also known as the Mediterranean subtype, occurs mainly in elderly men of Mediterranean or Jewish extraction. During the nineteenth century, classical KS was considered a rare disease, even though an increased rate was suggested at the turn of the century (Beral 1991). Whether such an

increase was the result of decreased diagnostic misclassification or a true phenomenon is not clear.

In North Americans and Europeans, the usual age of onset is between 50 and 70. The higher incidence rate of KS in men compared with women suggests that gender is a risk factor for KS. The predominance of the disease in males was noted in the earliest report of KS (Kaposi 1872). Prior to population based incidence rate registration, the reported male to female ratios were 10-15:1. In a recent population based incidence study by the United States Surveillance, Epidemiology and End Results (SEER) program prior to the AIDS epidemic, a gender ratio of 4:1 has been reported during the period 1970-1979.

Classical KS tumours usually present as one or more asymptomatic red, purple or brown patch, plaque, or nodular skin lesions. The disease is often localised to the lower extremities, especially involving the ankles and soles (Figure 1.1). It commonly runs a relatively benign, indolent course for 10 to 15 years or more with slow enlargement of the lesions and the gradual development of more lesions. Venous stasis and lymphoedema of the lower limbs are frequent complications. In longstanding cases, systemic lesions can develop along the gastrointestinal tract, lymph nodes and other organs. These visceral lesions are generally asymptomatic and are most often discovered only at autopsy. Rarely the gastrointestinal lesions can bleed and become symptomatic. Fatal complications from classical KS are uncommon. Interestingly up to one third of the patients with classical KS develop a second primary malignancy, most often

non-Hodgkin's lymphoma and cutaneous malignant melanoma (Safai, Mike, Giraldo, Beth and Good 1980). The exact mechanism of the increased risk of malignancy is unclear but HHV8 could play a role in this.

Figure 1.1 Classical Kaposi's sarcoma on the foot of a 66-year-old man of Mediterranean extraction.



1.2.2 African Kaposi's sarcoma

In the 1950s, KS was a relatively common endemic neoplasm in native populations in equatorial Africa, comprising approximately 9% of all cancers seen in Ugandan males (Taylor, Smith et al. 1972). African KS can present either as an indolent disease identical to the classical subtype seen in Europe or as an aggressive disease with fungating and exophytic tumours that may invade the subcutaneous and surrounding tissue including the underlying bone.

In Africa, both the indolent and the locally more aggressive forms of KS occur with a male to female ratio comparable to that observed with the classical KS seen in North America and Europe. In general, however, patients in Africa are younger. A lymphadenopathic form of KS is also seen in Africa, primarily in prepubescent children with male to female ratio of 3:1. In eastern and southern Africa, KS represents up to 25-50% of soft tissue sarcomas in children and 2-10% of all cancers in children (Athale, Patil, Chintu and Elem 1995). In these cases, the generalised lymphadenopathy is frequently associated with visceral organ involvement. The prognosis is very poor, with close to 100% mortality within 3 years (Templeton and Bhana 1975).

Whereas AIDS-related KS is typically negative for Epstein Barr Virus (EBV), both HHV8 and EBV have been detected in African KS of both AIDS-related and non-AIDS-related types (Chang, Cesarman, Pessin, Lee, Culpepper, Knowles and Moore 1994). These novel findings suggest that similar to African Burkitt's lymphoma, African KS may represent a biologically distinct subset of disease, in which a strong association with EBV may indicate a pathogenesis that differs from that occurring in most non-African KS cases (Hengge, Ruzicka, Tying, Stuschke, Roggendorf, Schwartz and Seeber 2002).

1.2.3 Kaposi's sarcoma secondary to immunosuppressive treatment

The first case of KS associated with immunosuppression was reported in a renal transplant patient in 1969 (Siegel, Janis, Alper, Schutte, Robbins and Blaufox 1969). Since then a number of renal and other organ allograft

recipients receiving prednisone and azathioprine have been reported to develop KS shortly after the start of immunosuppressive therapy (Penn 1979). The incidence of KS in immunosuppressed renal transplant recipients has been estimated at between 150 and 200 times the expected incidence of this tumour in the general population. The Collaborative Transplantation Research group of Ile de France reported a 0.5% overall risk of KS in 7923 organ transplant recipients (Farge 1993). In another series of 263 transplant patients, there were 14 cases of KS (5.3%) (Qunibi, Al-Furayh, Almeshari, Lin, Sun, Heston, Ross, Rigsby and Miller 1998).

HHV8 seems to be necessary but not sufficient to induce KS as KS was noticed in transplant patients who were HHV8 positive before transplantation, though the tumour occurred only upon post-transplant immunosuppression (Parravicini, Olsen, Capra, Poli, Sirchia, Gao, Berti, Nocera, Rossi, Bestetti, Pizzuto, Galli, Moroni, Moore and Corbellino 1997). The latent period to development of KS after transplantation is about 30 months (Montagnino, Bencini, Tarantino, Caputo and Ponticelli 1994). The male to female ratio in the subtypes is 2-4:1, similar to the ratio seen in classical KS (Lesnoni La Parola, Masini, Nanni, Diociaiuti, Panocchia and Cerimele 1997). Although the KS in these iatrogenically immunosuppressed patients often remain localised to the skin, widespread dissemination with mucocutaneous or visceral organ involvement is not uncommon and can be seen in up to 50% of the cases. In some cases, the KS can regress as a result of reduction in immunosuppressive therapy. Clinical management of transplant patients who develop KS is difficult. It requires

consideration of the risk of death from generalised KS versus the risk of graft rejection that may occur if the immunosuppressive therapy is reduced (Wijnveen, Persson, Bjorck and Blohme 1987; Tebbe, Mayer-da-Silva, Garbe, von Keyserlingk and Orfanos 1991).

1.2.4 AIDS-related Kaposi's sarcoma

In 1981, a fulminant and disseminated form of KS in young homosexual or bisexual men was first reported as part of the AIDS epidemic. The aetiology of AIDS is a T cell lymphotropic retrovirus known as HIV. The underlying immunological deficiency that characterises HIV infection is an acquired profound disorder of cell mediated immune functions. This immunological deficiency and immune dysregulation predisposes the host to a variety of opportunistic infections and neoplasms, especially KS. HIV itself may play an indirect role in the development of KS (Vogel, Hinrichs, Reynolds, Luciw and Jay 1988).

Approximately 95% of all the cases of epidemic KS in the United States have been diagnosed in homosexual or bisexual men. Analysis of 13,000 individuals with AIDS, reported to the US Centre for Disease Control and Prevention up to 1989, revealed that the incidence of KS in homosexual and bisexual men infected with HIV is ten times greater than in other seropositive transmission groups. In the past, approximately 26% of all homosexual males with HIV infection presented with, or eventually developed, KS during the course of their illness. In contrast, less than 3% of all heterosexual intravenous drug users with HIV infection developed KS. However, the proportion of patients with AIDS

with KS has steadily decreased since the epidemic was first described. About 48% of patients with AIDS in 1981 presented with KS as their AIDS-defining illness. By August 1987, the cumulative proportion of AIDS patients with KS had diminished to less than 20%. The introduction of highly active antiretroviral therapy (HAART) delayed or prevented the emergence of drug-resistant HIV strains, profoundly decreased the viral load, led to increased survival rate, and lessened the risk of opportunistic infections. The use of HAART also appeared to be related to the continuing downtrend (12%) in the incidence of KS as the AIDS-defining illness.

Epidemic KS is characterised by multifocal, widespread lesions at the onset of illness (Krigel, Laubenstein and Muggia 1983). These lesions may involve the skin, oral mucosa, lymph nodes and visceral organs (Figures 1.2, 1.3). Most patients with HIV infection who presented with mucocutaneous lesions are otherwise asymptomatic from the HIV infection compared with those patients who first developed an opportunistic infection. The sites of disease at presentation of epidemic KS are much more varied than those seen in the other clinical subtypes. In an early report on the clinical manifestation of the disease, 8% had no skin involvement, 27% had few localised skin lesions and 63% had innumerable skin lesions widely distributed over the entire body (Krigel, Laubenstein et al. 1983). Sixty-one per cent had generalised lymphadenopathy at the time of first presentation of KS. In 45% of the patients studied, KS lesions were found in one or more sites along the gastrointestinal tract. Thus, while most patients present with skin lesions, KS involvement of lymph nodes or

the gastrointestinal tract may occasionally precede the appearance of skin lesions.

Almost all patients with epidemic KS develop disseminated disease. Progression often proceeds in an orderly fashion from a few localised mucocutaneous lesions to numerous and generalised skin involvement with lymphadenopathy and disseminated visceral disease. The majority of patients with epidemic KS die of complicating opportunistic infections (Gill, Akil, Colletti, Rarick, Loureiro, Bernstein-Singer, Krailo and Levine 1989).

Figure 1.2 CT scan of the chest showing pulmonary infiltration with Kaposi's sarcoma in a HIV positive male patient

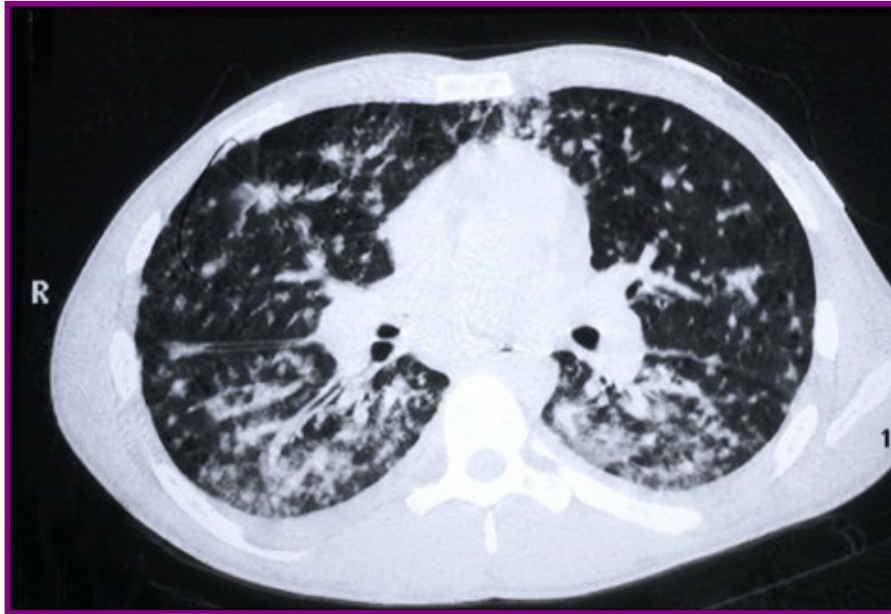


Figure 1.3 Extensive Kaposi's sarcoma in lower legs causing ulceration and oedema in a HIV positive male patient



1.2.5 Non AIDS-related homosexual related Kaposi's sarcoma

There have been several reports of KS in homosexual men with no evidence of HIV infection (Vandercam, Tennstedt, Gala, Bodeus, Louwagie and Burtonboy 1991). These patients have an indolent and cutaneous form of the disease, with new lesions appearing every few years. Lesions occur most commonly on the skin of extremities and genitalia but can occur anywhere on the skin. These cases may indicate the presence of causal factors, other than HIV, that homosexual men may be exposed to due to their lifestyle.

1.3 Treatment of Kaposi's sarcoma

1.3.1 Classical Kaposi's sarcoma

Once the diagnosis is established, observation is appropriate for immunocompetent and asymptomatic patients with little progression of the disease over a long period. Occasionally the disease regresses spontaneously and may not recur for long periods. For solitary lesions or lesions of limited extent, surgical excision may be of benefit. Multiple small excisions can be performed over the years and achieve good disease control. For multiple lesions within a well-defined area (e.g. lower limb), modest doses of radiation to the lesions with a 2-3 cm margin provide excellent local control. Thin, cutaneous lesions can be treated with a superficial x-ray beam (e.g. 100kV) or low energy electrons (4-6 MeV). Thick plaques or nodules are best treated with a high energy electron beam with bolus material on the skin surface to

encompass the entire lesion homogeneously but sparing the underlying normal structures. When substantial oedema is present, it is often necessary to use parallel opposed megavoltage beam to treat the deep tissue. Doses of 27.5 Gy in ten daily fractions was associated with an 85% long term local control rate (Cooper 1988). As the disease can be multifocal over a large area of skin surface in some patients, total skin electron beam therapy has been used (Margaretic, Faj, Tomas, Dmitrovic and Krajina 2002). Of 20 patients treated with total skin electron therapy, using 4 Gy once a week for 6 to 8 consecutive weeks, 17 (85%) had complete remission lasting a median of 48 months.

1.3.2 Immunosuppressive treatment related Kaposi's sarcoma

Some patients with this subtype of KS have achieved spontaneous and lasting remission following reduction or discontinuation of immunosuppressive therapy. The discontinuation of immunosuppressive therapy led to the resolution of KS in all twelve renal transplant recipients in one study (Duman, Toz, Asci, Alper, Ozkahya, Unal, Celik, Ok and Basci 2002). However, in another study, only 4 of 10 renal transplant recipients with KS had a complete response to a reduction in immunosuppressive therapy (Lesnoni La Parola, Masini et al. 1997). Discontinuation of immunosuppressive therapy is an option in renal transplant recipients since dialysis is available, but the dose can only be reduced in patients with heart or liver transplant. The decision to reduce immunosuppressive therapy must be balanced against the risk of graft rejection. Local modalities such as surgical excision or radiation therapy can also be used

in localised lesions and the response rate is similar to other clinical subtypes of KS.

1.3.3 AIDS-related Kaposi's sarcoma

Treatment may result in a disappearance or reduction in the size of specific skin lesions and thereby alleviate the discomfort caused by chronic oedema and ulceration that often accompany multiple skin tumours seen on the lower limbs and in controlling the symptoms associated with mucosal or visceral lesions. However there are no data to show that treatment improves survival rates (Safai 1997). In addition to anti-tumour treatment, essential components of an optimal KS treatment strategy include anti-retroviral treatment, prophylaxis for opportunistic infections and treatment of intercurrent infections.

Small, localized lesions of KS may be treated by cryotherapy, surgical excision or radiation therapy. AIDS-related KS is generally very radiosensitive and excellent palliation of local symptoms can be obtained with relatively low doses of radiation. Irradiated skin lesions sometimes are left with a purple haemosiderin stain, reminiscent of the colour of the original tumour but without the original tumour mass. For patients with a good performance status, 30 Gy in ten daily fractions is the preferred schedule whereas for patients with a poor performance status with significant co-morbidity, a single fraction of 8 Gy can be used. In the only randomised trial evaluating radiation therapy schedules in AIDS-related KS, Stelzer et al. reported a significant improvement in complete response rate and relapse rates and time to relapse with an extended fractionated course over a single fraction (Stelzer and Griffin 1993). The

nominal standard dose (NSD), an estimate of a biologically equivalent radiation dose for a fractionated regimen if delivered in one single fraction, has been calculated for each of the three radiation schedules used in the Stelzer study. The complete response rate was 83% after 40 Gy in 20 fractions (NSD=13.5), 79% after 20 Gy in 10 fractions (NSD=8.6) and only 50% after a single fraction of 8 Gy (NSD=8). Despite the similar NSD values for the 20 Gy in 10 fractions and the single 8 Gy, the complete response rate was significantly higher for the fractionated treatment. In vitro study has shown that fractionated radiation enhanced the production of the radiosensitising factors (interleukin-6 and oncostatin M) by KS cells and these factors enhanced the radiation response during subsequent fractions of radiation (Hong, Zhang, Leigh and Stevens 2001).

For patients with widespread AIDS-related KS, the immunosuppressive status of patients often limits the therapeutic use of systemic chemotherapy. The current first line chemotherapy is liposomal anthracyclines. Liposomal encapsulation prolongs the circulating half-life of the anthracyclines, increases drug concentration in the tumour and modifies the side effect profile (Gill, Espina, Muggia, Cabriaes, Tulpule, Esplin, Liebman, Forssen, Ross and Levine 1995). The overall response rate of pegylated liposomal doxorubicin was 45-58% (Northfelt, Dezube, Thommes, Miller, Fischl, Friedman-Kien, Kaplan, Du Mond, Mamelok and Henry 1998; Stewart, Jablonowski, Goebel, Arasteh, Spittle, Rios, Aboulafia, Galleshaw and Dezube 1998). Paclitaxel, a drug that stabilises microtubules, also has anti-angiogenic effects. In three studies of a

total of 105 patients who were treated with paclitaxel, response rates ranged from 49% to 71% (Saville, Lietzau, Pluda, Feuerstein, Odom, Wilson, Humphrey, Feigal, Steinberg, Broder and et al. 1995; Welles, Saville, Lietzau, Pluda, Wyvill, Feuerstein, Figg, Lush, Odom, Wilson, Fajardo, Humphrey, Feigal, Tuck, Steinberg, Broder and Yarchoan 1998; Gill, Tulpule, Espina, Cabriaes, Bresnahan, Ilaw, Louie, Gustafson, Brown, Orcutt, Winograd and Scadden 1999). Thus, paclitaxel is an excellent second line chemotherapy.

Subcutaneous, intravenous or intralesional interferon- α has been widely tested in AIDS-related KS, with a response rate of 40% (Groopman, Gottlieb, Goodman, Mitsuyasu, Conant, Prince, Fahey, Derezin, Weinstein, Casavante and et al. 1984; Real, Oettgen and Krown 1986). In these studies, the responses differed significantly according to the prognostic factors of the extent of disease, prior or coexistent opportunistic infections, prior treatment with chemotherapy, the CD4 cell count and an increase in beta-2 microglobulin. Several studies have combined interferon- α with chemotherapy agents. Overall, these studies have shown no additional benefit with chemotherapy compared to single agent interferon- α .

The feasibility of combining interferon- α with anti-retroviral therapy has been examined in a randomised study (Shepherd, Beaulieu, Gelmon, Thuot, Sawka, Read and Singer 1998). The rate of response was significantly higher in patients given interferon with zidovudine than interferon alone (31% vs 8%) and the median time to tumour progression was significantly longer (18 weeks vs 13

weeks). The incidence of side effects was similar in the two groups. A randomised study of two dose levels of interferon showed that the low dose of 1 million units per day with antiretroviral produced a response rate of 40% with the median duration of response of 110 weeks (Krown, Li, Von Roenn, Paredes, Huang and Testa 2002). This response rate was similar to the intermediate dose of 10 million units per day and the low dose was better tolerated.

Retinoids have been shown to have activity against KS in vitro. Retinoic acid and its synthetic analogues inhibited the proliferation of KS cells by inhibiting the production of interleukin-6, an autocrine growth factor for KS (Nagpal, Cai, Zheng, Patel, Masood, Lin, Friant, Johnson, Smith, Chandraratna and Gill 1997). In a series of 27 patients with mucocutaneous AIDS-related KS treated with all-trans-retinoic acid, 4 patients had a partial response after 12 to 28 weeks of therapy with the response lasting for 4 to 24 weeks (Gill, Espina, Moudgil, Kidane, Esplin, Tulpule and Levine 1994).

Anti-angiogenesis agents, such as thalidomide, have been tested against KS. In a study of 17 patients treated with oral thalidomide daily for 8 weeks, the overall response rate was 35% (Fife, Howard, Gracie, Phillips and Bower 1998). The level of HHV8 DNA titre became undetectable in 3 of the 5 patients with a response. The mechanism for the reduction of viral load is unclear. There is also preclinical data to support the use of toremifene in treatment of AIDS-related KS (Hong and Leigh 2002). The reported mechanism is via the up-regulation of the production of the inhibitory factor, transforming growth factor β -

1. The use of radioimmunotherapy using L6 antibody against KS has also been tested in preclinical setting with promising results (Leigh, Burke, Hong, O'Donnell, Howell, Miers, DeNardo and DeNardo 1999).

1.4 Histopathology

Lesions from all clinical subtypes of KS share the same histopathological features such as infiltration of spindle cells, presence of activated proliferating endothelial cells forming abnormal blood vessels (slit-like vessels), extravasation of red blood cells, infiltration of mononuclear cells, and the presence of neoangiogenesis and oedema. The first histological change of KS is the appearance of an inflammatory cell infiltrate that precedes spindle cell formation (Dorfman 1984; Fiorelli, Gendelman, Sirianni, Chang, Colombini, Markham, Monini, Sonnabend, Pintus, Gallo and Ensoli 1998). Immunohistochemical analysis of KS lesions shows the presence of T cells, particularly CD8⁺ T cells, monocytes, macrophages and dendritic cells, whereas B cells are few or absent (Regezi, MacPhail, Daniels, DeSouza, Greenspan and Greenspan 1993; Tabata, Langford, Becker and Reichart 1993; Uccini, Ruco, Monardo, Stoppacciaro, Dejana, La Parola, Cerimele and Baroni 1994; MacPhail, Dekker and Regezi 1996; Uccini, Sirianni, Vincenzi, Topino, Stoppacciaro, Lesnoni La Parola, Capuano, Masini, Cerimele, Cella, Lanzavecchia, Allavena, Mantovani, Baroni and Ruco 1997). These cells produce multiple cytokines including interleukin-6, interleukin-1 β and tumour necrosis factor- α (Miles, Rezai, Salazar-Gonzalez, Vander Meyden, Stevens,

Logan, Mitsuyasu, Taga, Hirano, Kishimoto and et al. 1990; Fiorelli, Gendelman et al. 1998). These cytokines activate endothelial cells to acquire the KS cell phenotype and to produce angiogenic factors that mediate angiogenesis, oedema and cell growth. In early lesions, KS spindle cells are few and are intermingled with stromal cells. However, with progression, the spindle cells fill the stroma between vascular spaces and the lesions acquire a more monomorphic characteristic, resembling a fibrosarcoma.

Histologically, KS can be classified into three stages. In the early patch stage, the histological changes are confined mainly to the reticular dermis usually in a periadnexal distribution. A proliferation of small, irregular and jagged endothelium-lined spaces surrounding normal dermal vessels and adnexal structures accompanied by a variable, inflammatory lymphocytic infiltrate (with or without plasma cells) is characteristic (Ackerman 1979).

In the intermediate plaque stage, the degree of vascular proliferation involving the whole thickness of the dermis increases with progressive disease. Within the deeper part of the dermis, spindle cell proliferation is seen with the beginnings of slit-like blood vessel formation. Haemosiderin deposits and eosinophilic hyaline globules are typically present. A perivascular inflammatory infiltrate is usually found. Histiocytes with the phenotype of either factor XIIIa or S-100 dermal dendritic cells as well as T cells and B cells including plasma cells are also present (McNutt, Fletcher and Conant 1983; Modlin, Hofman, Kempf, Taylor, Conant and Rea 1983).

In the most advanced nodular stage, the lesions are composed of fascicles of spindle cells with mild to moderate cytological atypia, single cell necrosis and entrapped erythrocytes within an extensive network of slit-like vascular spaces. Vessels lacking a prominent infiltration of pericytes have a fragmented basal lamina and frequent discontinuities in the endothelial lining. Erythrophagocytosis and necrosis of individual endothelial cells may also be present (McNutt, Fletcher et al. 1983).

Most of the spindle cells in KS lesions express endothelial markers including CD31 and CD34 (Sturzl, Brandstetter and Roth 1992). Factor VIII-related antigen is expressed by considerably fewer numbers of cells. However, KS spindle cells also express markers of smooth muscle cells, macrophages (CD68) and dendritic cells, suggesting that spindle cells either are derived from pluripotent mesenchymal precursors or represent a heterogeneous population of cells. Importantly, KS spindle cells do not stain with PAL-E, a marker expressed on vascular endothelium (Kaaya, Parravicini, Ordonez, Gendelman, Berti, Gallo and Biberfeld 1995).

The cellular origin of KS is difficult to determine because the lesions include several cell types (Dayan and Lewis 1967; Roth, Brandstetter and Sturzl 1992). Most studies indicate that KS consists of a heterogeneous cell population dominated by activated vascular and lymphatic endothelial cells mixed with cells of macrophage origin. Some of the spindle cells express markers of monocyte-

macrophages and endothelial cadherin, an adhesion molecule that is expressed by vascular endothelial cells (Uccini, Ruco et al. 1994; Uccini, Sirianni et al. 1997). Spindle cells with the endothelial and macrophages phenotype can also be cultured from the peripheral blood mononuclear cells of patients with KS or at risk of KS. These spindle cells proliferate particularly in the presence of the same cytokines that are produced by the peripheral blood mononuclear cells from patients with KS or by inflammatory cells infiltrating KS cells (Browning, Sechler, Kaplan, Washington, Gendelman, Yarchoan, Ensoli and Gallo 1994; Sirianni, Uccini, Angeloni, Faggioni, Cottoni and Ensoli 1997). These data suggest that the spindle cells in KS lesions and endothelial macrophages are related cell types and that circulating spindle cells may be the cell progenitors of KS. The simultaneous recruitment into tissues of the progenitors may lead to the appearance of multiple lesions at independent sites.

The histological differential diagnosis of cutaneous KS includes targetoid haemosiderotic haemangioma, benign lymphoangioendothelioma, acroangiodermatitis, cutaneous angiosarcoma, spindle cell haemangioendothelioma, angioliipoma, fibrous histiocytoma and bacillary angiomatosis (Gange and Jones 1979; Chor and Santa Cruz 1992; Larralde, Gonzalez, Marietti, Nussembaum, Peirano and Schroh 2001).

1.5 Initiation and development of Kaposi's sarcoma

The enhanced cytokine level, such as interleukin-6 and basic fibroblast growth factor, in individuals at risk of KS plays a key role in KS initiation (Lafeuillade,

Kaplanski and Allegre 1996). In fact experimental studies indicate that these cytokines induce several events associated with KS development, which include activation of endothelial cells to express adhesion molecules, induction of cytokines that recruit circulating cells into tissues, and induction of the endothelial cells to acquire the phenotypic properties of the KS spindle cells. The cytokines also induce the production of angiogenic factors that mediate endothelial cells and spindle cell proliferation, angiogenesis, and oedema.

In addition, the cytokines provide a major stimulus for HHV8 reactivation in circulating cells leading to viral spread and dissemination in tissues. In turn, HHV8 dissemination induces immune responses that are not capable of controlling virus infection and paradoxically exacerbate the reactive processes initiating KS lesions.

1.6 Natural history of Kaposi's sarcoma

KS initiation is the result of the complex interaction of several factors including cytokines induced by infectious agents such as HIV and HHV8 or other unknown factors. In time, KS progresses to a true sarcoma, most likely due to the deregulated expression of oncogenes, tumour suppressor genes, and the effects of viral genes endowed with proliferative and anti-apoptotic properties, including HHV8 latency genes and the HIV-1 Tat protein. In addition, overt immune suppression also plays a role in KS initiation and progression by allowing uncontrolled HHV8 replication and/or tumour growth.

Aside from homosexual men, HHV8 infection has only a weak or no association with sexual practices or HIV infection. This is in line with weak if any clustering of classical KS in spouses (Cottoni, Masia, Masala, Mulargia and Contu 1996). Mother to infant transmission of HHV8 has been reported in Africa (Andreoni, El-Sawaf, Rezza, Ensoli, Nicastri, Ventura, Ercoli, Sarmati and Rocchi 1999), which is plausible given the occurrence of KS in African children. Without HIV infection or iatrogenic immune deficiency, the risk of KS is low. For example, in parts of Italy where the HHV8 seroprevalence is 20%, the annual incidence of classical KS is 6.2 and 2.5 per 10,000 males and females respectively (Geddes, Franceschi, Barchielli, Falcini, Carli, Cocconi, Conti, Crosignani, Gafa, Giarelli and et al. 1994). With the assumption that classical KS occurs only with HHV8 seropositivity, the annual risk of KS is less than 1 per 1000.

In contrast, homosexual men with HIV have a very high risk of developing KS. Compared with the general population, KS risk is increased approximately 100,000-fold for homosexual men with AIDS with approximately 13,000-fold for others with AIDS. The seven-fold higher risk for homosexual men compared to other patients with AIDS approximately reflects their higher prevalence of HHV8. Likewise, the absolute risk of KS in patients with AIDS is great: 35% within ten years of HIV and HHV8 infection (O'Brien, Kedes, Ganem, Macrae, Rosenberg, Molden and Goedert 1999). The risk of KS in patients with AIDS is not due to HIV infection per se, but rather to the level of immune deficiency, as measured by CD4 lymphocyte count. In a prospective study, KS developed in

12.5% of patients with a CD4 count of less than 300 cells/ μ L, compared with 0% in patients with a CD4 count of more than 550 cells/ μ L (Goedert, Biggar, Melbye, Mann, Wilson, Gail, Grossman, DiGioia, Sanchez, Weiss and et al. 1987). Cofactors for AIDS and non-AIDS KS are poorly defined and must be re-examined in view of the recent discovery of HHV8. Despite similar HHV8 seroprevalence rates by gender, males are at higher risk of developing KS than females. This male predominance in all clinical subtypes remains unexplained. Early stage KS may not be a true malignancy, but rather a hyperplastic reactive inflammatory process. This is suggested by the simultaneous appearance of multiple lesions in different parts of the body and the regression of early KS lesions upon the withdrawal of immunosuppressive therapy (Zisbrod, Haimov, Schanzer, Ambinder and Burrows 1980; Brooks 1986). The reactive, non-neoplastic nature of KS is also indicated by the polyclonal nature of early lesions, as determined by the analysis of the methylation pattern of the androgen receptor gene (Gill, Tsai, Rao, Spruck, Zheng, Harrington, Cheung, Nathwani and Jones 1998). Studies with long term cultures of KS spindle cells suggest that KS is a cytokine-mediated disease, with an inflammatory and granulation reactive type of process that resembles hyperplastic infiltration rather than a malignant neoplasm (Costa and Rabson 1983). In agreement with these data, HHV8 genomic terminal repeats also show a polyclonal or oligoclonal pattern in most KS lesions analysed. These data support the concept that KS begins as a reactive process and suggest that, in the early stage, HHV8 acts by participating in the reactive process rather than by transforming the lesion to a malignant phenotype.

However, aneuploidy, microsatellite instability, and clonality have been found in advanced lesions (Rabkin, Bedi, Musaba, Sunkutu, Mwansa, Sidransky and Biggar 1995; Rabkin, Janz, Lash, Coleman, Musaba, Liotta, Biggar and Zhuang 1997). Transformed cell lines have also been established from nodular lesions (Lunardi-Iskandar, Gill, Lam, Zeman, Michaels, Mann, Reitz, Kaplan, Berneman, Carter and et al. 1995). These indicate that KS can be transformed into an advanced stage with the emergence of monoclonality. The long-term expression of the HHV8 latency genes associated with the deregulated expression of oncogenes or tumour suppressor genes, such as p53, Bcl2 and c-myc, may play a key role in the transformation of KS into a true malignancy. In addition, KS Tat protein may act as a progression factor in AIDS-related KS by inducing growth, migration and invasion of KS. Overt immunodeficiency may facilitate KS progression due to lack of control of tumour growth leading to chromosomal changes, as suggested by the presence of microsatellite instability in AIDS-related KS but not in other clinical subtypes.

1.7 Role of human herpes virus 8 in the development of Kaposi's sarcoma

1.7.1 Epidemiology and transmission of HHV8

The KS associated herpes virus was first identified in the tissue of a KS lesion from a patient with AIDS in 1994 (Chang, Cesarman et al. 1994). The virus is the eighth human herpes virus identified and is closely related to the Epstein Barr virus. It is now considered that the presence of HHV8 is the initiating factor

for the development of KS and that host immunosuppression is an important cofactor in the development of KS in HHV8 infected patients (Antman and Chang 2000).

The pattern of HHV8 infection parallels the geographical distribution of classical KS but following the emergence of the AIDS epidemic, the local rate of infection with HIV determines the rate of AIDS-related KS (Moore 2000). HHV8 infection is uncommon in Asia, North America and Northern Europe. The rate is moderate in Mediterranean and Eastern European countries. However, high HHV8 infection rates are present in Central and Southern Africa (Moore 2000).

Homosexual and bisexual men appear to have a higher risk of HHV8 infection in Western countries. HHV8 appears to be spread by sexual transmission, but there is emerging evidence that other routes of transmission may be significant also (Kedes, Operskalski, Busch, Kohn, Flood and Ganem 1996; Kedes, Ganem, Ameli, Bacchetti and Greenblatt 1997; Martin, Ganem, Osmond, Page-Shafer, Macrae and Kedes 1998). Similar to the Epstein Barr virus, HHV8 is not found in significant numbers in seminal fluid or the rectum but is usually found in the oral cavities of immunosuppressed patients (Moore 2000; Pauk, Huang, Brodie, Wald, Koelle, Schacker, Celum, Selke and Corey 2000). This suggests that transmission of the virus through exposure to saliva may be more important than sexual transmission. This may account for the high rates of infection in parts of Africa, where sexual transmission of HHV8 is a relatively unimportant route, as most infections occur through some form of casual contact among

young children and adults. However, oral transmission of the virus in North America appears to be more likely to be confined to homosexual men, particularly if they are immunocompromised. The relative sparing of other groups, such as women and children, suggests that there are other unidentified factors which cause the transmission of the virus (Pauk, Huang et al. 2000). More recently, HHV8 transmission has also been documented from infected donors to organ transplant recipients (Luppi, Barozzi, Schulz, Setti, Staskus, Trovato, Narni, Donelli, Maiorana, Marasca, Sandrini and Torelli 2000).

1.7.2 Detection of HHV8

HHV8 DNA can be detected in peripheral blood cells using standard polymerase chain reaction and Southern blot hybridisation (Whitby, Howard, Tenant-Flowers, Brink, Copas, Boshoff, Hatzioannou, Suggett, Aldam, Denton and et al. 1995; Moore, Kingsley, Holmberg, Spira, Gupta, Hoover, Parry, Conley, Jaffe and Chang 1996). However, serological assays using specific antibodies are more sensitive in detecting HHV8 and such methods are preferable to polymerase chain reaction for detecting past viral exposure. Persons who are exposed to HHV8 develop life-long antibody responses to the viral antigens, although these responses may be lost in patients with advanced stage of AIDS (Antman and Chang 2000). Indirect immunofluorescence assay for the latency-associated nuclear antigen of the virus and enzyme-linked immunosorbent assay, which uses recombinant antigens made from the open-reading-frame proteins 65 and K8.1A of the HHV8, can be used in combination

for diagnostic purposes. In tissue sections, HHV8 can be detected by immunohistochemistry (Katano, Sata, Suda, Nakamura, Tachikawa, Nishizumi, Sakurada, Hayashi, Koike, Iwamoto, Kurata and Mori 1999; Katano, Sato, Kurata, Mori and Sata 1999) or in situ hybridisation (Sturzl, Wunderlich, Ascherl, Hohenadl, Monini, Zietz, Browning, Neipel, Biberfeld and Ensoli 1999).

The HHV8 virus can also be detected on electron microscopy as virions with the size and shape characteristic of those seen in the herpes virus family. The ultrastructural identification of the virus can be difficult due to the frequent presence of other herpes viruses, particularly the cytomegalovirus (CMV). However, there are a few specific ultrastructural features that allow for the distinction of HHV8 from the cytomegalovirus (Said, Chien, Tasaka and Koeffler 1997). HHV8 viral intranuclear nucleocapsids are about 110 nm in diameter and smaller than CMV particles (150-200 nm). The viral envelope of HHV8 is less pleomorphic than that of CMV. These ultrastructural features can be used to identify HHV8 in suitably preserved tissues (Said, Chien et al. 1997).

1.7.3 Molecular genetics of HHV8 in neoplasia

HHV8 is a gamma herpes virus which is known to be important in carcinogenesis, particularly in lymphoproliferative disorders and lymphomas (Moore, Gao, Dominguez, Cesarman, Lungu, Knowles, Garber, Pellett, McGeoch and Chang 1996). More specifically, HHV8 is a rhadinovirus with more than 80 open reading frames and has genes with transforming potential (Lee, Veazey, Williams, Li, Guo, Neipel, Fleckenstein, Lackner, Desrosiers and

Jung 1998; Kuper, Adami and Trichopoulos 2000). The virus has a 165 kb genome and its entire coding region is flanked by terminal-repeat sequences (Russo, Bohenzky, Chien, Chen, Yan, Maddalena, Parry, Peruzzi, Edelman, Chang and Moore 1996).

It has recently become evident that there are essentially three known mechanisms in which HHV8 can contribute to the neoplastic process. The first two pathways involve inhibition of the two principal tumour suppressor genes in cells: retinoblastoma (Rb) gene and p53, which regulate cellular senescence and apoptosis respectively (Sarid, Sato, Bohenzky, Russo and Chang 1997). The third mechanism involves the prevention of interferon from repressing the c-myc oncogene by the HHV8 viral interferon regulatory factor (Gao, Boshoff, Jayachandra, Weiss, Chang and Moore 1997; Jayachandra, Low, Thlick, Yu, Ling, Chang and Moore 1999).

The HHV8 gene encodes numerous proteins that are homologous to cell signalling and regulatory pathway proteins. One of the virus genes encodes proteins that are homologous to human oncoprotein is v-cyclin, which can promote phosphorylation and inactivation of the Rb tumour suppressor gene and is important in inhibiting apoptosis (Chang, Moore, Talbot, Boshoff, Zarkowska, Godden, Paterson, Weiss and Mitnacht 1996; Li, Lee, Yoon, Albrecht, Fleckenstein, Neipel and Jung 1997). The kinase activity triggered by this viral cyclin is sensitive to the cdk inhibitor p27, due to activation of cdk6, and may abrogate cell cycle checkpoints by phosphorylating not only the Rb

protein but also histone H1 (Godden-Kent, Talbot, Boshoff, Chang, Moore, Weiss and Mitnacht 1997).

The virus also encodes a bcl-2-like protein that can prevent apoptosis (Cheng, Nicholas, Bellows, Hayward, Guo, Reitz and Hardwick 1997; Sarid, Sato et al. 1997). The major latency-associated nuclear antigen of the virus can interact with p53 and inhibit transcriptional activity mediated by p53 (Friborg, Kong, Hottiger and Nabel 1999; Ojala, Yamamoto, Castanos-Velez, Biberfeld, Korsmeyer and Makela 2000; Rivas, Thlick, Parravicini, Moore and Chang 2001). Other proteins produced by HHV8 that regulate cellular proliferation include a G-protein-coupled receptor, the Fas-associated death domain-like interleukin-1 β converting enzyme mediated apoptotic inhibitor, an active immunoreceptor and an interferon signalling inhibitor (Gao, Boshoff et al. 1997; Thome, Schneider, Hofmann, Fickenscher, Meini, Neipel, Mattmann, Burns, Bodmer, Schroter, Scaffidi, Krammer, Peter and Tschopp 1997; Bais, Santomasso, Coso, Arvanitakis, Raaka, Gutkind, Asch, Cesarman, Gershengorn, Mesri and Gerhengorn 1998; Lee, Veazey et al. 1998; Sturzl, Hohenadl, Zietz, Castanos-Velez, Wunderlich, Ascherl, Biberfeld, Monini, Browning and Ensoli 1999). HHV8 encodes an interleukin-6 that can induce B cell proliferation and chemokines that can activate angiogenesis and inhibit the immune type 1 helper T cell responses (Molden, Chang, You, Moore and Goldsmith 1997; Stine, Wood, Hill, Epp, Raport, Schweickart, Endo, Sasaki, Simmons, Boshoff, Clapham, Chang, Moore, Gray and Chantry 2000). The interaction between angiogenic factors and cytokines has been postulated to be

responsible for the development of KS (Ensoli, Nakamura, Salahuddin, Wong-Staal and Gallo 1989; Cai, Gill, Masood, Chandrasoma, Jung, Law and Radka 1994; Hong, Zhang et al. 2001).

However, expression of the different HHV8 proteins appears to be dependent on infected cell types and the diseases produced. Viral interleukin-6 is only found in HHV8 infected B cells but the viral latency-associated nuclear antigen is expressed in all cells infected by HHV8. The virus infects all the spindle shaped cells in KS but only a small minority contains the actively replicating virus (Moore, Boshoff, Weiss and Chang 1996; Zhong, Wang, Herndier and Ganem 1996).

1.7.4 HHV8 infection and Kaposi's sarcoma

KS is the commonest tumour associated with HHV8 and the virus has been detected in more than 95 per cent of these lesions (Antman and Chang 2000). HHV8 is found in the tumour spindle cells, in normal appearing endothelial cells within lesions and in tumour-infiltrating leukocytes in KS lesions (Blauvelt 1999). HHV8 can cause all clinical subtypes of KS and the virus can be detected in AIDS-related KS, classical KS and the KS that occurs in HIV negative homosexual men (Moore and Chang 1995). In addition, the risk of developing KS increases with increasing antibody titre to HHV8 although for a given titre, the risk is greater in HIV positive patients compared with HIV negative patients (Sitas and Newton 2001).

The role of HHV8 in the pathogenesis of KS appears to be related to some of the proteins produced by the virus. HHV8 is found to produce cytokines that can activate endothelial cells, which in turn leads to adhesion and tissue extravasation of lymphocytes and monocytes, spindle cell formation and angiogenesis (Ensoli, Sgadari, Barillari, Sirianni, Sturzl and Monini 2000). The cytokine, viral IL-6, produced by HHV8, is structurally homologous to human and murine IL-6 and has been shown to increase haematopoiesis and plasmacytosis in spleen and lymph nodes, and cause an increase in expression of vascular endothelial growth factor, which can result in angiogenesis. Thus, viral IL-6 is a multifunctional cytokine and its expression appears to play a significant role in the pathogenesis of HHV8 associated disorders (Aoki, Jaffe, Chang, Jones, Teruya-Feldstein, Moore and Tosato 1999). In addition, both IL-6 and platelet-derived growth factor appear to be important for the survival of KS cells (Ascoli, Scalzo, Andreoni, Manente, Pistilli and Lo Coco 1999).

During the lytic phase of viral infection, HHV8 expresses chemokine-like proteins that can activate the highly angiogenic chemokine receptors, CCR8 and CCR3, suggesting a possible pathogenic role in KS (Boshoff, Endo, Collins, Takeuchi, Reeves, Schweickart, Siani, Sasaki, Williams, Gray, Moore, Chang and Weiss 1997; Dairaghi, Fan, McMaster, Hanley and Schall 1999; Benelli, Barbero, Buffa, Aluigi, Masiello, Morbidelli, Ziche, Albin and Noonan 2000; Luttichau, Stine, Boesen, Johnsen, Chantry, Gerstoft and Schwartz 2000; Luttichau, Lewis, Gerstoft and Schwartz 2001). Another chemokine, vMIP-III,

encoded by the virus and expressed in KS lesions, also stimulates angiogenesis and selectively chemoattracts TH2 cells (Stine, Wood et al. 2000).

It is now believed that KS most likely begins as a reactive process of a polyclonal nature, and early KS can regress. However, with deregulated expression of oncogenes and tumour suppressor genes, continued expression of the HHV8 latency genes and, in the case of AIDS-related KS, the proliferative and angiogenic effects of the HIV-1 Tat protein, KS can in time progress to a true malignant sarcoma (Ensoli and Sturzl 1998; Ensoli, Sgadari et al. 2000). In addition, reduced expression of p27, due to HHV8 infection, occurs in KS progression through its various histopathological stages, leading to eventual extracutaneous spread (Swanton, Mann, Fleckenstein, Neipel, Peters and Jones 1997; Fernandez-Figueras, Puig, Penin, Mate, Bigata and Ariza 2000). This is because HHV8 encoded v-cyclin complexes with cyclin-dependent kinase 6 that can result in phosphorylation and proteasome-mediated degradation of p27. Down-regulation of p27 expression is known to facilitate metastatic dissemination in a number of other human neoplasms (Fernandez-Figueras, Puig et al. 2000).

Another HHV8 product that acts via the vascular endothelial growth factor pathway, which may explain the angioproliferative nature of KS, is the homologue of the Epstein Barr virus SM protein, KS-SM. KS-SM enhances the expression of KDR/flk-1, which is a receptor for VEGF. KDR/flk-1 expression is increased in KS and its stimulatory effect on VEGF enhances the proliferation of

HHV8-infected cells (Mesri 1999; Gupta, Ruvolo, Patterson and Swaminathan 2000).

1.8 Role of HIV in the development of Kaposi's sarcoma

Before the discovery of HHV8, it was thought that cellular cytokines, growth factors and the HIV-1 Tat protein initiated and promoted the growth of KS spindle cells (Ensoli, Barillari and Gallo 1992). The fact that the incidence of KS is much higher in HIV-infected individuals than in iatrogenically immunosuppressed patients probably reflects the prevalence of HHV8 in different populations, rather than HIV itself playing a role in the pathogenesis. KS, like HHV8, is uncommon in patients who acquired HIV infection through intravenous drug use or from blood transfusion. Moreover, rapidly progressive KS affecting extensive areas of skin and viscera is seldom seen in HIV-negative patients, even in those who have been iatrogenically immunosuppressed for prolonged periods. This observation favours a synergistic role for HHV8 and HIV-1 in promoting the growth of KS spindle cells. A role for HIV-1 in the pathogenesis of KS is supported by the observation that KS can rapidly resolve with highly active anti-retroviral therapy. Whether these responses are purely due to a restoration of cellular immunity against HHV8 or to the lower circulating HIV viral load is not known. In contrast, HHV8 positive Castleman's disease does not often resolve with HAART and can progress to fatal lymphoma. These differential responses may reflect the behaviour of cytokine-driven neoplasia compared with clonal tumours.

1.9 Cell cycle regulation

Cell proliferation is an ordered, tightly regulated process involving multiple checkpoints that assess extracellular growth signals, cell size and DNA integrity. The somatic cell cycle is divided into an interphase, designated for cellular growth and DNA synthesis, and a mitotic phase, in which a single cell divides into two daughter cells. Interphase is further subdivided into two gap phases (G1 and G2) separated by a phase of DNA synthesis (S phase). The vast majority of human cells exist in a non-dividing, terminally differentiated state. In contrast, stem cells exist in a resting G0 quiescent state. Upon appropriate external stimulation, G0 cells can enter the cell cycle into early G1, likewise cycling cells present in early G1 can exit into G0 when deprived of external growth stimulus (Zetterberg, Larsson and Wiman 1995).

There are three parameters that are critical to cancer growth: first, the duration of a single cell cycle; second, the proportion of cells actively proliferating (growth fraction); and third, the rate of cell loss as a result of death or apoptosis (Tubiana 1989). The lengths of tumour cell cycle measured in vivo and in vitro are not significantly shorter than those of normal cells, suggesting that neoplastic cells are acquiring ability that either enhance the growth fraction and/or minimal cell loss, rather than simply decreasing the cell cycling duration. Consequently, processes that govern cell cycle entrance/exit and metabolism are perhaps more important to tumour growth than those events that regulate the rate of individual cell division.

The orderly progression of cells through the G1, S, G2 and M phases of the cell cycle is governed by a series of proteins called cyclins, which exert their effects by binding to and activating a series of specific cyclin dependent kinases (CDKs). This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and specific CDK inhibitor proteins such as p16, p21 and p27 (Sgambato, Flamini, Cittadini and Weinstein 1998).

The point between the early G1 and late G1 phase passage represents an irreversible commitment to undergo cell division is termed the “restriction point”. One of the important regulating factors for this restriction point is retinoblastoma (Rb) protein. The Rb gene is a tumour suppressor gene and acts as a negative regulator at this restriction point. Phosphorylation of the Rb protein by the cyclin D1 can release E2F transcription factors, which enhance the G1-S transition and the onset of DNA synthesis in the S phase (Ezhevsky, Ho, Becker-Hapak, Davis and Dowdy 2001). Moreover, genetic studies in *Drosophila* point to a role for cyclin D1/cdk4 in promoting cell growth and metabolism during G1, a vital role to sustain uncontrolled divisions in neoplastic cells. The following is a brief overview on the individual cell cycle proteins examined in this thesis and the details on the function of each protein will be presented later in the thesis.

p53 is also involved in the control of cell cycle check points. p53 protects cell, from neoplasia by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of stresses (el-Deiry, Harper, O'Connor, Velculescu,

Canman, Jackman, Pietenpol, Burrell, Hill, Wang and et al. 1994). Part of the mechanism by which p53 blocks cells at the G2 checkpoint involves the inhibition of CDK/p34, the cyclin dependent kinase required for entry into mitosis. One of the target genes of p53 is cyclin G1. The function of cyclin G1 is to serve as a negative regulator of p53 by activating Mdm2 through dephosphorylation. It has been shown that cyclin G1 null mice had fewer and smaller carcinogenic tumours (Okamoto and Beach 1994).

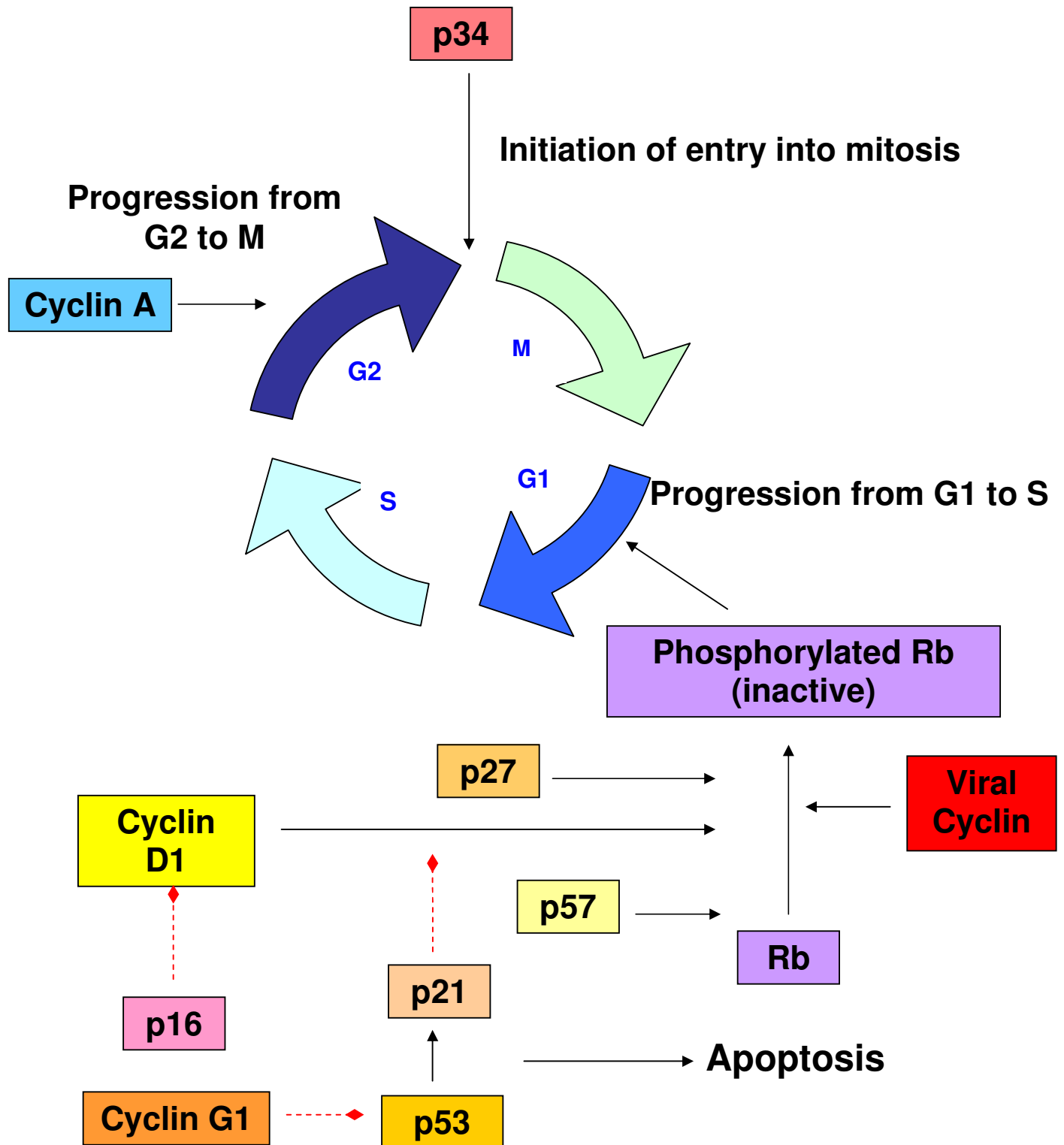
p27KIP1 is a cyclin dependent kinase inhibitor that plays an important role in the control of cellular progression from G1 to S phase of the cell cycle. Cytosolic localisation of p27 has been correlated with increased tumour aggressiveness (Jiang, Zhao and Verfaillie 2000). Alexander et al. have demonstrated that p27 is a down stream regulator of Rb-mediated senescence and this suggest that p27 plays a critical role in mediating cell cycle exit (Alexander and Hinds 2001).

Cyclin A is a protein kinase and has a pivotal role in the G2/M phase transition as a mitotic cyclin during the cell cycle. It binds to cdk2 and can modulate the DNA replication in the S phase and the initiation of mitosis (Pagano, Pepperkok, Verde, Ansorge and Draetta 1992; Rosenblatt, Gu and Morgan 1992). Studies have suggested that cyclin A is associated with malignant transformation of cells and is a useful prognostic indicator for some cancers (Pines and Hunter 1990; Wang, Zindy, Chenivesse, Lamas, Henglein and Brechot 1992). p34 is also a mitotic cyclin. It is a serine/threonin kinase which is involved in cell cycle

regulation. When complexed with cyclins, p34 can initiate the entry of cells into mitosis by promoting nuclear envelope breakdown (Lukas, Draetta and Bartek 1992).

The relationships of the aforementioned proteins to the cell cycle are summarised in Figure 1.4.

Figure 1.4 Regulation of the cell cycle by cell cycle proteins



1.10 Hypothesis and aims

The work in this thesis can generally be summarised as aiming towards the understanding of the factors responsible for the pathogenesis and disease progression of the two main clinical subtypes of KS, using clinically available tissue samples. The reason for mainly using tissue samples for the experimental work in this thesis is that the results generated may better reflect the in vivo processes involved in KS development and progression. The following hypotheses were generated:

1. During the early phase of the candidate's work in 1999, there were suggestions that an infectious agent, probably from the herpes family of viruses, may be the initiating factor for the development of KS. It was the initial hypothesis and plan of this thesis to elucidate the association of KS with the human herpes viruses including the Epstein Barr virus. However, by the year 2000, there was conclusive evidence that the HHV8 was directly responsible for initiating the development of KS. Despite the discovery of HHV8 as the initiating agent for the development of KS, the virus is not seen in parts of Africa where KS is endemic. Therefore, it was hypothesised that there may be a small subgroup of cases in which development of the KS lesions may not be related to HHV8 infection.
2. The different clinical presentation and disease progression seen in AIDS-related KS and classical KS suggest that there may be different underlying mechanisms for the progression and manifestation of the disease. It is only

logical to hypothesize, that the different presentations of the various clinical subtypes of KS may be related to these different cell cycle pathways that the HHV8 virus, the HIV infection and/or the immunosuppression associated with HIV infection have channelled into the affected cells.

3. Since the HHV8 viral genome has an open reading frame (ORF 72) with a sequence similar to the human cyclin D protein and in view of the importance of the cyclins in the regulation of the cell cycling pathway, especially in the development of malignancy, it is hypothesised that the cell cycle proteins are important in the progression of the HHV8 infected and non HHV8 infected KS.

The aims of this thesis were:

1. To document the clinical and pathological features of a cohort of patients in a tertiary referral centre that includes both classical and AIDS-related KS cases.
2. To demonstrate the incidence of HHV8 infection in the KS in the above cohort.
3. To investigate the expression of cell cycle proteins of the Rb-p21 pathway in KS lesions.
4. To examine the expression of cell cycle proteins of the p53-apoptotic pathway in KS.
5. To demonstrate the frequency of expression of the mitotic cyclins, cyclin A and p34^{cdc2}.
6. To demonstrate any difference in expression of the above cell cycle proteins in classical and AIDS-related KS lesions.
7. To demonstrate any difference in the expression of the above cell cycle proteins in HHV8 infected and non-HHV8 infected KS lesions.

8. To demonstrate by in vitro study that non-HHV8 infected KS lesions progress through a different molecular route that may not involve the cyclin D1 pathway.

Chapter 2: Methods and Materials

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2.1 Clinico-pathological study

The study cohort consisted of 43 patients diagnosed with KS by histology between 1990 to 1999 at the Royal Prince Alfred Hospital, Sydney, Australia. Eligible patients were identified using the database from departments of Anatomical Pathology and Radiation Oncology. Ethics approval for this study was obtained from the Ethics Review Committee of the Central Sydney Area Health Service, Protocol No. X00-0041.

The clinical data were obtained from the Medical Records Department of the Central Sydney Area Health Service. Each individual case history was reviewed to obtain the demographic and clinical data (Table 2.1). Paraffin-embedded specimens were retrieved from the archive for this study. The original slides were reviewed to confirm the diagnosis of KS and the histological features were further defined (Table 2.2). All results were entered into a computer database (Filemaker Pro) and statistical analysis was performed using the Excel and Statistica programs.

Table 2.1 Demographic and clinical data

Demographic Data

Age at first diagnosis of KS

Sex

Country of birth

Ethnic origin

Clinical Data

Date of first diagnosis of KS

Involved sites

HIV status

Treatment for KS

Response

Recurrence

If HIV positive

Risk factor for HIV infection

Treatment for HIV infection

CD4 count at diagnosis of KS

Concurrent infection

Other Malignancy

Other AIDS-related illness

Table 2.2 Pathological data

Histological Stage

Lesional cell mitosis

Single cell necrosis

Apoptosis

Single cell anaplasia

Presence of dissecting blood vessel

Presence of plasma cell

Protuberant blood vessel

Hyaline globules

Red cell extravasation

Presence of haemosiderin

2.2 Fixation of specimens

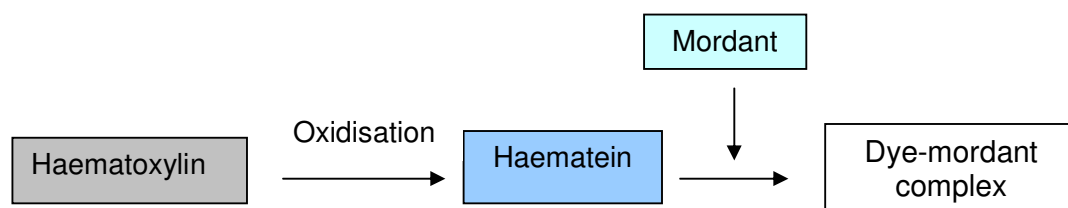
The archival material obtained for this study was fixed in 10% buffered formaldehyde. After fixation, the specimens were blocked and processed for dehydration. The blocks were then embedded in paraffin wax and sectioned to 4µm thickness for haematoxylin and eosin staining.

Fixation prevents autolysis and degradation of the specimen by bacteria. Fixation also preserves the shape and volume of the specimen, hence as close to their in vivo state as possible (Durgun-Yucel, Hopwood and Yucel 1996). The aldehydes (formaldehyde and glutaraldehyde) are known to form cross-links with protein molecules with the reaction being basic amino acid lysine. The reaction is pH dependent and the reaction occurs more rapidly at a higher pH. The most commonly used aldehyde is formaldehyde (10% buffered solution), which causes relatively little cross-linking under fixation conditions, whereas glutaraldehyde is most effective at cross-linking with proteins. The reaction between formaldehyde and the tissue specimen is reversible, whereas with glutaraldehyde, the reaction is rapid and irreversible. Fixation with glutaraldehyde gives a better morphological preservation than formaldehyde but it does not preserve the enzymes in the specimen and hence is unsuitable for immunohistochemistry.

2.3 Haematoxylin and eosin staining

Haematoxylin is an extract from the Haematoxylin Campechianum tree. Its use as a biological stain was first reported in 1863. Haematoxylin needs to be oxidised into haematein for tissue staining. Oxidisation can occur either naturally by exposure to light and air (e.g. Ehrlich and Delafield's haematoxylin) or by the use of an oxidising agent such as mercuric oxide in Harris's haematoxylin. A mordant, such as aluminium, iron or tungsten, is added to form a positive charge dye mordant complex. This positive charge complex can then bind to nuclear chromatin which is anionic or negatively charged. Haematoxylin-aluminium complex is the most commonly used as it produces strong nuclear staining.

Figure 2.1 Reaction of Haematoxylin dye



Haematoxylin staining can be performed by progressive or regressive methods. With the progression method, the section is stained up to an optimum point to provide adequate staining of the nuclei but leaving the surrounding tissue relatively unstained (e.g. Mayer's haematoxylin). With the regressive method,

such as Harris's haematoxylin, the section is overstained and then washed with acid alcohol to obtain optimum stain intensity.

Eosin is combined with haematoxylin in order to demonstrate cytoplasmic changes in cells and inclusion bodies. All specimens used in this study were stained with haematoxylin and eosin to define the location of the KS and normal surrounding tissue.

2.4 Immunohistochemistry

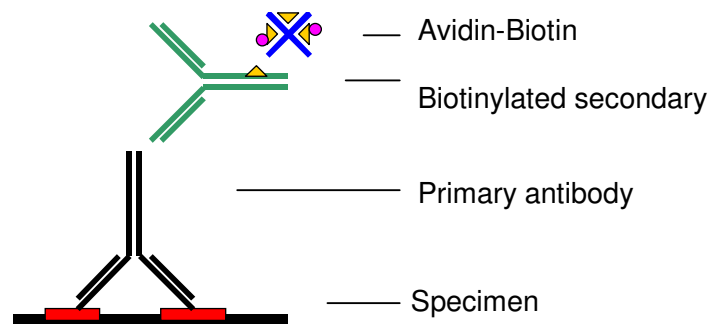
Immunohistochemical staining was first used in 1941. The principle of the immunoperoxidase (IPX) method is to seek and identify specific antigens in tissues with an antibody. The antigen-antibody complex is then made visible under the light microscope. The IPX method can be used in specimens fixed with formaldehyde or on frozen specimen. The labelling of the antigen sites is permanent and the immunoperoxidase methods show the distribution of cellular components. The tissue sections can also be stored for future retrieval. IPX may also be used for ultrastructural studies (Mesa-Tejada, Pascal and Fenoglio 1977).

Avidin Biotin Complex (ABC) method

The ABC method depends on the high affinity that avidin has for biotin. Avidin is a large glycoprotein derived from egg white and comprises four binding sites per molecule for biotin. The ABC method has been shown to be more sensitive than the peroxidase-antiperoxidase method (Hsu, Raine and Fanger 1981).

There are three essential steps to this method. Firstly, the primary antibody is added to the specimen, then a biotinylated secondary antibody is added, and finally the avidin-biotin peroxidase complex is added (Figure 2.2).

Figure 2.2 Avidin-biotin complex method



Antibody

Immunoglobulins (Ig) are produced by B lymphocytes and secreted into plasma. The main Ig classes are IgA, IgE, IgG and IgM. Class identity is determined by class specific sequences in the Fc region of the heavy chain. Polyclonal antibodies are produced by immunizing a rabbit with an immunogen bearing the target antigen. The antibodies are collected from the serum after the rabbit mounts a humoral response. Each clone of the polyclonal antibodies will demonstrate a slightly different specificity for the target antigen. Monoclonal antibodies recognise only a single epitope on the antigen. To produce a monoclonal antibody, a mouse is immunised with the target antigen. Once a large amount of antibody has been produced, the spleen is removed for collection of B lymphocytes, which are subsequently fused with myeloma cells

and grown in cell culture. The hybridomas are tested to determine which clone is producing the antibody against the desired epitope. The selected hybridoma can then be grown in tissue culture or can be injected back into the mouse model.

Monoclonal antibodies have a higher specificity and affinity to antigen than polyclonal antibodies. There is also less background staining of the tissue with monoclonal antibodies as polyclonal antibodies can produce contamination by the presence of irrelevant clones. However specific epitope recognised by monoclonal antibodies may be masked by formalin fixation and therefore require antigen retrieval for unmasking.

In this study, the paraffin blocks were cut at 4 μm , mounted on 5-aminopropyltriethoxysilan coated slides and allowed to dry overnight. The sections were dewaxed in xylene and were treated with microwave heating at 60°C for 20 minutes in a citrate buffer (2.1g/1000 ml; pH 6.0) for antigen retrieval. After the blocking of endogenous peroxidase, washing in phosphate-buffered saline, and the blocking of non-specific binding of secondary antibody with normal swine serum, routine streptavidin-biotin-peroxidase immunostaining with diaminobenzidine was applied to the sections incubated overnight with a murine monoclonal antibody (table 2.3). The primary antibody was substituted with phosphate-buffered saline in sections used as negative controls. Histologically normal dermal tissues located away from the primary lesions were used as normal non-neoplastic controls. The sections were counterstained with

Harris's haematoxylin. A minimum of 50 cells were examined to determine the positivity.

All the slides were coded to mask the clinical data and hence avoiding possible bias. All the slides were assessed by the candidate and an independent investigator. There was good correlation between the assessments by the two readers.

Table 2.3 List of primary antibody tested			
Primary Antibody	Ig Class	Clone	Source
Ki-67	IgG1		Dako
Cyclin A	IgG1	6E6	Novocastra
Cyclin D1	IgG2a	DCS-6	Novocastra
Cyclin G1	IgG2B	11C8	Novocastra
Cdc2/p34	IgG2a	POH-1	Novocastra
HHV8	IgG1	13B10	Novocastra
Mutant p53	IgG1	NCL-p53-505	Novocastra
p16	IgG2b	NCL-p16-432	Santa Cruz
Retinoblastoma	IgG1	13A10	Sigma
p57	IgG1	25B2	Novocastra
p27	IgG2	1B4	Novocastra

2.5 Statistical analysis

Correlations between IPX staining results and clinicopathological factors were analysed using the chi-squared (χ^2) contingency test. Multivariate analysis was performed using the stepwise multiple linear regression model by assessing the IPX staining result status in the KS lesions with the following baseline covariates: age, gender, HIV status, tumour stage, recurrent lesion, multiplicity of the lesion and involved site. Only p values of less than 0.05 were considered significant.

2.6 Detection of HHV8 in Kaposi's sarcoma cell culture

KS Y-1 was used for in vitro examination of cell cycle protein expression. KS Y-1 is an established immortal human KS cell line from cutaneous KS lesions from a patient with AIDS (Lunardi-Iskandar, Gill et al. 1995). The HHV8 status of this patient is unknown and the HHV8 status of the cell line has not been documented previously. The cells were grown in T100 flasks in RPMI 1640 with 5% fetal calf serum and incubated at 37°C in 5% CO₂ incubator. The culture was subcultured twice a week with a ratio of 1:8 to 1:10. The culture was checked regularly for mycoplasma contamination.

For the IPX staining, KS cells were harvested from confluent culture and a thin layer of cells was spread on a glass slide, allowed to airdry for 10 minutes and then fixed in ethanol for 10 minutes. The slides were blocked with 1.5% goat serum in 0.5% bovine serum albumin and incubated overnight at 4°C with

antibodies against HHV8 and human cyclin D1. The slides were then washed extensively and incubated with biotinylated goat anti-rabbit and the avidin-enzyme complex. For negative controls, the primary antibodies were replaced with normal rabbit serum immunoglobulin.

Chapter 3: Clinico-pathological Analysis

Chapter 3: Clinico-pathological Analysis

3.1 Histopathology and classification of Kaposi's sarcoma lesions at different stages of development

All the original slides were reviewed and classified into three different histological stages. Patch stage KS lesions are characterised by a poorly circumscribed collection of thin-walled vessels with some surrounding spindle cells dissecting into the stromal connective tissue associated with extravasation of erythrocytes, some lymphoplasmacytic inflammatory cells and oedema (Figure 3.1). Plaque stage KS lesions show similar features to those of the patch stage KS but with a more prominent number of spindle cells (Figure 3.2). The nodular KS lesion is featured by a circumscribed collection of spindle cell fascicles with some intervening thin-walled vessels, extravasation of red blood cells, haemosiderin collections and spindle cells with intracytoplasmic, PAS positive and diastase-resistant globules (Figures 3.3, 3.4, 3.5). There were 43 specimens of KS from 43 patients consisting of early or patch stage KS (21%), plaque stage (24%) and nodular stage (55%).

3.2 Distribution of lesions

The majority of the cases were cutaneous KS (28/43; 67%) and these were distributed at the following sites:

Table 3.1 Anatomical distribution of cutaneous lesions	
Legs:	10 (3 patch, 1 plaque , 6 nodular)
Feet:	5 (1 patch, 4 nodular)
Thigh/groin:	3 (2 patch, 1 plaque)
Upper limbs:	5 (3 patch, 2 nodular)
Face:	3 (1 patch, 1 plaque, 1 nodular)
Chest:	1 (patch lesion)
Penis:	1 (patch lesion)

Non-cutaneous KS were distributed at the following sites:

Table 3.2 Anatomical distribution of non-cutaneous lesions	
Oropharynx:	5 (2 plaque, 3 nodular)
Lungs:	3 (1 plaque, 2 nodular)
Small intestine:	4 (1 patch, 3 plaque)
Colon:	1 (plaque)
Anus:	1 (nodular)

Of the 43 cases, 13 had multiple lesions in the sites similar to those in the aforementioned table. Another additional site of involvement was the lymph node, which was seen in two of the cases with multiple lesions.

3.3 HIV status and histopathological features in Kaposi's sarcoma

The patients with known HIV positive status showed a relatively uniform range in the recognised specific histological types of KS. Of the 33 cases of HIV positive cases, the majority of cases (23/33; 70%) were late nodular stage KS but all of the HIV negative cases (14/14; 100%) were nodular stage lesions ($\chi^2=5.39$, $p=0.020$; χ^2 -test) (Table 3.3). Of the various histopathological features examined, the presence of lesional cell mitosis ($\chi^2=13.94$, $p=0.0002$; χ^2 -test), single cell necrosis ($\chi^2=10.79$, $p=0.001$; χ^2 -test), apoptosis ($\chi^2=14.77$, $p=0.0001$; χ^2 -test), and single anaplasia ($\chi^2=18.59$, $p=0.0001$; χ^2 -test) were more likely to be found in the KS lesions of HIV negative patients rather than in HIV-positive patients (Table 3.4). However, KS lesions in HIV positive patients tended to have dissecting blood vessels (14/33 cases; 42%) unlike those seen in HIV-negative patients (0/14 cases; 0%) ($\chi^2=8.46$, $p=0.004$; χ^2 -test).

There was no significant difference in the presence of plasma cells, newly formed blood vessels protruding into vascular lumens ("protuberant vessels", which are the promontory sign of early KS), hyaline globules, red cell extravasation and haemosiderin in the lesions between HIV positive and HIV negative patients (Table 3.3).

3.4 HIV status and relationship to clinical features in patients with KS

Of the 47 cases studied, 43 had adequate clinical follow-up information. There were 33 HIV positive patients in which the majority (30/32; 90%) was male (Table 3.4). This was in contrast with the HIV-negative cases with almost equal distribution between males (6/11; 55%) and females (5/11; 45%) ($\chi^2=7.33$, $p=0.0068$; χ^2 -test).

All of the patients with AIDS (33/33 cases; 100%) were <60 years old, with a median age of 35 years (range: 24-54 years). This contrasted significantly with HIV negative patients, in which the majority (9/10 cases; 90%) were ≥ 60 years of age ($\chi^2=38.8$, $p=0.0001$; χ^2 -test), with a median age of 70.5 years (range: 60-86).

Of the 43 cases with adequate follow-up in the study group, 12 (36%) had multiple lesions. However, there was no significant difference between the HIV positive and HIV negative groups in the occurrence of multiplicity of KS lesions. In addition, neither group of patients was more likely to develop recurrence of the KS lesions after treatment.

The majority of the cases were cutaneous KS (33/43; 75%). However, HIV positive patients were more likely than HIV negative patients to have non-cutaneous, or visceral, KS lesion ($\chi^2=4.89$, $p=0.027$; χ^2 -test).

3.5 Incidence of Kaposi's sarcoma over the ten-year period (1990-1999)

The incidence of histologically proven KS cases has decreased markedly over the 10-year period between 1990 and 1999. The number of cases over the five-year period between 1990-1994 was 33 whereas that 1995-1999 was 14, which was less than half of the number seen in the preceding five-year period.

Figure 3.1 Light microscopy of a patch stage Kaposi's sarcoma (H&E, x40)

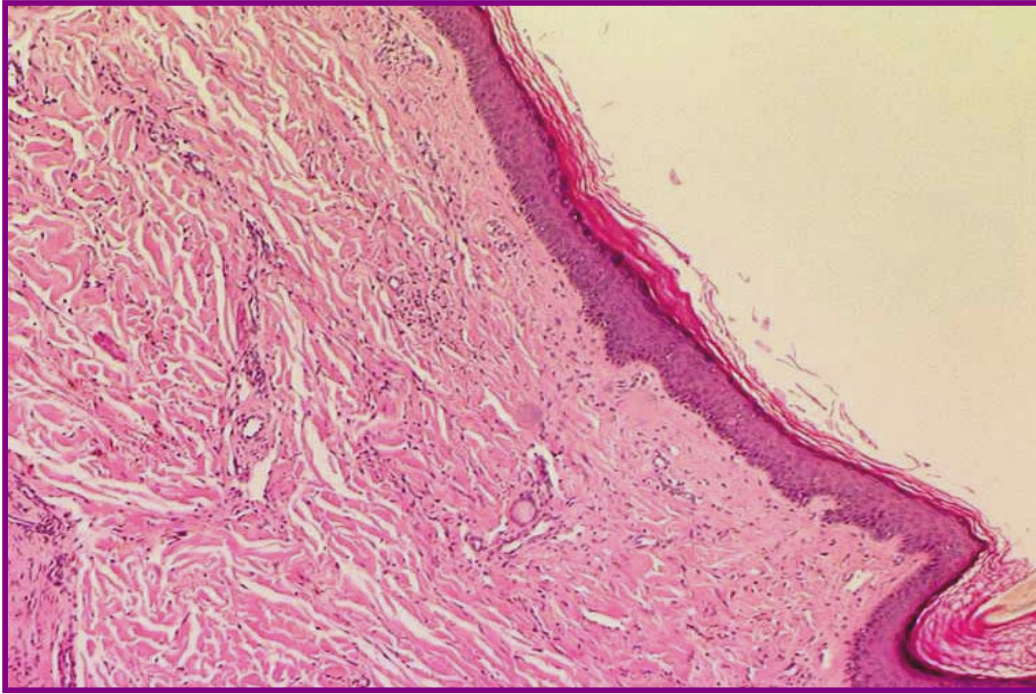


Figure 3.2 Light microscopy of a plaque stage Kaposi's sarcoma (H&E, x40)

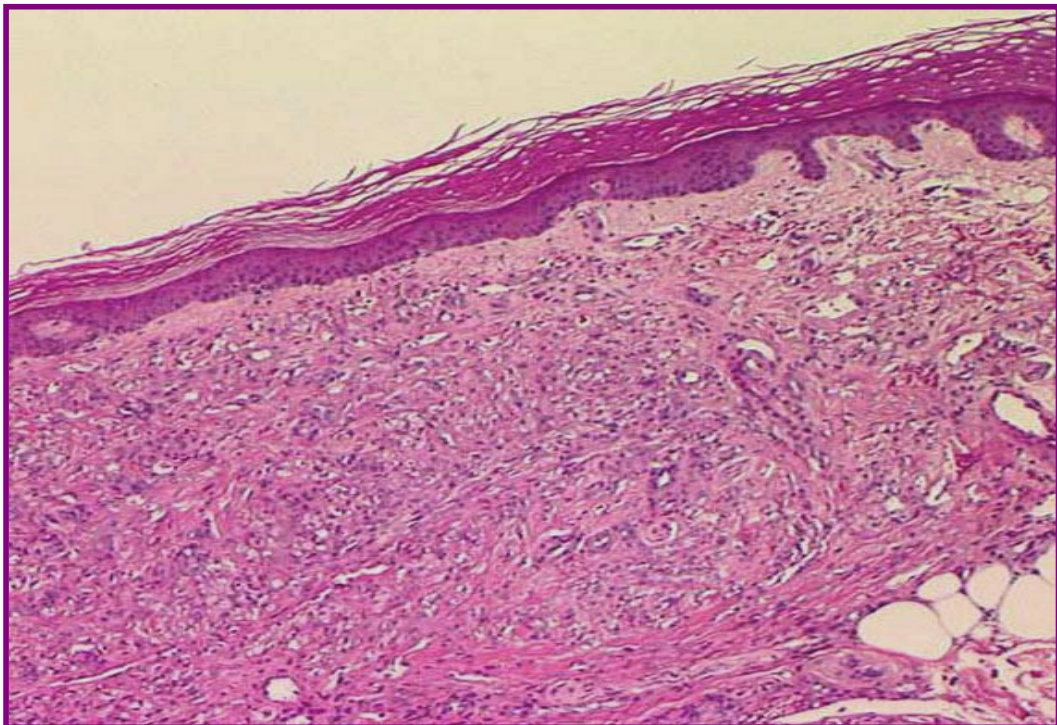


Figure 3.3 Light microscopy of a nodular Kaposi's sarcoma (H&E, x20)

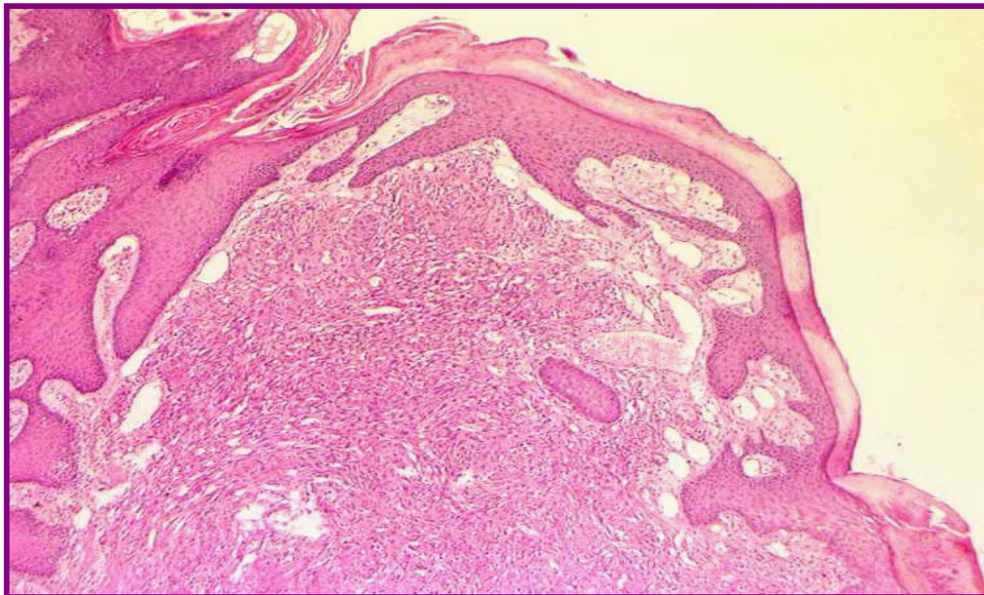


Figure 3.4 Light microscopy of a nodular Kaposi's sarcoma (H&E, x40)

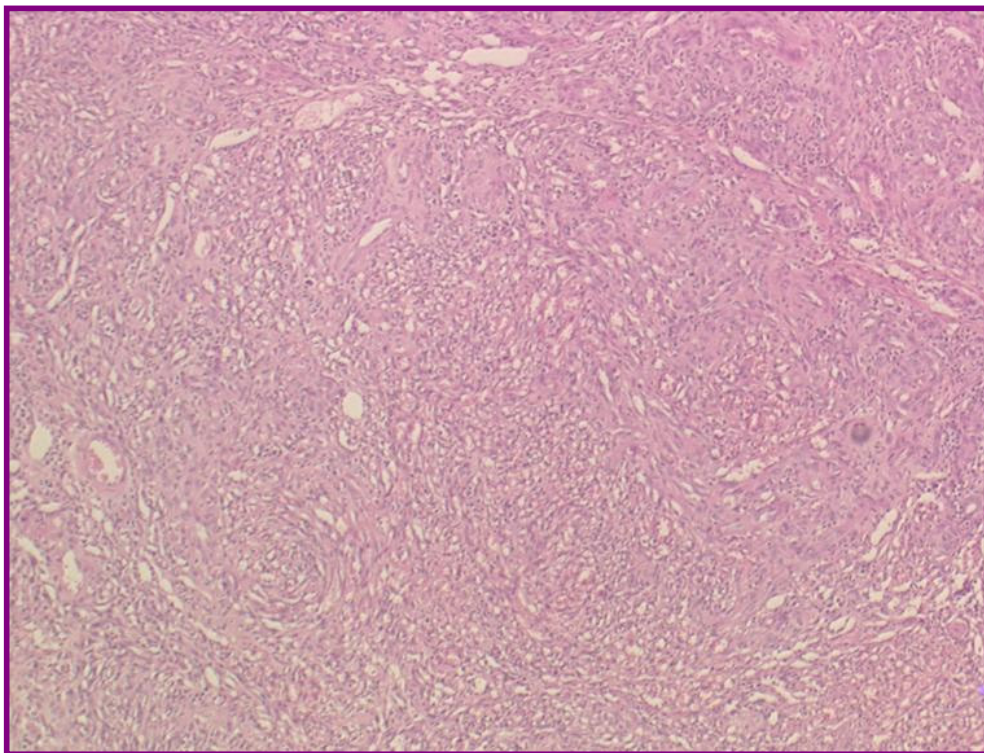
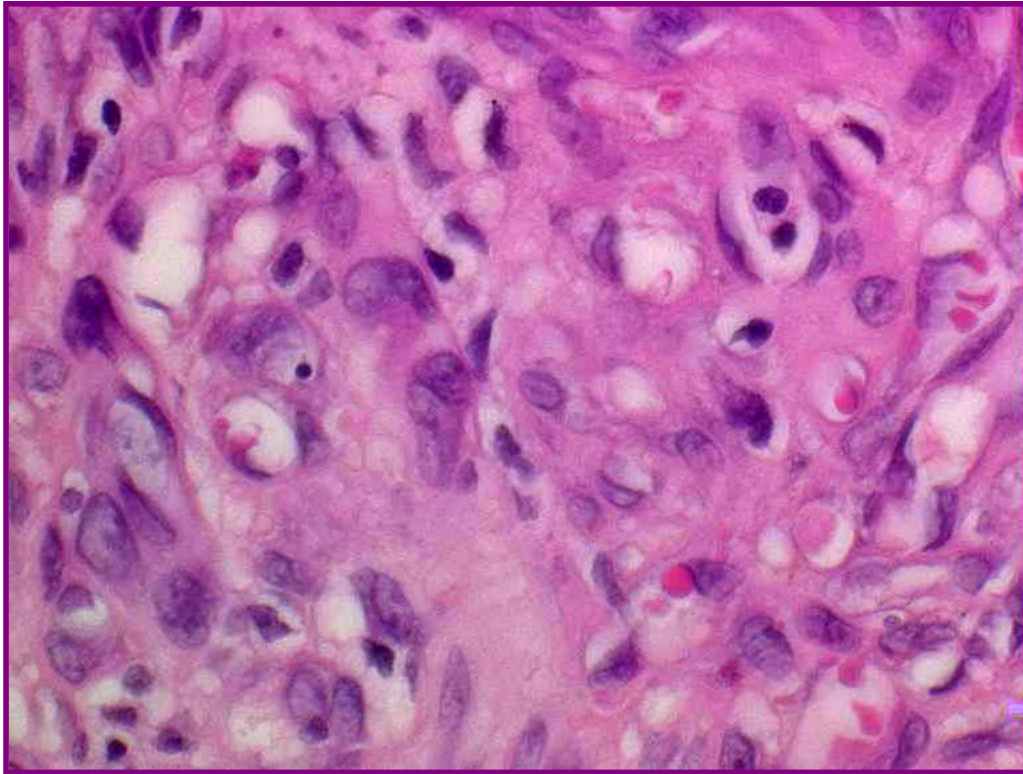


Figure 3.5 High power view of a nodular Kaposi's sarcoma (H&E, x400)



3.6 Discussion

Classical KS is uncommon and is seen mostly in elderly men of Mediterranean or Ashkenazic Jewish descent (DiGiovanna and Safai 1981). The increased risk of developing KS in immunosuppressed patients receiving immunosuppressive therapy for transplants, or those who have haematological malignancy, is well documented (Gange and Jones 1978; Gupta, Cardella and Haberman 1986; Hoshaw and Schwartz 1980; Klepp, Dahl and Stenwig 1978). It is also well known that KS is one of the commonest malignancy seen in patients with AIDS (Safai et al. 1985).

The histopathological appearances of the KS lesions in patients with AIDS were described to be different from those of HIV negative patients only in relation to the progression of the lesions from early patch stage to developed plaque or late stage nodular lesions. However, the histological features seen in the lesions between HIV positive and HIV negative patients were in fact similar in each stage of development (Safai et al. 1985; Santucci, Pimpinelli, Moretti and Giannotti 1988).

In this part of the thesis, we documented that in the combined cohort of HIV and non-HIV patients, the majority of the lesions (33/43 cases; 70%) were late stage lesions at the time of clinical presentation. In the HIV positive group of patients, the majority of the KS lesions were late stage although early stage lesions were more common in this group than in the HIV negative group of patients. Because there were more early stage KS in HIV positive patients than in the

HIV negative patients, the lesions in the former group were more likely to have dissecting vessels. These findings are consistent with those found by others (Gottlieb and Ackerman 1982). In contrast, these results showed that the KS lesions in HIV negative cases were exclusively of the late stage, and not surprisingly the presence of lesional cell mitosis, single cell necrosis, apoptosis and single cell anaplasia were found to occur significantly more frequently than in the KS lesions of HIV positive patients.

There was no significant difference in the presence of plasma cells, protuberant blood vessels, hyaline globules, red cell extravasation and haemosiderin in the lesions between HIV positive and HIV negative patients. It was of interest, however to note that plasma cells were found in a minority (4/10 cases; 40%) of the early KS lesions in this study despite its reported usefulness as a clue to the histological diagnosis of KS (Chor and Santa Cruz 1992). However, previous studies made by others also found that the presence of plasma cell was a minor finding in early AIDS-related KS lesions (Gottlieb et al. 1982; Santucci et al. 1988).

In the evaluation of HIV status and the various clinical parameters, it was found that there were significant differences in the age and sex of the patients between HIV positive and HIV negative patients, a result which is expected due to the epidemiological influence of HIV infection. It was also found that patients who were HIV positive were far more likely to develop extracutaneous or visceral KS lesions in this study.

The recent discovery of the human HHV8 as the initiating factor for the development of KS (Antman et al. 2000) has provided important insights into the pathogenesis of this tumour in both HIV positive and HIV negative patients. High HHV8 infection rates are found in central and southern Africa with intermediate infection rates in Mediterranean and eastern European countries and low rates in Asia, North America and northern Europe (Moore 2000; Hong and Lee 2001). Homosexual and bisexual men have a higher risk of HHV8 infection in Western societies and the virus appears to be spread by sexual transmission although there is evidence that other routes of transmission may be important (Kedes et al. 1996; Kedes et al. 1997; Martin and Osmond 2000; Hong et al. 2001). It appears that HHV8 initiates and promotes the development of KS lesions through the viral interleukin-6, a cyclin, and vascular endothelial growth factor that it produces. In HIV positive cases, the Tat protein of the HIV virus induces various cytokines that synergistically interact with the products of HHV8, which may account for the development and progression of KS (Prakash, Tang, He, Ali, Coleman and Gill 2000; Weiss and Boshoff 2000). It is interesting to note that the HHV8 virus is found in over 95 per cent of all KS lesions in various studies and it would be expected that these lesions should be similar in both HIV positive and HIV negative patients. It is obvious that this is not the case in the current study and in those of others (Modlin, Crissey and Rea 1983; Santucci et al. 1988). This suggests that the evolution and progression of KS in HIV positive and HIV negative patients may require different cofactors.

In summary, there are distinct differences in the clinical and histopathological features of KS lesions in HIV positive and HIV negative patients. Despite the recent discovery of the HHV8 virus as the initiating factor for all subtypes of KS, these differences highlighted the possibility of different mechanisms of tumour progression in the presence or absence of HIV infection.

Table 3.3 Histological subtypes of Kaposi's sarcoma and correlation with pathological features

	HIV positive	HIV negative	Chi square	p value
Histological subtypes				
Patch	10 (30%)	0 (0%)		
Plaque/Nodular	23 (70%)	14 (100%)	5.39	0.02
Plasma cells				
Present	15 (45%)	8 (57%)		
Absent	18 (55%)	6 (43%)	0.54	0.46
Dissecting vessels				
Present	14 (42%)	0 (0%)		
Absent	19 (58%)	14 (100%)	8.46	0.004
Protruding vessels				
Present	2 (6%)	0 (0%)		
Absent	31 (94%)	14 (100%)	0.89	0.347
Hyaline globules				
Present	25 (76%)	13 (93%)		
Absent	8 (24%)	1 (7%)	1.86	0.173
Red cell extravasation				
Present	33 (100%)	14 (100%)		
Absent	0 (0%)	0 (0%)	NA	NS

Table 3.3 Histological subtypes of Kaposi's sarcoma and correlation with pathological features

	HIV positive	HIV negative	Chi square	p value
Haemosiderin				
Present	31 (94%)	14 (100%)		
Absent	2 (6%)	0 (0%)	0.89	0.347
Mitosis				
Present	11 (33%)	13 (93%)		
Absent	22 (67%)	1 (7%)	13.94	0.0002
Single cell necrosis				
Present	11 (33%)	12 (86%)		
Absent	22 (67%)	2 (14%)	10.79	0.001
Apoptosis				
Present	13 (39%)	14 (100%)		
Absent	20 (61%)	0 (0%)	14.77	0.0001
Spindle cell anaplasia				
Present	1 (3%)	8(57%)		
Absent	32 (97%)	6 (43%0	18.59	0.001

Table 3.4 Histological subtypes of Kaposi's sarcoma and correlation with clinical features

	HIV positive	HIV negative	Chi square	p value
Gender				
Male	30 (90%)	6 (55%)		
Female	3 (10%)	5 (45%)	7.33	0.0068
Age				
<60	33 (100%)	1 (95)		
≥60	0 (0%)	10 (91%)	38.8	0.0001
Multiplicity of lesions				
Single	21 (64%)	7 (64%)		
Multiple	12 (36%)	4 (36%)	NE	0.717
Recurrence				
Absent	28 (85%)	7 (64%)		
Present	5 (15%)	4 (36%)	2.28	0.13
Site				
Cutaneous	22 (67%)	11 (100%)		
Non-cutaneous	11 (33%)	0 (0%)	4.89	0.027

NE=not estimable

Chapter 4: Detection of HHV8 in KS by immunohistochemistry

Chapter 4: Detection of HHV8 in Kaposi's sarcoma

4.1 Background

The HHV8 virus can be detected in AIDS-related KS, classical KS and the KS that occurs in HIV negative homosexual men (Moore et al. 1995). In addition, the risk of developing KS increases with rising antibody titre to HHV8, although for a given titre, the risk is greater in HIV positive compared with HIV negative patients (Sitas et al. 2001).

Recently, a monoclonal antibody against the latent nuclear antigen-1 molecule of HHV8 has been made available commercially. The aim of this part of the thesis was to assess the usefulness of IPX detection of the HHV8 latent antigen for the diagnosis of KS, particularly in its early stage of development. The prevalence of HHV8 in the study cohort was defined.

4.2 Methods

For the detailed protocol of IPX staining, please refer to Chapter 2 and Appendix 1. Paraffin sections were cut at 4 μ m, mounted on 5-aminopropyltriethoxysilane coated slides and allowed to dry overnight. The sections were dewaxed in xylene and were treated with microwave heating at 60°C for 20 minutes in a citrate buffer (2.1 g/1000 ml; pH 6.0) for antigen retrieval. After the blocking of endogenous peroxidase, washing in phosphate-buffered saline and the blocking of non-specific

binding of secondary antibody with normal swine serum, routine streptavidin-biotin-peroxidase immunostaining with diaminobenzidine was applied to the sections incubated overnight with a murine monoclonal antibody directed against latent nuclear antigen of HHV8 (clone 13B10; Novocastra) at 1:50 dilution. The primary antibody was substituted with phosphate buffered saline in sections used as negative controls. The sections were counterstained with Harris haematoxylin. Cells were classified positive with any positive nuclear staining. The percentage of positive cells was noted. Three cases of Merkel cell carcinoma and the non-neoplastic components of the KS specimens were used as negative control.

4.3 Results

HHV8 was detected in the nuclei of the spindle and endothelial cells of the vascular channels of infected KS lesions (Figures 4.1, 4.2, 4.3). Positive HHV8 nuclear staining was detected in 78% of all cases. More than 50% (9/19 cases) of the nodular stage lesions showed positive staining in more than 50% of the neoplastic cells. None of the plaque stage lesions and only 12.5% (1/8 cases) of the patch stage showed positive staining in more than 50% of the cells. In contrast, in all the cases examined, the HHV8 was absent in the non-neoplastic vessels in the adjacent tissue ($p=0.0001$, $X^2=44.46$; X^2 -test with continuity correction). The three cases of merkel cell carcinoma did not show any positive staining.

However, there was no significant difference in HHV8 positivity between HIV positive and HIV negative patients (77.8% vs 80%, $p=0.88$). HHV8 was detected in 80% (8/10 cases) of the patch stage, 88% (7/8 cases) of the plaque stage and 74% (14/19 cases) of the late stage ($p=0.61$, Table 4.1). There was also no statistically significant association recognised between the presence of HHV8 and other clinicopathological features such as age, gender, tumour recurrence, multiplicity and site of the lesions (Table 4.2).

Figure 4.1 Positive HHV8 staining in a patch stage lesion (x400)



Figure 4.2 Positive HHV8 staining in a plaque stage lesion (x400)

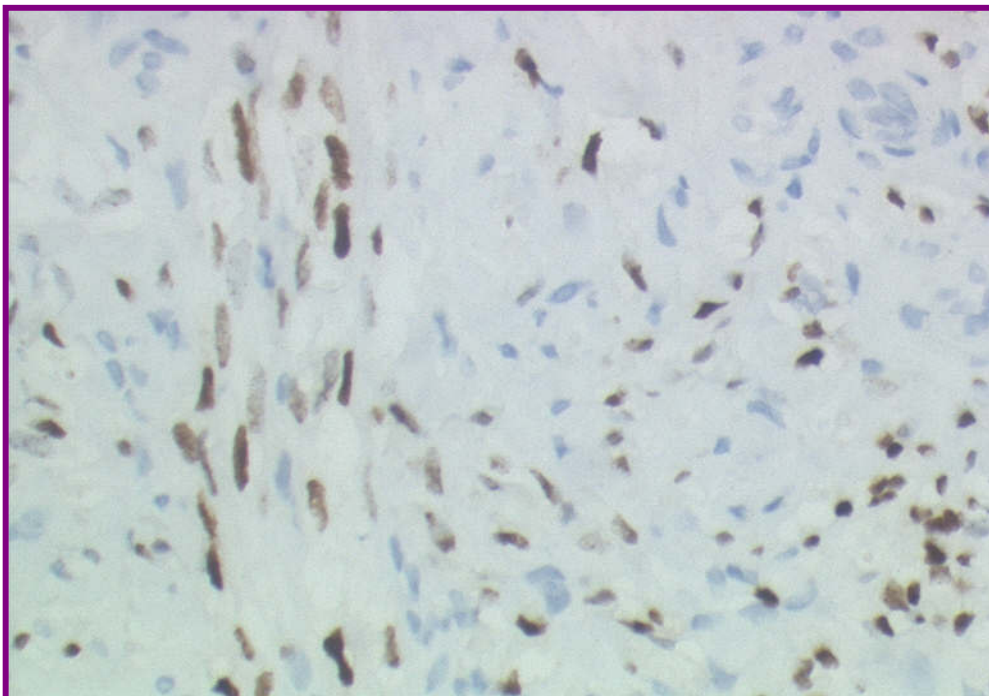


Figure 4.3 Positive HHV8 staining in a nodular lesion (x400)

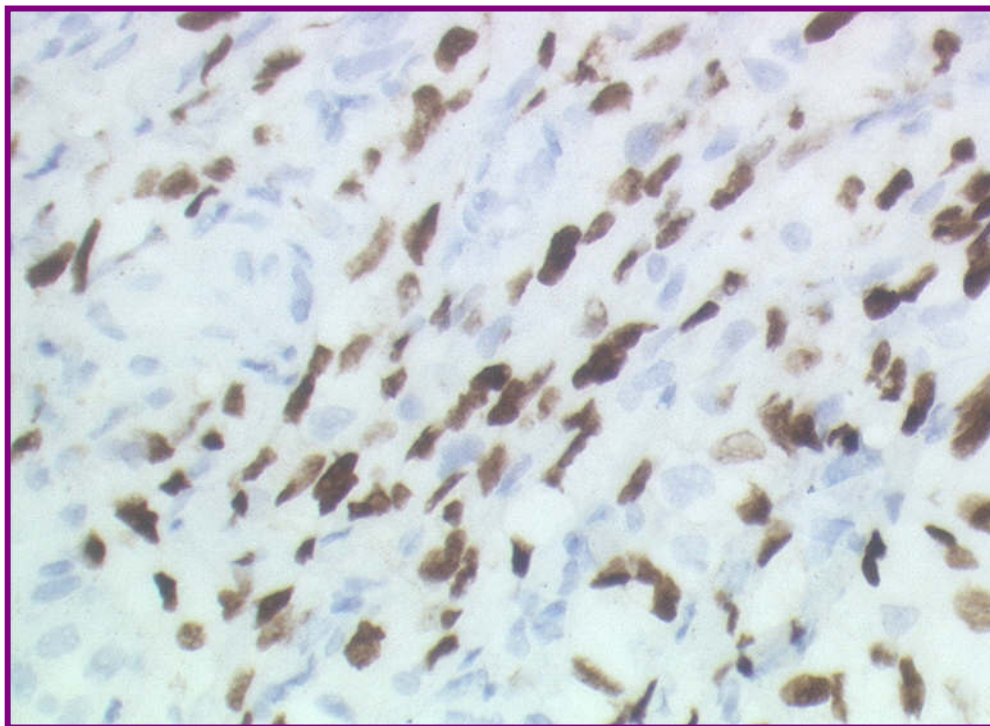


Table 4.1: Detection of HHV8 in Kaposi's sarcoma

Histological subtype	Total No.	HHV8 Positive	HHV8 Negative
Patch	10	8 (80%)	2 (20%)
Plaque	8	7 (87.5%)	1 (12.5%)
Nodular	19	14 (73.7%)	5 (26.3%)

Table 4.2: HHV8 expression in Kaposi's sarcoma and the correlation with clinico-pathological data

Clinico-pathological parameters	Total no.	HHV8 Positive	HHV8 Negative
HIV status			
Positive	27	21 (77.8%)	6 (21.2%)
Negative	10	8 (80%)	2 (20%)
Age			
≤60	28	22 (78.6%)	6 (21.4%)
>60	9	7 (77.8%)	2 (21.2%)
Sex			
Male	31	24 (77.5%)	7 (22.5%)
Female	6	5 (83.3%)	1 (16.7%)
Multiplicity of lesions			
Single	22	16 (72.7%)	6 (27.3%)
Multiple	15	13 (86.7%)	2 (13.3%)
Recurrence			
Absent	29	22 (75.9%)	7 (24.1%)
Present	8	7 (87.5%)	1 (12.5%)
Site			
Cutaneous	28	22 (78.6%)	6 (21.4%)
Non-cutaneous	9	7 (77.8%)	2 (22.2%)

4.4 Discussion

Histological recognition of KS can be difficult at times. The advanced nodular stage of KS can often be diagnosed without difficulty on histology. However, the florid vascular proliferation can sometimes be mistaken for a pyogenic granuloma or bacillary angiomatosis, although the latter two conditions lack the spindle cell component seen in KS (Templeton 1981; Blumenfeld et al. 1985; Cockerell, Whitlow, Webster and Friedman-Kien 1987). Occasionally, spindle cell anaplasia which can be seen in KS (Hong and Lee 2002) together with the spindle cell proliferation, can be confused with an angiosarcoma but the latter also shows anastomosing vascular channels that are only rarely seen in KS (Templeton 1981).

In the early stage of KS development, the often non-descript proliferation of thin-walled angulated vessels with a scant infiltrate of chronic inflammatory cells and haemosiderin deposits can be confused with an inflammatory dermatosis or angiodermatitis (Blumenfeld et al. 1985). It is this early stage of KS that can be most difficult to diagnosis and, as the current study suggests, detection of HHV8 can be useful to confirm the diagnosis.

Overall, 78% of the lesions showed positive HHV8 nuclear staining and there was no significant variation according to the HIV status, histological stage, age, gender, site of lesions and the presence of recurrent disease. Previous reports have shown the presence of the HHV8 virus in more than 95% of lesions by polymerase

chain reaction and electron microscopy. Our yield of IPX staining for the detection of HHV8 is lower than is reported in the literature. This was unlikely to be a false negative result as the positive controls were always positive during the experiments and the positive staining was easily identifiable nuclear staining. A previous study has shown that there was 100% correlation between the detection of HHV8 by IPX and in vitro polymerase chain reaction for HHV8 DNA (Courville, Simon, Le Pessot, Tallet, Debab and Metayer 2002). More importantly, in that study, there were eight cases of uncertain histology on routine histology but were HHV8 negative on both IPX and polymerase chain reaction and subsequently all lesions developed a self-healing evolution consistent with a diagnosis of pyogenic granuloma. The only other possible explanation for a false negative result would be the loss of the HHV8 antigen during the fixation and processing of some of the archival tissues in which the virus was not detected. Unfortunately, there is no possible way to prove or disprove this.

A lower incidence of HHV8 positive lesions in this study is probably a true reflection of the incidence of HHV8 infected KS in Australia. The incidence of HHV8 infection in KS in Australia has not been reported previously. Genetic fingerprinting of HHV8 stains based on variations in the HHV8 Ki glycoprotein B and glycogen protein H genes showed a geographic variation of the genotype of HHV8 (Meng, Spira, Bhat, Birch, Druce, Edlin, Edwards, Gunthel, Newton, Stamey, Wood and Pellett 1999). The majority of HHV8 strains from the United States were relatively closely related (genotype I and II) whereas some strains in Australia were

phylogenetically distant (genotype III and IV). Among the sequences analysed, genotypes I (subtypes A, B, C, D and E) and II were found in the United States and genotypes I (subtype A), II and III found in Australia and New Zealand. The unique distribution of the HHV8 subtype in Australia might explain the immunohistochemistry findings. Possible links between HHV8 subtypes and particular diseases have been suggested. A study on the HHV8 variants from KS in France has suggested HHV8 subtype A was associated with more aggressive diseases (Boralevi, Masquelier, Denayrolles, Dupon, Pellegrin, Ragnaud and Fleury 1998). The subtype A group displayed significantly more frequent mucosal and/or visceral lesions and a higher mean CD4 cell count at the onset of KS. Luppi et al. suggested that the distribution of the HHV8 genomes might be related to disease conditions with the subtype A mainly associated with KS and subtypes B and C with lymphoproliferative malignancy (Luppi, Barozzi, Marasca, Ferrari and Torelli 1997).

In conclusion, this part of the thesis shows that the latent nuclear antigen 1 of the HHV8 can be detected by IPX in a high proportion of KS cases, and the simple technique may be used to guide the diagnosis of KS, especially the patch stage lesion. The rate of HHV8 infection in a cohort of KS lesions in Australia has been documented.

Chapter 5: Ki-67 expression in Kaposi's sarcoma

Chapter 5: Ki-67 expression in Kaposi's sarcoma

5.1 Introduction

Excessive proliferation is one of the first steps in oncogenic activation and one of the most important biological features defining the aggressiveness of tumours. Quantifying the proportion of tumour cells in the S phase of the cell cycle by means of flow cytometry has shown to be useful for defining high risk subgroups in many human malignancies. One of the markers of cell proliferation is the Ki-67 antigen. Monoclonal antibodies against Ki-67 antigen were first described in 1983. The Ki-67 antigen is closely associated with proliferation, and IPX detection of the antigen in tissue sections is an easier alternative to flow cytometry for measuring tumour cell proliferation. Since its discovery, there has been a steady increase in the use of Ki-67 antibody in diagnostic pathology (Brown and Gatter 2002). Ki-67 is of prognostic value in a number of malignancies. For example, in breast cancer most studies show a strong, statistically significant correlation with clinical outcomes, both on univariate and multivariate analysis (Locker, Birrell, Bell, Nicholson, Elston, Blamey and Ellis 1992; Jacquemier, Penault-Llorca, Bertucci, Sun, Houvenaeghel, Geneix, Puig, Bardou, Hassoun, Birnbaum and Viens 1998; Midulla, De Iorio, Nagar, Pisani, Cenci, Valli, Nofroni and Vecchione 1999).

This part of the thesis examined the level of Ki-67 in classical and AIDS-related KS using IPX staining. We hypothesised that the more advanced nodular stage lesions express a higher level of Ki-67 than the patch stage lesions. The

correlation between Ki-67 expression and other clinicopathological factors was examined.

5.2 Methods

Standard immunohistochemistry was performed as described in Chapter 2 for the detection of the Ki-67 antigen. Ki-67 antibody (Dako) was used at a dilution for 1:200. Ki-67 staining was classified into 0 (0%), 1 (1-10%), 2 (11-25%), 3 (26-50%) and 4 (>50%) (Saiz, Olvera, Rezk, Florentine, McCourty and Brynes 2002).

5.3 Results

Classical KS lesions had a higher Ki-67 staining score than AIDS-related lesions (Figure 5.1); 50% of the classical lesions had a staining score 3 or 4 compared with 16% of the AIDS-related lesions ($p=0.02$). The more advanced nodular lesions were more likely to have a higher Ki-67 staining score than earlier stage lesions (Figure 5.2); 47.1% of the nodular lesions had a staining score 3 or 4 whereas none of the patch or plaque stage lesions had a 3 or 4 staining score ($p<0.001$).

However, there was no correlation between Ki-67 staining score and gender, site of lesion and recurrence. On multivariate analysis, the nodular stage was independent predictor for Ki-67 positivity. Furthermore, there was no correlation between Ki-67, HIV status and HHV8 status.

Figure 5.1 Ki-67 staining score in classical and AIDS-related lesions

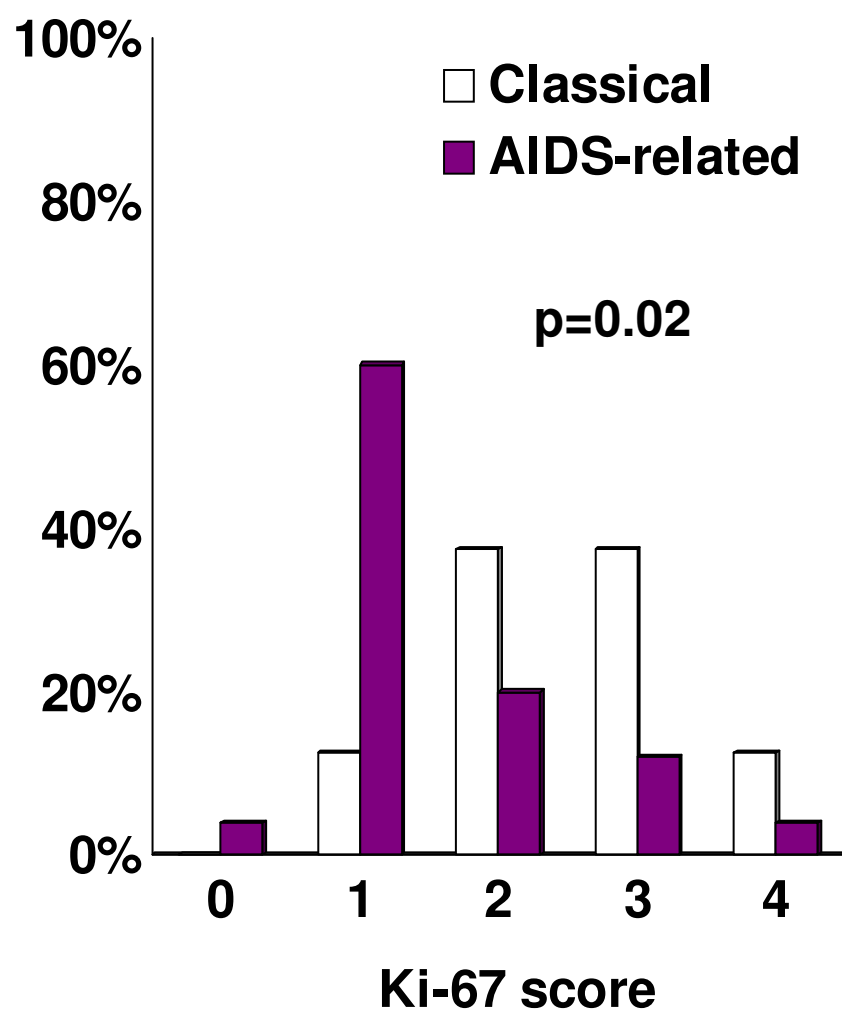
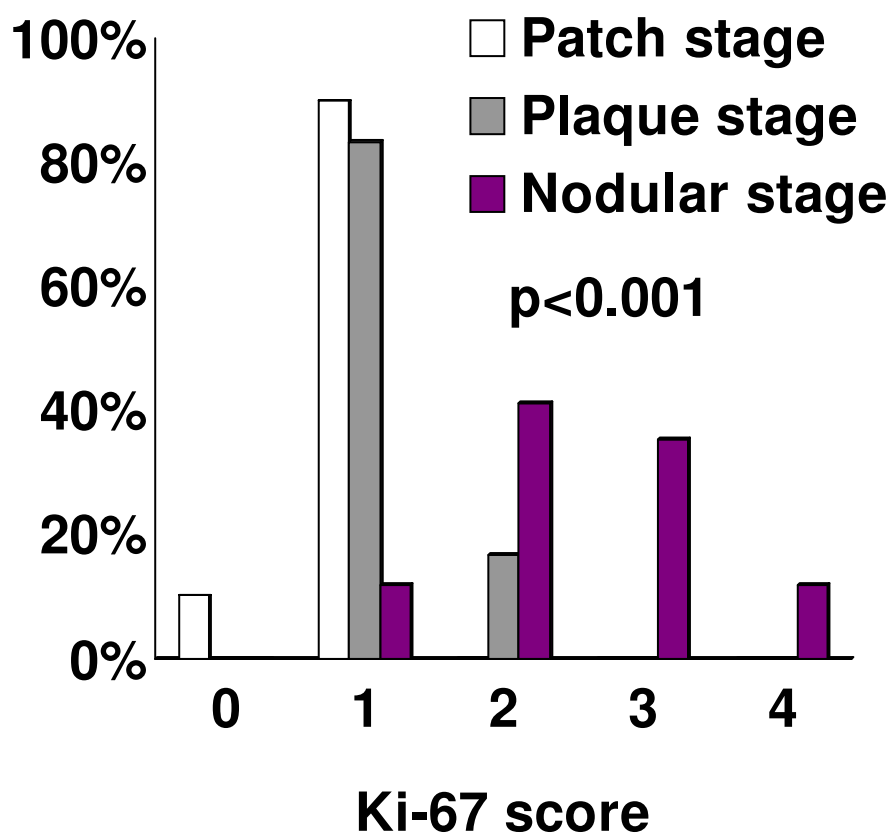


Figure 5.2 Ki-67 staining score in different histological subtypes



5.4 Discussion

The Ki-67 antigen is a large nuclear non-histone protein of approximately 395 kDa, encoded by approximately 30,000 base pairs of cDNA nucleotides (Schluter, Duchrow, Wohlenberg, Becker, Key, Flad and Gerdes 1993). The protein undergoes phosphorylation and dephosphorylation during mitosis and is susceptible to proteases. It has a very large complex and specific localisation pattern within the nucleus. The Ki-67 protein moves from within the nucleolus to the perichromosomal layer during mitosis (Endl and Gerdes 2000). Despite the vast amount of information on the structure, localisation and regulation of the function of this protein, little is known about its exact function. Ki-67 appears to be vital for cell proliferation, since its removal by antisense nucleotides prevents cell proliferation (Schluter et al. 1993). Progress on identifying the function of Ki-67 has been hindered by the lack of obvious homology with other proteins (Scholzen and Gerdes 2000). Based on its localisation to a zone with late ribosomal RNA processing, Ki-67 is an essential factor in the synthesis of ribosome during cell division. This is supported by the observation that Ki-67 protein expression correlated with the rate of protein synthesis, a function of ribosomes (Plaat, Kole, Mastik, Hoekstra, Molenaar and Vaalburg 1999).

As expected, the Ki-67 level was significantly higher in the nodular stage than in the plaque and patch stages. This finding validates the histological staging system of KS as an *in vivo* model of its progression as a tumour, since excessive

proliferation is regarded as one of the first steps in oncogenic activation and one of the most important biological features defining the aggressiveness of tumours. However, HIV status, HHV8 status and recurrent lesions were not independent predictors of Ki-67 expression. The results are consistent with the findings of the study by Hodak et al. (Hodak, Hammel, Feinmesser, Zelinger, Maron, Sulkes and David 1999), IPX staining using antibody against Ki-67 was performed on 26 cases of classical KS and 19 cases of AIDS-related KS. They demonstrated that Ki-67 immunoreactivity correlated significantly with histopathological stage, and classical KS had a significantly higher level of Ki-67 compared with AIDS-related KS.

However, two other studies have shown no correlation between Ki-67 and the histological stage. A study of 40 cases of AIDS-related KS showed a low mean staining score of 8% (range 1-20%) and there was no significant difference among the different stages of the disease (De Thier, Simonart, Hermans, Andre, Van Vooren and Noel 1999). However, the Ki-67 staining score in the latter study was very low and this might represent problems with the staining technique or the primary antibody. A more recent study by Penin et al. showed that Ki-67 expression was independent of disease stage (Penin, Fernandez-Figueras, Puig, Rex, Ferrandiz and Ariza 2002). No statistically significant differences were found in regard to gender, HIV status and Ki-67 staining.

The prognostic value of Ki-67 in KS is unknown, as all the published studies including the current study do not have sufficient clinical outcome data. This is

probably due to the overall extremely poor prognosis of AIDS until the recent introduction of highly active anti-retroviral therapy. The exact cause of death might not be documented accurately and some patients were lost to follow up. However, it is clear that Ki-67 is of prognostic value for many types of malignancy even after taking into consideration the publication bias in favour of studies showing significance. Table 5.1 shows the current literature on the prognostic value of Ki-67 in malignancy. In breast cancer, only one out of a total 24 studies showed no prognostic value for Ki-67 (Paradiso, Tommasi, Barletta, Leone, Lacava, Vellejo, Labriola, Marzullo, Altieri, Schittulli and De Lena 1999). In this particular study, only women with advanced disease were included and hence a small difference in survival is difficult to detect statistically.

Other studies reveal an unclear and often contradictory pattern for certain tumour types, e.g. non-Hodgkin's lymphoma (Grogan, Lippman, Spier, Slymen, Rybski, Rangel, Richter and Miller 1988; Hall, Crocker, Watts and Stansfeld 1988; Nakamura, Akazawa, Yao and Tsuneyoshi 1995; Czader, Mazur, Pettersson, Liliemark, Stromberg, Christensson, Tribukait, Auer, Ost and Porwit 1996). The prognostic value of Ki-67 in non-Hodgkin's lymphoma is complicated by the heterogeneity of the different subtypes of the disease and localisation of the primary tumours. In colorectal cancer, the majority of studies did not show a correlation between Ki-67 expression and survival. This implies that the proliferation rate is only one of the factors that may influence outcomes in malignancy.

In conclusion, the more advanced nodular stage lesions have a significantly higher level of cellular proliferation index. The histological stage of KS can be at times difficult to define. In view of the finding of a parallel increase in cell proliferation activity in the stepwise stage progression of KS, Ki-67 immunostaining as a marker of cell proliferation may be a useful marker in the histological staging of KS. The sequential progression of the KS lesion observed on the histology can be regarded as a reasonable model of tumour progression since it was demonstrated that the rate of Ki-67 positivity, which is generally regarded as a reliable indicator of cellular proliferation, was higher with more advanced stage nodular lesions.

Table 5.1 Summary of studies in the current literature on the prognostic value of Ki-67 in malignancy

Disease	No. of studies showing Ki-67 with prognostic value	No. of studies showing Ki-67 with no prognostic value
Group 1 (>75% of studies show that Ki-67 has a prognostic value)		
Breast Cancer	23	1
Soft Tissue Sarcoma	11	3
Lung Cancer	10	2
Astrocytoma	8	2
Meningioma	6	0
Group 2 (25-75% of studies show that Ki-67 has a prognostic value)		
Cervical Cancer	5	6
Prostate Cancer	15	7
Kaposi's Sarcoma	2 (including findings from this thesis)	2
Group 3 (<25% of studies show that Ki-67 has a prognostic value)		
Colorectal Cancer	2	10

Chapter 6: Expression of the Rb protein and cell cycle proteins acting upstream of Rb in Kaposi's sarcoma

Chapter 6: Expression of the Rb protein and cell cycle proteins acting upstream of Rb in Kaposi's sarcoma

6.1 Background

Cyclins are important protein molecules involved in the induction and control of the cell cycle. Factors modulating exit from the G0 phase and progression through to the G1 phase of the cell cycle are critical in determining overall growth rates of cells and, more importantly, cellular sensitivity to cytotoxic agents and radiation therapy. Cyclin D1 regulates G1 phase progression by inactivation of the Rb protein via phosphorylation (Weinberg 1995). Overexpression of the cyclin D1 protein has been reported in many different malignancies (Arber, Doki, Han, Sgambato, Zhou, Kim, Delohery, Klein, Holt and Weinstein 1997; Ikeda, Isaji, Das, Watanabe and Kawarada 1999) and is associated with more advanced stage diseases (Michalides, Van Veelen, Hart, Loftus, Wientjens and Balm 1995; Gansauge, Gansauge, Ramadani, Stobbe, Rau, Harada and Beger 1997; Barbieri, Cagnoli, Ragni, Foglia, Bruzzo, Pedulla and Alama 1999).

The p16 is a tumour suppressor gene located on chromosome 9p21-22 (Kamb, Gruis, Weaver-Feldhaus, Liu, Harshman, Tavitian, Stockert, Day, Johnson and Skolnick 1994). The p16 protein functions in a regulatory pathway controlling G1 progression. p16 can inhibit the activities of cyclin dependent kinases Cdk4 or Cdk6 which, in association with cyclin D1, promote cell cycle progression through the G1 phase.

The Rb gene is a tumour suppressor gene and lies downstream to cyclin D1 and p16 (Lukas, Parry, Aagaard, Mann, Bartkova, Strauss, Peters and Bartek 1995). Inactivation of the Rb gene and p16 has been observed in many different cancers (Azechi, Nishida, Fukuda, Nishimura, Minata, Katsuma, Kuno, Ito, Komeda, Kita, Takahashi and Nakao 2001; Backus, van Riel, van Groeningen, Vos, Dukers, Bloemena, Wouters, Pinedo and Peters 2001; Zamparelli, Masciullo, Bovicelli, Santini, Ferrandina, Minimo, Terzano, Costa, Cinti, Ceccarelli, Mancuso, Scambia, Bovicelli and Giordano 2001). In patients with liver metastasis from colorectal cancer, a low level of expression of Rb protein detected by IPX was associated with poorer survival. In non-small cell lung cancer, patients with p16 positive tumours had significantly better prognosis than patients with p16 negative tumours (Jin, Inoue, Umemura, Moriya, Arakawa, Nagashima and Kato 2001). In hepatocellular carcinoma, inactivation of p16 by hypermethylation or homozygous deletion was detected in 60% of cases (Jin, Piao, Kim, Park, Shin, Park, Jung, Kim and Kim 2000).

Thus, loss of p16, loss of Rb and overexpression of cyclin D1 have similar effects on G1 phase progression and may represent a common pathway to carcinogenesis. This part of the thesis examined the level of cyclin D1, p16 and Rb in classical and AIDS-related KS.

6.2 Methods

The protocol of IPX staining is presented in Chapter 2 and Appendix 1. The primary antibody was substituted with saline in sections used as negative controls. The sections were counterstained with Harris's haematoxylin. Cells were classified as cyclin D1 positive when there was nuclear staining. A semi-quantitative scoring system based on the staining intensity and the percentage of positive cells was used (Vos, Ter Haar, Peters, Cornelisse and Van De Vijver 1999). The staining intensity was scored as 0 (none), 1 (weak), 2 (moderate) and 3 (strong), and the percentage of positive cells was scored as 0 (0%), 1 (1-25%), 2 (>25-50%), 3 (>50-75%) and 4 (>75%). The cyclin D1 staining score was calculated as the sum of the intensity and the percentage of positive cells. A cyclin D1 staining score of ≥ 4 has been shown to correlate with cyclin D1 overexpression detected by Western blot analysis (Vos et al. 1999).

For Rb protein, a cut-off level of 10% for positive nucleus staining was used (Zamparelli et al. 2001). For p16, $\geq 5\%$ nucleus staining was counted as positive (Lee, Capodiceci, Osman, Fazzari, Ferrara, Scher and Cordon-Cardo 1999). Histologically normal dermal tissues located away from the primary lesions were used as normal non-neoplastic controls.

Correlation between cyclin D1, p16, Rb and clinicopathological factors were analysed using the chi-squared (χ^2) contingency test. Multivariate analysis was

performed using the stepwise multiple linear regression model by assessing the cyclin D1 status in KS lesions with the following baseline covariates: age, gender, HIV status, tumour stage, recurrent lesion, multiplicity of the lesion and involved site. Only p values of less than 0.05 were considered significant.

6.3 Results

Cyclin D1 overexpression was detected in 29% (12/41) of all KS cases (Figures 6.1, 6.2). The regions of the lesions containing the spindle-shaped cells displayed most immunoreactivity. However, proliferating blood vessels with prominent activated endothelial cells were immunoreactive as well. There was a statistically significant correlation between overexpression of cyclin D1 protein and histological subtypes (Table 6.1). None of the patch lesions had a cyclin D1 staining score of ≥ 4 . Thirteen per cent (1/8) of the plaque lesions scored ≥ 4 and 50% (11/22) of the nodular lesions scored ≥ 4 ($p=0.0017$).

The histological stage at presentation varied significantly among the two clinical subtypes. Ninety per cent of the classical KS cases presented as nodular stage. In contrast, only 49% of the AIDS-related KS presented as nodular stage. The classical KS lesions were more likely to show cyclin D1 overexpression (70%; 7/10) than AIDS-related lesion (16%, 5/31) ($\chi^2=10.6$, $p=0.0011$) (Table 6.2). Among the nodular subtype, classical lesions had a significantly higher level of cyclin D1 overexpression (78%; 7/9) than AIDS-related lesions (31%, 4/13, $p=0.03$) (Table 6.2).

Cyclin D1 expression was correlated with clinicopathological features (Table 6.3). Older patients were more likely to have cyclin D1 overexpressed lesions. The majority of patients >60 years old had KS lesions that demonstrated

overexpression of cyclin D1 (75%; 6/8 cases). This contrasted significantly with patients of ≤ 60 years, wherein only 18% (6/33 cases) of the lesions had cyclin D1 overexpression ($\chi^2=10.1$; $p=0.006$). Female patients were more likely to demonstrate cyclin D1 overexpression in their KS lesions (67%; 4/6) than male patients (23%; 8/35). This difference in cyclin D1 expression in KS lesions and gender status was statistically significant ($\chi^2=4.75$; $p=0.03$).

Patients who had tumour recurrences were more likely to have cyclin D1 overexpressed KS lesions (78%; 7/9) compared with those with no tumour recurrence detected at the time of clinical follow-up (16%; 5/32) ($\chi^2=13.1$; $p=0.0003$). There was no significant difference in cyclin D1 expression in the KS lesions from patients who presented with single lesions compared with those who presented with multiple lesions ($\chi^2=0.099$; $p=0.75$). In both categories of patients with single (25%; 6/24) and those with multiple (29%; 5/17) lesions, only a minority of cases showed cyclin D1 overexpression. There was no significant difference in cyclin D1 expression in the KS lesions of the skin compared with those from non-cutaneous sites ($\chi^2=0.029$; $p=0.87$). Only a minority of both cutaneous (30%; 9/30) and non-cutaneous (27%; 3/11) lesions showed cyclin D1 overexpression. Female patients were more likely to have cyclin D1 overexpression in their KS lesions than male patients (67% vs 23%, $p=0.029$).

The effects of variables on cyclin D1 overexpression were examined by multivariate analysis with stepwise linear regression model. Among the seven

factors tested, negative HIV status ($p<0.001$) and nodular histological subtypes ($p=0.007$) were significant and independent predictors of cyclin D1 overexpression. No statistically significant relevance was recognised for age, gender, tumour recurrence, multiplicity of lesions and the site of the lesions on multivariate analysis.

Functional Rb protein was detected in 75.75% of cases (Figure 6.3, Table 6.4). The level of expression did not differ significantly between classical lesions and AIDS-related lesions (75% vs 76%, $p=ns$). The levels were not significantly different among the histological subtypes (patch=76.5%, plaque=66.6% and nodular=80%). However, there was a significant difference in the level of RB protein between HHV8 positive cases and HHV8 negative cases. The majority (83.3%) of the HHV8 positive cases showed positive Rb staining whereas only 42.8% of the HHV8 negative cases were positive for RB. In addition, there was no significant difference between gender and the site of lesions.

Two thirds of the KS showed positive staining for p16 (Table 6.5). There was no significant difference between classical and AIDS-related lesions (87.5% vs 60.7%, $p=0.3$). However, it was noted that there was a progressive increase in p16 level with more advanced histological stages. The difference between the patch and nodular stages was statistically significant (50% vs 77.8%, $p=0.04$). There was no significant difference between gender and the site of lesion. On multivariate

analysis, the histological stage did not predict for p16 expression. There was no correlation between the expression of Rb and p16.

Figure 6.1 Positive staining of cyclin D1 in a patch stage lesion (x400)

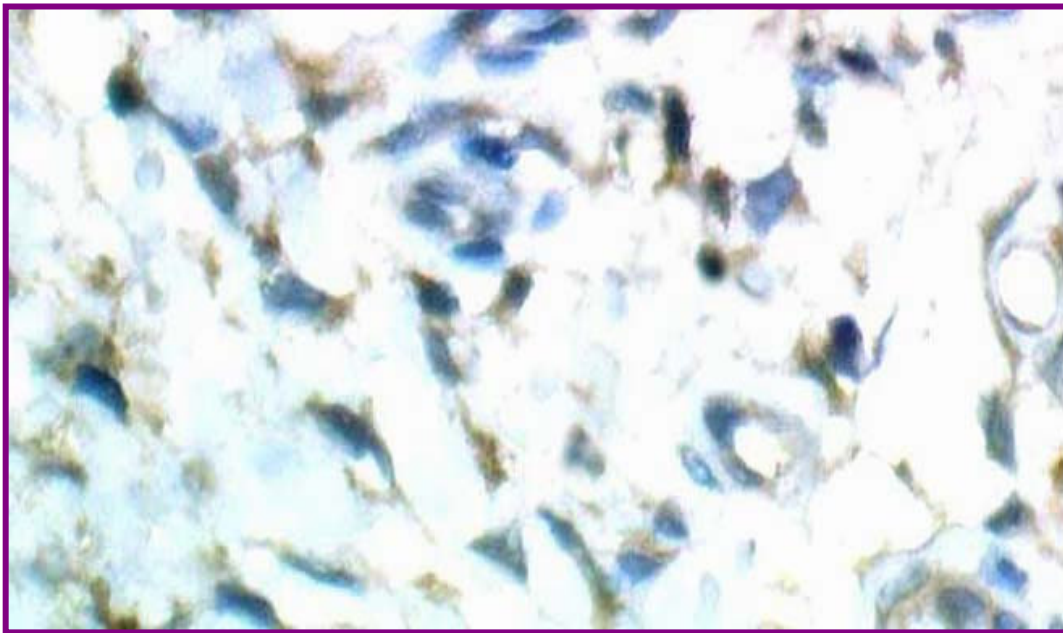


Figure 6.2 Positive staining of cyclin D1 in a nodular stage lesion (x400)



Figure 6.3 Positive staining of Rb in a nodular lesion (x400)

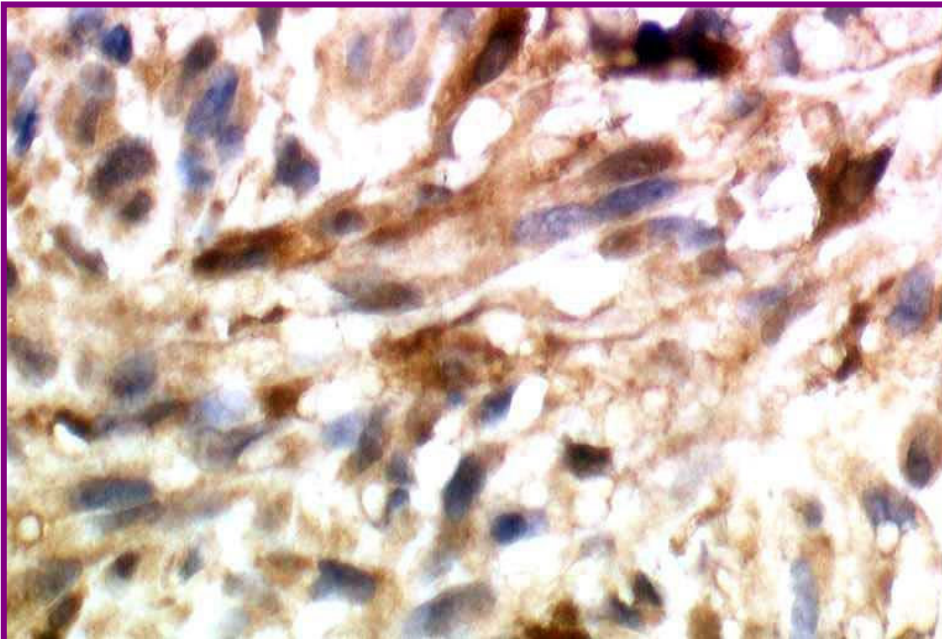


Table 6.1 Expression of cyclin D1 in different histological subtypes			
Pathological subtypes	Cyclin D1 staining score <4	Cyclin D1 staining score ≥4	p value
Patch (n=11)	11 (100%)	0 (0%)	0.0017
Plaque (n=8)	7 (87.5%)	1 (12.5%)	
Nodular (n=22)	11 (50%)	11 (50%)	

Table 6.2 Expression of cyclin D1 in nodular stage lesions			
Cyclin D1 staining score	HIV +ve (n=13)	HIV –ve (n=9)	p value
<4	9 (69.2%)	2 (22.2%)	0.03
≥4	4 (30.8%)	7 (77.8%)	

Table 6.3 Cyclin D1 expression and correlation with clinicopathological data

Parameter	Total No.	Cyclin D1 score <4	Cyclin D1 score ≥4	X ²	p value	G-statistic	Contingency coefficient
HIV status							
Positive	31	26 (84%)	5 (16%)				
Negative	10	3 (30%)	7 (70%)	10.6	0.0011	9.96	0.45
HHV8 status							
Positive	24	16 (66.7%)	8 (33.3%)				
Negative	8	4 (50%)	4 (50%)	2.1	0.85	0.043	0.76
Age							
≥60	33	27 (82%)	6 (18%)				
>60	8	2 (25%)	6 (75%)	10.1	0.0065	9.31	0.44
Gender							
Male	35	27 (77%)	8 (23%)				
Female	6	2 (33%)	4 (67%)	4.75	0.029	4.3	0.32
Multiplicity of lesions							
Single	24	18 (75%)	6 (25%)				
Multiple	17	12 (71%)	5 (29%)	0.099	0.750	0.098	0.049
Recurrence							
Absent	32	27 (84%)	5 (16%)				
Present	9	2 (22%)	7 (78%)	13.1	0.0003	12.3	0.49
Site							
Cutaneous	30	21 (70%)	9 (30%)				
Non-cutaneous	11	8 (73%)	3 (27%)	0.029	0.865	0.029	0.77

Table 6.4 Expression of Rb in Kaposi's sarcoma

	Rb positive	Rb negative	p value
HIV status			
Positive	19 (76%)	6 (24%)	
Negative	6 (75%)	2 (25%)	ns
HHV8 status			
Positive	20 (83.3%)	4 (16.7%)	
Negative	3 (42.8%)	4 (57.2%)	0.03
Histological subtype			
Patch	13 (76.5%)	4 (23.5%)	
Plaque	4 (66.6%)	2 (33.3%)	
Nodular	8 (80%)	2 (20%)	ns
Site			
Cutaneous	21 (84%)	4 (16%)	
Non-cutaneous	4 (50%)	4 (50%)	ns
Gender			
Male	22 (75.8%)	7 (24.2%)	
Female	3 (75%)	1 (25%)	0.08

Table 6.5 Expression of p16 in Kaposi's sarcoma

	p16 positive	p16 negative	p value
HIV status			
Positive	17 (60.7%)	11 (39.3%)	
Negative	7 (87.5%)	1 (12.5%)	0.1
HHV8 status			
Positive	69.9%	57.2%	
Negative	30.4%	42.8%	0.08
Histological subtype			
Patch	5 (45.5%)	6 (54.5%)	
Plaque	5 (71.5%)	2 (28.5%)	
Nodular	14(77.8%)	4 (22.2%)	0.3
Site			
Cutaneous	18 (75%)	4 (25%)	
Non-cutaneous	4 (50%)	4 (50%)	0.09
Gender			
Male	19 (63.3%)	11 (36.7%)	
Female	5 (83.3%)	1 (16.7%)	0.08

6.4 Discussion

The cyclin D1 protein has an important regulatory function in the control of cell cycle progression in the G1 phase by activating cellular kinase to phosphorylate checkpoint molecules such as the Rb protein. The cyclin D1 gene is localised to chromosome 11q13 and encodes a putative oncogenic nuclear protein of approximately 34 kD that appears to be significant in tumourigenesis (Motokura, Bloom and Kim 1991). Amplification of the cyclin D1 gene with protein overexpression can allow cancer cells to progress from the resting G0 phase to G1 and/or G1 to S phases of the cell cycle, resulting in enhanced DNA replication.

Immunostaining revealed a strong correlation between cyclin D1 overexpression, negative HIV status and nodular subtypes. It seems that for the progression of KS to a more advanced, nodular stage lesion, the effect of overexpressed cyclin D1 protein and its consequential effect on the cell cycle is important in classical lesions. This is supported by the findings that cyclin D1 overexpression was more prevalent in the HIV-negative, classical nodular lesions. Similarly, in a smaller study by Horenstein et al., which employed immunohistochemistry and monoclonal antibodies against cyclin D1 in 24 cases of KS (21 AIDS-related, 3 classical), enhanced cyclin D1 expression was found to correlate with tumour progression (Horenstein, Cesarman, Wang, Linkov, Prieto and Louie 1997). Cyclin D1 was detected in 1 of 13 cases of early, patch or plaque stage lesions, in 4 of 5 cases of nodular stage lesions and in 3 of 6 cases of visceral KS lesions. Another study

from the United Kingdom, using immunohistochemistry staining on 17 KS cases (11 AIDS-related, 3 endemic and 2 HIV status unknown), found that only one case, which was a nodular lesion in a lymph node in a HIV-negative male, showed moderately strong staining (Kennedy, Biddolph, Lucas, Howells, Picton, O'D McGee, Silva, Uhlmann, Luttich and O'Leary 1999). However, this study only examined a small number of cases and may not be representative.

These findings raise the possibility of a different pathogenetic mechanism in the progression of KS in HIV-negative patients from that in HIV-positive patients. It appears that the role of cyclin D1 overexpression in the progression of KS in HIV positive patients may not be as critical as in HIV negative patients. A possible explanation for these observations is that HIV infection together with the various cytokines concomitantly induced by the Tat protein of HIV virus could have a synergistic effect with KS associated HHV8, therefore progressing through a different molecular route for the development and advancement of KS (Prakash, Tang, He, Ali, Coleman and Gill 2000; Weiss and Boshoff 2000). IPX studies of KS biopsy specimens have shown an increased level of interleukin-6 in the tumour areas and to a lesser extent in the surrounding normal epidermis (Oxholm, Oxholm, Permin and Bendtzen 1989; Oxholm 1992). In cell culture, interleukin-6 has been shown to be a growth factor for KS cells and the results were presented in the candidate's Master of Medicine thesis (Hong, Zhang, Leigh and Stevens 2001).

The cyclin D1 protein shares significant homology to the HHV8, which is now regarded as the initiating factor in the development of KS (Moore and Chang 1995). The virus contains an open reading frame (ORF 72) with a sequence similar to the human cyclin D1 protein (Cesarman, Chang, Moore, Said and Knowles 1995). It has been previously shown that KSHV-cyclin latently infected cells can overcome Rb mediated cell cycle arrest (Chang et al. 1996) and hence contribute to tumour development. Advanced KS lesions also demonstrate a range of clonality with monoclonal, oligoclonal and polyclonal patterns. This suggests that cells with latent HHV8 infection are probably initially polyclonal but evolution to monoclonality occurs as the lesion progresses (Judde, Lacoste, Briere, Kassa-Kelembho, Clyti, Couppie, Buchrieser, Tulliez, Morvan and Gessain 2000). The latter part of this may proceed through a molecular pathway involving the regulatory roles of cyclin D1.

Deregulated expression of cyclin D1 is linked to the development and progression of a number of human cancers (Lammie, Fantl, Smith, Schuuring, Brookes, Michalides, Dickson, Arnold and Peters 1991; Barbieri, Cagnoli et al. 1999). In common with this, our study shows that cyclin D1 overexpression correlates with tumour recurrence in KS. In extremity soft tissue sarcoma, cyclin D1 overexpression was noted in 29% of cases (Kim, Lewis, Brennan, Woodruff, Dudas and Cordon-Cardo 1998). Using IPX followed by Southern blot analysis, overexpression of cyclin D1 correlated significantly with a higher tumour grade. On multivariate analysis, cyclin D1 was significantly associated with a worse overall

survival. In oesophageal carcinoma, cyclin D1 was involved in tumour progression. Overexpression of the protein detected by IPX has been shown to be an important prognostic factor (Naitoh, Shibata, Kawaguchi, Kodama and Hattori 1995). In pancreatic adenocarcinoma, cyclin D1 mRNA levels were 2.1-fold higher in the pancreatic cancers than in normal pancreatic tissues ($p < 0.0002$). Patients with lower cyclin D1 levels ($n=16$) had a median survival of 15.5 months whereas patients with higher levels ($n=16$) had a median survival of 6.5 months ($p < 0.007$). These data indicate that cyclin D1 expression may be useful as a predictor of survival in pancreatic cancer (Kornmann, Ishiwata, Itakura, Tangvoranuntakul, Beger and Korc 1998). In addition, inhibition of cyclin D1 expression not only suppresses the growth of pancreatic cancer cells in vitro, but it also enhances their responsiveness to chemotherapy. This effect may be related to the altered expression of the chemoresistance gene (Kornmann, Danenberg, Arber, Beger, Danenberg and Korc 1999). These data support the concept that cyclin D1 overexpression may determine the evolution of a particularly advanced subset of tumours. Furthermore, inhibition of cyclin D1 expression in colonic cancer cells with antisense can reverse the tumorigenicity of these cells (Arber, Doki et al. 1997). In this study, an antisense cyclin D1 cDNA construct was overexpressed in the human colon cancer cell line SW480E8. The integration and expression of the antisense construct resulted in a decreased expression of the cyclin D1 protein. This was associated with decreased levels of the Rb and p27Kip1 proteins. In addition, the hypophosphorylated form of Rb was increased in these cells. The SW480E8 antisense cyclin D1 cells displayed an increased doubling time, a

decreased saturation density, a decreased plating efficiency, a decreased anchorage-independent growth, and a loss of tumourigenicity in nude mice. The results of these studies suggest that cyclin D1 may be a potentially useful target in the treatment of malignancies that overexpressed the protein. Perhaps in the future, identifying lesions with abnormal cyclin D1 expression may be helpful in allowing more aggressive treatment or trials of treatment with novel therapeutic agents, such as vaccines or cytokine therapies.

Amplification of the 11q13 region is found in 20-40% of ovarian and squamous cell carcinoma (Lammie, Fantl et al. 1991; Barbieri, Cagnoli, Ragni, Pedulla, Foglia and Alama 1997). The t(11;14) (q13;q32) chromosomal translocation is associated with approximately 70 to 90% of mantle cell lymphoma and is associated with cyclin D1 overexpression (Raynaud, Bekri, Leroux, Grosgeorge, Klein, Bastard, Gaudray and Simon 1993; de Boer, van Krieken, Kluin-Nelemans, Kluin and Schuurin 1995). The 11q13 breakpoints are clustered in a 1-kb region, known as the major translocation cluster of the BCL-1 locus, which is located approximately 120 kb centromeric of the cyclin D1 gene. In a study of 48 cases of myeloma, the t(11;14) has been reported in 17-25% of the cases (Avet-Loiseau, Li, Facon, Brigaudeau, Morineau, Maloisel, Rapp, Talmant, Trimoreau, Jaccard, Harousseau and Bataille 1998; Pruneri, Fabris, Baldini, Carboni, Zagano, Colombi, Ciceri, Lombardi, Rocchi, Buffa, Maiolo and Neri 2000). IPX staining and fluorescence in situ hybridisation were used to detect cyclin D1 expression and the presence of chromosome 11 abnormalities. Cyclin D1 positivity was detected in all 7 cases

bearing the t(11;14) translocation, 2 of the 13 cases with trisomy 11, and 1 of the 19 cases with no apparent abnormalities of chromosome 11. The author concluded that t(11;14) translocation in multiple myeloma leads to cyclin D1 overexpression. The 3 cases with cyclin D1 expression but no apparent t(11;14) translocation might be secondary to breakpoints occurring outside the region detected by fluorescent in-situ hybridisation or other unknown mechanisms responsible for the induction of cyclin D1.

Recently, it has been shown that cyclin D1 overexpression can enhance the radiosensitivity in a breast cancer cell line (Coco Martin, Balkenende, Verschoor, Lallemand and Michalides 1999). Cyclin D1 expressing MCF7 cells treated with a dose of 5 Gy resulted in a rapid increase of p53 and p21 whereas cyclin D1 non-expressing cells showed a more transient expression of these proteins after radiation. It was found that there was an increase in exit from G2-M phases of the cell cycle which occurred following radiation and hence entry into the next cell cycle when the radiation induced DNA damage was not fully repaired. This may lead to reinduction of apoptosis and formation of micronuclei. This increased apoptosis was not related to a decline of the apoptosis protecting protein bcl-2 or induction of the apoptosis promoting protein, BAX. An increased radiation induced apoptosis was also demonstrated in rat embryo cells transfected with cyclin D1 (Pardo, Su and Borek 1996) However the overexpression of cyclin D1 was not associated with enhanced radiosensitivity in the haematopoietic mouse cell line 32Ccl3 (Epperly, Berry, Halloran and Greenberger 1995). In this study, there was

no induction of p53 and p21 proteins by radiation, indicating that the cell line lacked wild type p53. Overexpression of cyclin D in hematopoietic cells induces detectable effects on hematopoietic cell radiation biology including a broadening of the shoulder on the radiation survival curve and a decrease in radiation-induced G1/S-phase arrest. Clinically, KS is a radiosensitive tumour with an 80% response rate after moderate doses of radiation (Stelzer et al. 1993). This may be related to the high level of cyclin D1 present and hence the enhanced induction of p53 after radiation. The high level of p53 can then lead to an increased apoptotic cell death and hence reduction in the overall cell survival after radiation.

The paucity of KS in females was initially attributed to lower androgenic hormones. However, KS occurs infrequently in females in the western countries probably because females are rarely infected with HHV8. In contrast, in Africa where HIV is essentially a heterosexually transmitted disease rather than through homosexual contacts, KS occurs almost equally in males and females. Nevertheless, there are in vitro studies to support a protective effect of the human chorio-gonadotrophic hormone (Lunardi-Iskandar, Bryant, Zeman, Lam, Samaniego, Besnier, Hermans, Thierry, Gill and Gallo 1995). The putative mechanism of action is not clear and it may relate to a reduction in follicle-stimulating hormone and luteinizing hormone. Despite the in vitro findings, clinical trials with human chorio-gonadotrophic hormone as a treatment modality for KS yielded inconsistent results (Gill, Tsai et al. 1998; Tavio, Nasti, Simonelli, Vaccher, De Paoli, Giacca and Tirelli 1998; Darzynkiewicz 1999). A significant difference in cyclin D1 expression was detected

between males and females in this study cohort: 67% of the KS lesions from females showed cyclin D1 expression, whereas only 23% of the lesions from males showed cyclin D1 expression. However, gender is not an independent predictor for cyclin D1 expression on multivariate analysis. This finding is probably due to the fact that females in the study cohort were more likely to be HIV negative and presented with nodular lesions rather than a true difference in the cyclin D1 expression in lesions from males.

About half of the HHV8 negative lesions showed overexpression of cyclin D1, and this finding suggests that many of these tumours, which were not initiated by HHV8, can develop and progress along a similar cell cycling route that involves the Rb/cyclin D1/p16 pathway. In other words, the HHV8 negative lesions can also progress through a route that is similar to the Rb/cyclin D1/p16 pathway imparted by HHV8. For those HHV8 negative that were also cyclin D1 negative, their progression is probably through a different molecular route apart from the cyclin D1 pathway. The interaction between other viral proteins and regulation of the cell cycle transition has been reported. The E6 protein of the human papilloma virus has been shown to have the ability to deregulate the G1/S phase transition (Malanchi, Caldeira, Krutzfeldt, Giarre, Alunni-Fabbroni and Tommasino 2002). The E6 viral protein acted upstream of Rb and its mechanism in promoting cell cycle progression is dependent on Rb phosphorylation.

The level of p16 in KS has not been reported previously. Our results indicate that loss of p16 function might not be responsible for the differing clinical picture of classical and AIDS-related KS. The p16 protein forms binary complexes exclusively with Cdk4 and Cdk6, inhibiting their kinase activity and subsequent pRb phosphorylation during the G1 phase of the cell cycle (Serrano, Hannon and Beach 1993). The p16 gene is mutated in a wide variety of primary tumours and tumour cell lines. The inactivation of this tumour suppressor gene involves three major mechanisms. The first mechanism of inactivation is homologous deletion of the gene and this is seen in T cell acute lymphoblastic leukaemia (Ohnishi, Kawamura, Ida, Sheng, Hanada, Nobori, Yamamori and Hayashi 1995), glioma (Giani and Finocchiaro 1994) and non-small cell lung cancer (Shapiro, Edwards, Kobzik, Godleski, Richards, Sugarbaker and Rollins 1995). Using polymerase chain reaction single-strand conformation polymorphism and Southern blot analysis, homozygous deletions were detected in 39% of the 56 T cell acute lymphoblastic leukaemia samples and in 50% of the 17 cell lines. In the 32 cases of malignant glioma, homozygous deletions were detected in 42%. In primary lung cancer, there is a reciprocal relationship between Rb inactivation and p16 expression and this finding may define differential p16 expression as a fundamental distinction between non-small cell lung cancer and small cell lung cancer.

The second mechanism for p16 gene inactivation is small deletions. These have been reported in familial melanoma, oesophageal carcinoma and bile duct carcinoma. Eight p16 germline substitutions (one nonsense, one splice donor site

and six missense) were detected in 13/18 familial melanoma kindreds (Hussussian, Struewing, Goldstein, Higgins, Ally, Sheahan, Clark, Tucker and Dracopoli 1994). In a study of 25 primary esophageal squamous cell carcinomas, 4 of 25 primary lesions were found to be mutated, one in exon 1 and three in exon 2 (Igaki, Sasaki, Tachimori, Kato, Watanabe, Kimura, Harada, Sugimura and Terada 1995). Similarly, point mutation of p16 was frequently detected in bile tract carcinoma (Yoshida, Todoroki, Ichikawa, Hanai, Suzuki, Hori, Fukao, Miwa and Uchida 1995). The third mechanism is hypermethylation of the gene and its promoter region. Primary tumours of the breast (31%) and colon (40%) displayed de novo methylation of the CpG islands of the p16 gene (Merlo, Herman, Mao, Lee, Gabrielson, Burger, Baylin and Sidransky 1995).

Loss of p16 expression is generally associated with more aggressive tumours and a poorer survival. Using IPX staining of p16 in 202 primary melanomas and 68 corresponding metastases, 45% of the primary cases showed absent or minimal staining. These negative cases were significantly associated with higher levels of Ki-67 expression, ulceration of the primary lesions and vascular invasions (Straume, Sviland and Akslen 2000). An even higher level of loss of p16 was observed in the corresponding metastatic lesions (77%). On multivariate analysis, p16 staining was an independent prognostic factor and absence of p16 was associated with a poorer ten-year survival. In adenocarcinoma of the pancreas, mutation in p16 was found to be a predictor for poorer survival (Bartsch, Shevlin, Callery, Norton, Wells and Goodfellow 1996). Southern blot analysis was used to

detect p16 mutation in 114 samples of T-cell acute leukemia (Yamada, Hatta, Murata, Sugawara, Ikeda, Mine, Maeda, Hirakata, Kamihira, Tsukasaki, Ogawa, Hirai, Koeffler and Tomonaga 1997), 24.6% of the cases showing deletions of p16 gene. Patients with deleted p16 gene had a significantly shorter survival than for those patients in whom the genes was preserved ($P<0.001$). Using the Cox proportional hazard model, the deletion of the p16 gene emerged as an independent prognostic indicator. However, in a study of 88 patients with prostate cancer, overexpression of p16 was associated with tumour recurrence and poorer clinical outcome (Lee, Capodieci et al. 1999). In situ hybridisation and IPX staining were used to determine the status of the p16 protein. p16 was undetectable in normal prostate and benign hyperplastic tissues. p16 overexpression was observed in 43% of prostate carcinomas and was correlated with a higher pretreatment PSA level and a shorter time to relapse. Although p16 acts as a negative cell cycle regulator, specific mechanisms may contribute to its altered expression, overcoming the p16-mediated tumour suppressor activities.

It has been reported that hypophosphorylated active Rb can repress p16 expression (Li, Nichols, Shay and Xiong 1994). Consistent with these findings, p16 gene deletion and Rb deficiency are reported to be inversely correlated in many primary tumours as well as in cancer cell lines (Shapiro, Edwards et al. 1995; Yeager, Stadler, Belair, Puthenveetil, Olopade and Reznikoff 1995), because a defect in either of the two tumour suppressor genes is sufficient to deregulate G1

progression. In this thesis, such a reciprocal relationship between p16 and pRb was not elucidated in KS.

The Rb gene was the first identified tumour suppressor gene. It was initially cloned as a result of its frequent mutation in retinoblastoma (Friend, Bernards, Rogelj, Weinberg, Rapaport, Albert and Dryja 1986). It has now been shown to play a fundamental role in cell cycle regulation and is a target of genetic mutation in many malignancies. Structurally, pRb contains several functional domains. Domains A and B are highly conserved from humans to plants and they interact with each other to form a central pocket (Chow and Dean 1996). This pocket is disrupted by most naturally occurring germ line mutation (hereditary retinoblastoma) and somatic mutation (Horowitz, Park, Bogenmann, Cheng, Yandell, Kaye, Minna, Dryja and Weinberg 1990; Harbour 1998).

Our finding of Rb expression in 76% of cases is in keeping with the result from another study (Horenstein, Cesarman et al. 1997). Horenstein et al. demonstrated Rb protein by IPX in all 24 cases of KS examined. The consistently high level of Rb protein found in both the classical and AIDS-related KS would imply that the Rb gene mutation might not be important in the progression of KS. Positive staining of the nucleus using monoclonal antibody is an indication of a functional Rb gene as tumours having homozygous mutations of this gene do not show expression of the protein. This indicated that the hyperproliferative state in KS cannot be attributed to a defect of this growth restriction factor, as is the case in several other tumours.

Using IPX staining for RB protein in vulvar squamous cell carcinoma, abnormal staining was detected in 37% of 547 cases and was associated with poor tumour grade in one study (Rolfe, Crow, Benjamin, Reid, Maclean and Perrett 2001). In liver metastases from colorectal carcinoma, lesions with Rb staining detected by IPX staining was associated with a significantly longer median survival (79 weeks vs 44 weeks, $p=0.03$) (Backus, van Riel et al. 2001).

The majority of HHV8 positive lesions (83.3%) were Rb positive compared with 57.2% of the HHV8 negative lesions. This suggests that the HHV8 negative lesions are more likely to progress through a deficient Rb pathway whereas the role of RB in the progression of HHV8 positive lesions may not be as important. This is an unexpected finding given that one of the postulated mechanisms of tumour initiation of the HHV8 virus is via the viral cyclin it produces. The viral cyclin produced by HHV8 acts through the Rb pathway much the same as cyclin D1 (Chang and Moore 1996) and one would have expected that HHV8 positive cases are less likely to be positive for the Rb protein.

In conclusion, along the Rb/cyclin D1/p16 pathway (the “Rb tumour suppression pathway”), cyclin D1 overexpression and its effect on the cell cycle might be responsible for the progression of classical KS. In particular, differences in cyclin D1 expression seem crucial in transitions from the early patch stage to the late nodular stage. The present observations suggest that the role of cyclin D1 in the pathogenesis and aggressiveness of KS varies in the different clinical subtypes of

KS. The rates of p16 and Rb expression do not appear to vary between classical and AIDS-related lesions.

Chapter 7: The role of mitotic cyclins in Kaposi's sarcoma

- Cyclin A and p34^{cdc2} expression in Kaposi's sarcoma

Chapter 7: The role of mitotic cyclins in Kaposi's sarcoma - Cyclin A and p34^{cdc2} expression in Kaposi's sarcoma

7.1 Background

It is now known that cell proliferation is precisely regulated by cell cycle control mechanisms that depend on the activities of various cyclins and cyclin dependent complexes. Deregulated expression of cyclins has been found in different types of malignancy. Cyclin A is detectable in the S phase, increasing during progression into the G2 phase, and is a useful marker of proliferation (Schulze, Zerfab, Spitkovsky, Middendorp, Berges, Helin, Jansen-Durr and Henglein 1995). The majority of breast tumours with increased cyclin A expression has been shown to correspond with an increase in the proliferative status of tumour cells (Dutta, Chandra, Leiter and Lester 1995).

The cyclin dependent kinase, p34^{cdc2}, plays a critical role in the cell cycle by combining with cyclin B during the transition phase from G2 to M to form the mitosis promoting factor. Mitosis promoting factor is required for cells to enter the M phase and without p34^{cdc2} activity, cells are not capable of dividing. Currently, there is no documented study on the possible significance of p34^{cdc2} in the pathogenesis of KS. This part of the thesis examined the role of cyclin A and p34^{cdc2} in the pathogenesis of classical and AIDS-related KS.

7.2 Methods

The method for IPX staining is presented in Chapter 2 and Appendix 1. Murine monoclonal antibody directed against human cyclin A, clone 6E6 (Novocastra) at 1:50 dilution was used. The antibody against cyclin A was an IgG1, kappa antibody with a nuclear staining pattern. The primary antibody was substituted with PBS in sections used as negative controls. The sections were counterstained with Harris's haematoxylin.

Cells were classified as cyclin A positive when there was nuclear staining. A semiquantitative scoring system, based on the staining intensity and the percentage of positive cells, was used (Vos, Ter Haar et al. 1999). The staining intensity was scored as 0 (none), 1 (weak), 2 (moderate) and 3 (strong) and the percentage of positive cells was scored as 0 (0%), 1 (1-25%), 2 (>25-50%), 3 (>50-75%) and 4 (>75%). The cyclin A staining score was calculated as the sum of the intensity and the percentage of positive cells. For p34^{cdc2} staining was classified using a standard evaluation method as described previously (Schadendorf, Makki and Stahr 1995; Georgieva, Sinha and Schadendorf 2001). The proportion of positive cells was scored according to five categories (- 0-5%; + 5-25%; ++, 26-50%; +++, 51-75%; +++, 75-100%). Assessment of the staining score was performed by investigators blinded to the clinical details of the cases. Histologically normal dermal tissues located away from the primary lesions were used as normal non-neoplastic controls.

Correlations between cyclin A, p34^{cdc2} staining score and clinicopathological factors were analysed using chi-squared (χ^2) contingency test. Multivariate analysis was performed using the stepwise multiple linear regression model by assessing the cyclin A and p34^{cdc2} in KS lesions with the following baseline covariates: age, gender, HIV status, tumour stage, recurrent lesion, multiplicity of the lesion and involved site. Only p values of less than 0.05 were considered significant.

7.3 Results

Cyclin A expression was detected in 68.2% (28/41) of all KS cases (Table 7.1, Figures 7.1, 7.2). There was a statistically significant correlation between the histological stage and expression of cyclin A. Cyclin A expression was detected in 36.4% of the patch stage lesions and 45.5% of the plaque stage lesions. In contrast, 78.9% of the nodular stage lesions showed cyclin A expression (patch stage vs nodular stage, $p=0.03$). Classical KS lesions had a higher incidence of cyclin A expression than AIDS-related lesions (90.9% vs 53.3%, $p=0.004$; Table 7.2). Within the nodular stage, cyclin A expression was significantly higher in classical lesions than in AIDS-related lesions (85.7% vs 72.7%, $p=0.04$).

Patients who had tumour recurrence were more likely to have cyclin A overexpressed lesions (100% vs 61%, $p=0.025$; Table 7.2) when compared with those with no tumour recurrence detected at the time of clinical follow up. Cutaneous lesions were significantly more likely to show cyclin A expression (78% vs 35%, $p=0.03$) than non-cutaneous lesions. The rates of cyclin A expression were not significantly different in the <60 age group vs the >60 age group (63% vs 88%, $p=0.13$). Similarly, the rates of cyclin A expression between males and females were not significantly different either (64% vs 88%, $p=0.19$).

Since Ki-67 is also a marker of proliferation, the relationship between cyclin A and Ki-67 was explored (Figure 7.5). Cyclin A positive lesions had a significantly higher

Ki-67 staining score than cyclin A negative lesions (staining score of 3 or 4, 33.3% vs 0%, $p=0.01$).

p34^{cdc2} expression was found in all histological stages of KS (17/39 cases; 44%) with the highest rate in the early patch stage (64%) followed by the nodular stage (47%) and then the plaque stage (18%) (Figures 7.3, 7.4 Table 7.3). In contrast, none of the matching normal tissues showed any staining in blood vessels or fibroblasts ($p<0.05$). However, clinicopathological correlation with HIV status, age, gender, site of lesion and recurrence did not show any relationship with p34^{cdc2} expression.

Figure 7.1 Lack of cyclin A expression in a patch lesion (x 400)

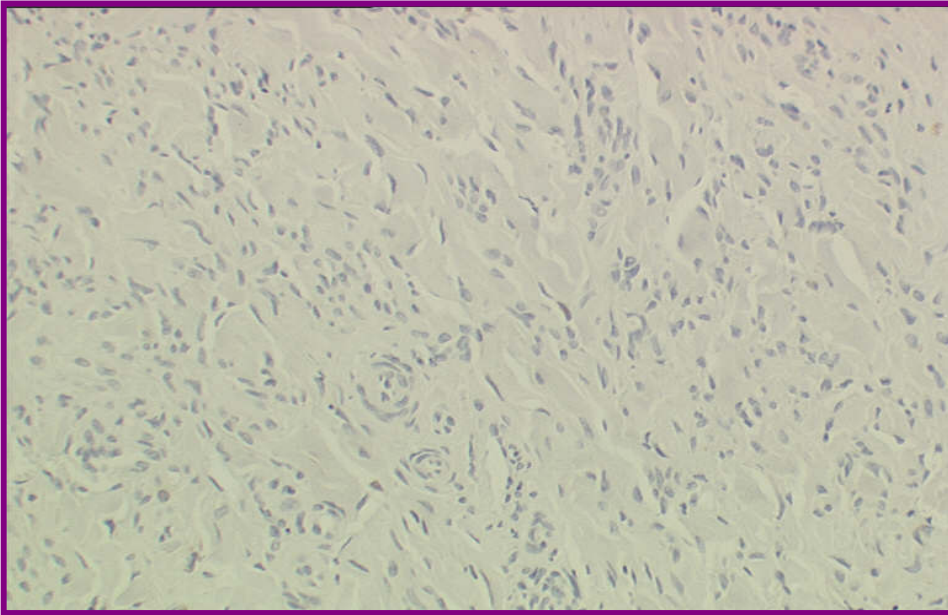


Figure 7.2 Cyclin A expression in several cells in a nodular KS (x 200)

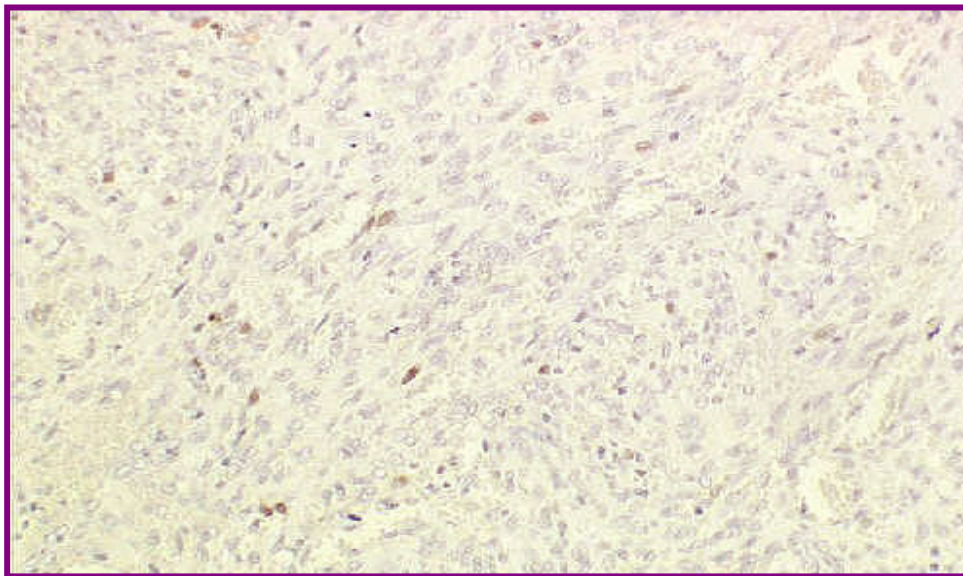


Figure 7.3 Positive p34^{cdc2} staining in a patch lesion (x 200)

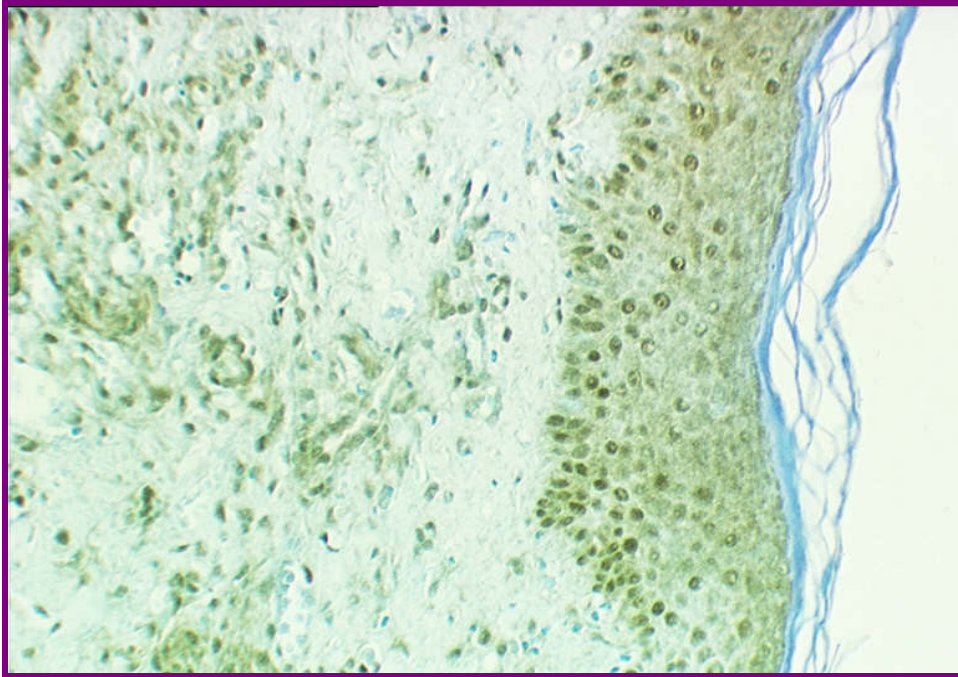


Figure 7.4 High power view of lesional cells positive for p34^{cdc2} in a patch lesion (x 400)

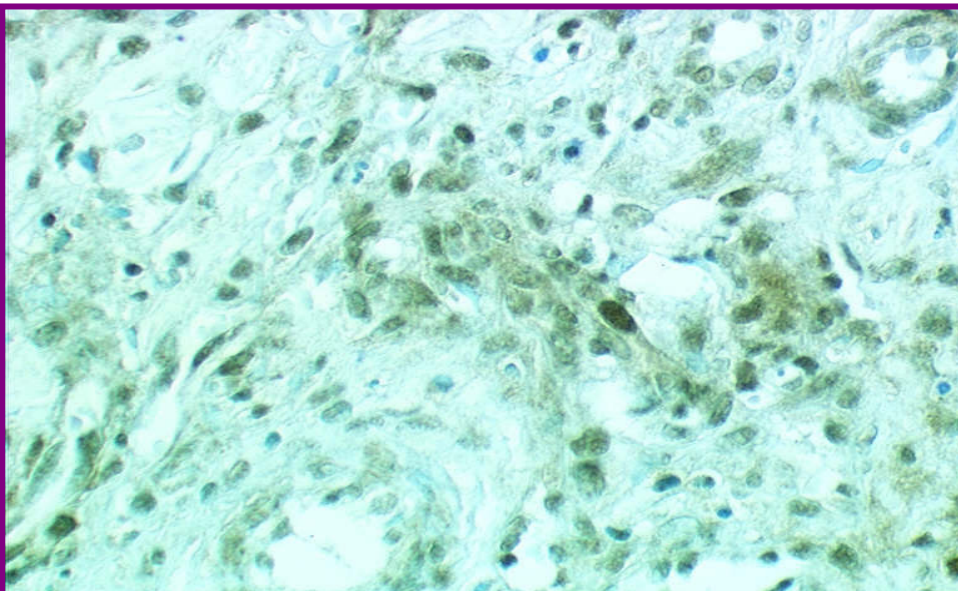


Table 7.1 Expression of cyclin A in Kaposi's sarcoma

Histological subtypes	No.	Cyclin A expression		Chi square	p value
		>10%	≤10%		
Patch	11	4 (22.2%)	7 (77.8%)		
Plaque	8	5 (62.5%)	3 (37.5%)		
Nodular	22	19 (86.4%)	3 (13.6%)	9.11	0.011

Table 7.2: Cyclin A expression and correlation with clinico-pathological data

Parameter	No.	Cyclin A+ve	Cyclin A -ve	χ^2	p value	G	Contingency
HIV status							
Positive	30	18 (60%)	12 (40%)				
Negative	12	11 (90%)	1 (10%)	4.02	0.045	4.71	0.30
HHV8 status							
Positive	23	15 (65.2%)	8 (34.8%)				
Negative	8	5 (62.5%)	3 (37.5%)	0.94	0.63	0.43	0.13
Age							
<60	32	20 (63%)	12 (37%)				
≥60	9	8 (87%)	1 (13.5%)	2.26	0.13	2.62	0.23
Gender							
Male	33	21 (64%)	12 (36%)				
Female	8	7 (88%)	1 (12%)	1.69	0.19	1.9	0.20
Multiplicity							
Single	16	12 (75%)	4 (25%)				
Multiple	25	16 (64%)	9 (36%)	0.55	0.46	0.56	0.12
Recurrence							
Absent	33	20 (61%)	13 (39%)				
Present	8	8 (100%)	0 (0%)	4.62	0.03	N/A	0.32
Site							
Cutaneous	32	25 (78%)	7 (22%)				
Non-cutaneous	9	3 (35%)	6 (65%)	6.51	0.01	6.14	0.37

Figure 7.5 Ki-67 staining in cyclin A positive and cyclin A negative lesions

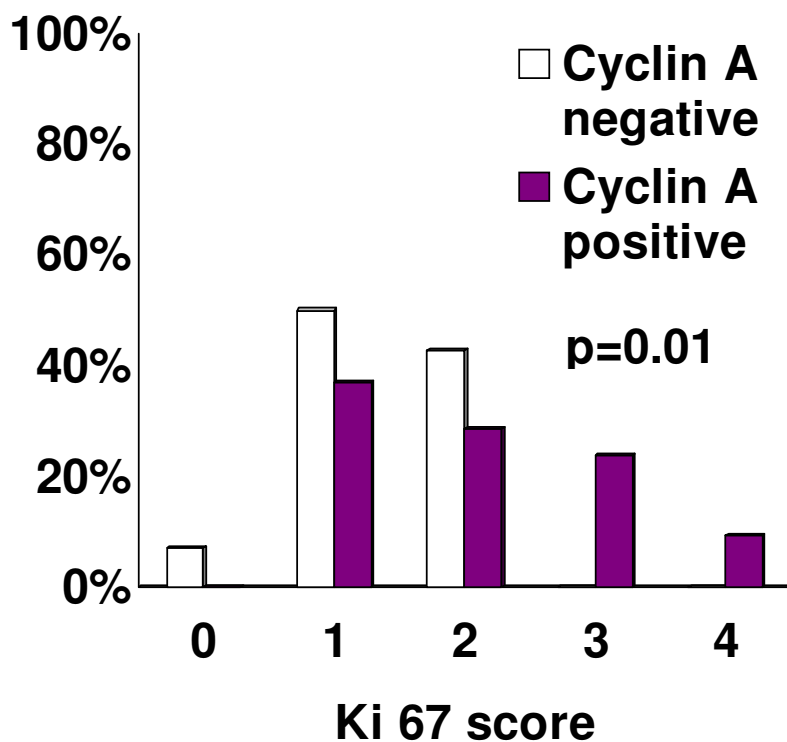


Table 7.3 p34^{cdc2} expression in Kaposi's sarcoma

	No.	p34 ^{cdc2} positive	p34 ^{cdc2} negative	Chi-square	p value
Normal skin	39	0 (0%)	39 (100%)		
Kaposi' sarcoma	39	17 (43.6%)	22 (56.4%)	21.7	0.0001
Patch	11	7 (63.6%)	4 (36.4%)		
Plaque	11	2 (18.2%)	9 (81.8%)	4.7	0.03
Nodular	17	8 (47.1%)	9 (52.9%)		

7.4 Discussion

Recent work has elucidated that many genes are involved in the regulation of the cell cycle. These genes can be classified into two main groups: cyclins and cyclin dependent kinases (cdks). The cdks regulate the cell cycle via the phosphorylation of the Rb gene product, thus facilitating the transcription of genes needed for DNA synthesis (Gillett and Barnes 1998). The cyclins provide the cell cycle specificity to the cdk phosphorylation signal. The cyclins are transiently produced during various phases of the cell cycle and, when present, activate the cdks, allowing for progression through the cell cycle. Cyclin A is a 60 kd protein that is involved in DNA replication and mitosis. It is produced and transported to the nucleus during the S and G2 phases of the cell cycle. It is a mitotic cyclin (like cyclin B) and binds to both cdk2 and cdc2. In the normal cell, cyclin A complexes with p21^{WAF1/CIP1} (Zhang, Xiong and Beach 1993) but the latter is released from the complex during cell transformation (Zhang, Kobayashi, Galaktionov and Beach 1995). Degradation of cyclin A occurs after the cells enter the M phase (Desdouets, Sobczak-Thepot, Murphy and Brechot 1995).

The role of cyclin A in the pathogenesis of the different subtypes of KS has not been previously reported. The higher incidence of cyclin A expression in classical KS in the current study suggests a different pathogenetic mechanism in the progression of KS in HIV negative and HIV positive patients. A possible explanation for these observations is that HIV infection together with various

cytokines concomitantly induced by the Tat protein of HIV virus could have a synergistic effect with HHV8, therefore progressing through a different molecular route for the development and progression of AIDS-related KS (Prakash, Tang et al. 2000; Weiss and Boshoff 2000). Cyclin A expression may play a role in the progression of KS from the patch to nodular stage as supported by the results of higher incidence of cyclin A positivity in the nodular stage. For the progression from the patch stage to the more advanced nodular stage, overexpression of cyclin A, and perhaps its consequential effect on cell cycle, is important in classical KS. A previous study has suggested that cyclin A might be a useful marker of proliferation (Schulze, Zerfab et al. 1995). This thesis found a positive correlation between cyclin A and Ki-67 which confirmed that cyclin A can also be regarded as a marker of proliferation.

Overexpression of cyclin A has also been shown to be associated with more aggressive disease in other malignancies. In non-small cell lung carcinoma, immunohistochemical positivity for cyclin A was associated with a shorter survival (Dobashi, Shoji, Jiang, Kobayashi, Kawakubo and Kameya 1998). IPX staining was used to examine the level of cyclin A expression in 190 cases of primary lung carcinoma. In the non-small cell lung cancer patients, the cyclin A positive group showed a statistically significant lower survival rate compared with the negative group. The level of cyclin A expression was low in small cell lung cancer and therefore the correlation with survival rate could not be assessed. In a study of 170 cases of primary breast cancer, cyclin A expression was found to be associated

with a poorer prognosis (Bukholm, Bukholm and Nesland 2001). A highly statistically significant association was observed between cyclin A expression and early relapse ($p=0.001$ univariate analysis, $p=0.006$ multivariate analysis) as well as cancer specific death ($p<0.0001$) during the follow-up period. Florenes et al. examined the level of cyclin A expression in 172 cases of primary melanoma by IPX (Florenes, Maelandsmo, Faye, Nesland and Holm 2001), and they reported that in superficial spreading melanomas, the cyclin A level was related to tumour thickness, with less expression in thinner lesions ($p<0.001$), and to Ki-67 ($p<0.001$). Multivariate analysis showed that in addition to the depth of the primary tumour, the cyclin A protein level was an independent indicator of relapse-free period (thickness, $p<0.001$; cyclin A, $p=0.03$). In contrast, in nodular melanoma, the cyclin A level was associated with Ki-67 expression, but neither cyclin A nor Ki-67 was related to tumour thickness (cyclin A, $p=0.06$; Ki-67, $p=0.61$) and neither had any impact on relapse-free survival (cyclin A, $p=0.64$; Ki-67, $p=0.32$) or overall survival (cyclin A, $p=0.94$; Ki-67, $p=0.45$).

This thesis demonstrated cyclin A expression in 68% of KS cases, which is considerably higher than the reported level of expression in other malignancies. Dutta et al. reported a 22% cyclin A positivity in breast cancer cells (Dutta, Chandra et al. 1995). Other studies reported a 6.7% positive rate in prostate cancer and a similar 6% in renal cell carcinoma (Mashal, Lester, Corless, Ritchie, Chandra and Propert 1996; Renshaw, Loughlin and Dutta 1998). A high level of cyclin A expression in KS would indicate that more cells have passed the G1

restriction point and already in the S and G2 phases of the cell cycle. As the G1 restriction point is responsible for the repair of DNA damage, cells that are in the S and G2 phases cannot repair any DNA damage such as that induced by radiation. This high fraction of cells in the S and G2 phases may explain why KS has a high sensitivity to radiation (Stelzer and Griffin 1993). In addition, the G2 phase is known to be the radiosensitive part of the cell cycle. A high proportion of KS cells in this radiosensitive part of the cell cycle may also explain the radiosensitivity of KS seen clinically.

The exact mechanism, by which cyclin A leads to a more aggressive disease, and hence poor survival, is not known. Expressed discontinuously during the cell cycle, cyclins promote the phosphorylation of specific substances and allow cells to proceed through the sequential phases of the cell cycle. Biochemical studies on the function of cyclin A have shown that it is required to initiate DNA replication (Adachi and Laemmli 1994). Since the presence of cyclin A leads to the initiation of DNA replication, a higher level of cyclin A in cancer cells may lead to more rapid growth of the tumours at the primary and metastatic sites.

p34^{cdc2} is a serine/threonine kinase which is involved in cell cycle regulation. It is a 34 kD catalytic subunit maturation promoting factor, the other subunit being cyclin B1 (Lukas, Draetta et al. 1992). When complexed with cyclins, the p34^{cdc2} is activated and it initiates the entry of cells into mitosis, where it acts to promote nuclear envelope breakdown. Expression of p34^{cdc2} reflects the proliferating

potential of positively staining tissue and in concordance with this; p34^{cdc2} was not detected in normal tissue. The early patch stage had the highest staining rate and this probably relates to the maturation promoting effect of p34^{cdc2} rather than implying that the patch stage had the greatest proliferating potential. The lower positive staining rate in the plaque stage than nodular stage might not be a true finding due to the small number of cases.

In conclusion, a high level of cyclin A expression has been demonstrated in KS. Specifically, a differential expression of cyclin A in classical and AIDS-related KS has been found. The results suggest that cyclin A may play a role in the pathogenesis and progression of different clinical subtypes of KS. The other mitotic cyclin, p34^{cdc2}, has not been demonstrated to have a role in the variable clinical behaviours of classical and AIDS-related KS.

Chapter 8: Exploring the p53 pathway in Kaposi's sarcoma - Cyclin G1 and mutant p53 expression in Kaposi's sarcoma

Chapter 8: Exploring the p53 pathway in Kaposi's sarcoma - Cyclin G1 and mutant p53 expression in Kaposi's sarcoma

8.1 Background

Cyclin G1, a proto-oncogene, is one of the target genes of the transcription factor p53. It contains two p53 binding sites within its promoter and its up regulation coincides with activation of p53 by various DNA damaging agents (Okamoto and Beach 1994; Zauberman, Lupo and Oren 1995). Cyclin G1 is also induced by transforming growth factor- β , p63 and p73, and is often detected in cells lacking p53 (Bates, Rowan and Vousden 1996; Reimer, Borrás, Kurdistaní, Garreau, Chung, Aaronson and Lee 1999). It appears to be important in G2/M cell cycle arrest recovery and growth promotion after cellular stress (Kimura, Ikawa, Ito, Okabe and Nojima 2001). However, conflicting roles have been assigned to cyclin G1 in growth control and cancer development. Some studies indicate that cyclin G1 promotes growth, based on observation that its overexpression enhances the growth of cancer cell lines, and the introduction of antisense construct suppresses their growth (Skotzko, Wu, Anderson, Gordon and Hall 1995; Smith, Kontny, Bortnick and Fornace 1997; Gordon, Liu, Chen, Liu, Whitley, Gee, Groshen, Hinton, Beart and Hall 2000; Kampmeier, Behrens, Wang, Yee, Anderson, Hall, Gordon and McDonnell 2000). Conversely, it has been suggested that cyclin G1 may have growth inhibitory effects. This is because cyclin G1 expression is high in differentiated tissues (Horne, Goolsby, Donaldson, Tran, Neubauer and Wahl 1996).

Alterations in the p53 gene are the most common genetic defects found in tumours (Hainaut, Soussi, Shomer, Hollstein, Greenblatt, Hovig, Harris and Montesano 1997). Taking into account that p53 is a powerful inducer of cell death, it is not surprising that the abolition of its function occurs almost universally during tumour development. There are several features of p53 inactivation in tumours that are unique (Selivanova 2001). Firstly, mutations occur at high frequency in the p53 gene, that is around 50% of human tumours carry p53 mutations. Secondly, mutations are largely of the same type: 87% of them are point missense mutations resulting in a substitution of one amino acid residue. Thirdly, the majority of mutations occur in the DNA binding domain of p53. Finally, mutant p53 proteins accumulate at high levels in tumour cells.

In this part of the thesis, the levels of cyclin G1 and mutant p53 in classical and AIDS-related KS were examined by IPX.

8.2 Methods

For the method of immunohistochemistry, please refer to Chapter 2 and Appendix 1. Antibodies against human cyclin G1 (Novocastra) at 1:50 dilution and mutant p53 (Novocastra) at 1:100 dilution were used. The primary antibody was substituted with saline in sections used as negative controls. The sections were counterstained with Harris's haematoxylin. Cells were classified as cyclin G1 positive when there was >10% nuclear staining. For p53, lesions were considered

positively stained when >10% of cell nuclei were stained (Takahashi, Munakata, Ohtsuka, Nisihara, Nasuhara, Kamachi-Satoh, Dosaka-Akita, Homma and Kawakami 2002). Histologically normal tissues located away from the primary lesions were used as normal non-neoplastic controls.

8.3 Results

Cyclin G1 was detected in 81% (30/37) of all KS cases (Table 8.1, Figures 8.1, 8.2). The normal skin epithelium showed positive staining in the basal layer of all the skin cases. Classical KS had a statistically significantly higher rate of cyclin G1 expression than in AIDS-related KS (90% vs 76.9%, $p=0.013$). There was a significant correlation between histological stage and the rate of cyclin G1 expression. The more advanced nodular and plaque stages showed cyclin G1 positivity in 90% and 85.7% of the cases respectively. In contrast, only 55.6% of the patch stage had cyclin G1 positive staining ($p<0.05$). Lesions from skin were more likely to have cyclin G1 expression compared with lesions from extra-cutaneous sites (83.3% vs 64.7%, $p<0.05$). There was no significant difference in cyclin G1 expression between males and females (80% vs 85.7%, $p=0.24$). The rate of cyclin G1 also did not differ between HHV8 positive and HHV8 negative lesions (80% in the two groups).

The effect of cyclin G1 expression on clinicopathological variables was examined by multivariate analysis with the stepwise linear regression model. Among all the

variables, classical lesions and the more advanced histological stage (plaque and nodular) were significant independent predictors of cyclin G1 expression. No statistically significant relevance was found for gender and site of lesions.

Positive staining for mutant p53 was detected in 45.5% of cases (Figures 8.3, 8.4 Table 8.2). The pattern of mutant p53 positivity was similar to the pattern of cyclin G1. Classical lesions were more likely to be mutant p53 positive than AIDS-related lesions (75% vs 36%, $p=0.056$). Histologically more advanced lesions had a significantly higher rate of mutant p53 expression. Only 10% of the patch stage lesions were positive of mutant p53 compared to 70.5% of the nodular stage lesions ($p<0.005$). There was no significant difference in the rate of mutant p53 expression between HHV8 positive and HHV8 negative lesions (50% vs 42.8%), males and females (44.8% vs 50%), and cutaneous and non-cutaneous lesions (48% vs 37.5%). On multivariate analysis, the classical lesion and nodular subtype were independent predictors for mutant p53 expression.

Figure 8.1 Lack of cyclin G1 staining in a patch stage KS lesion (x200)

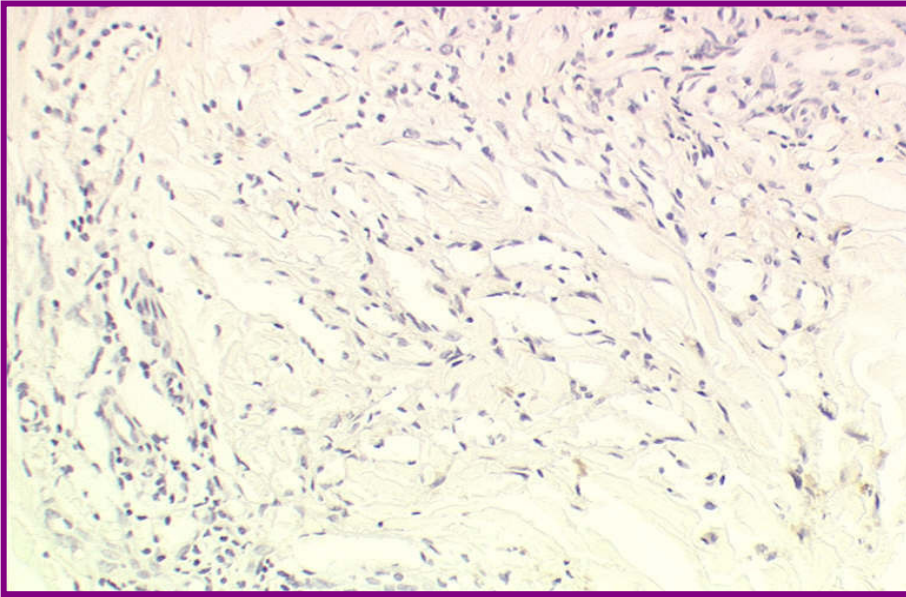


Figure 8.2 Positive staining of cyclin G1 in a nodular lesion (x400)

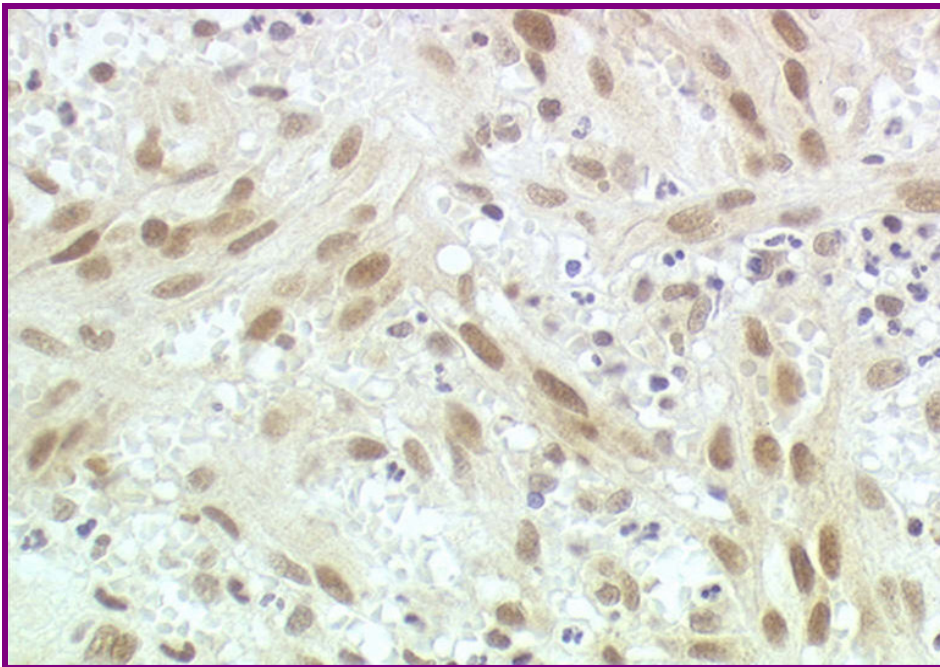


Figure 8.3 Lack of mutant p53 staining in a patch stage KS lesion (x200)

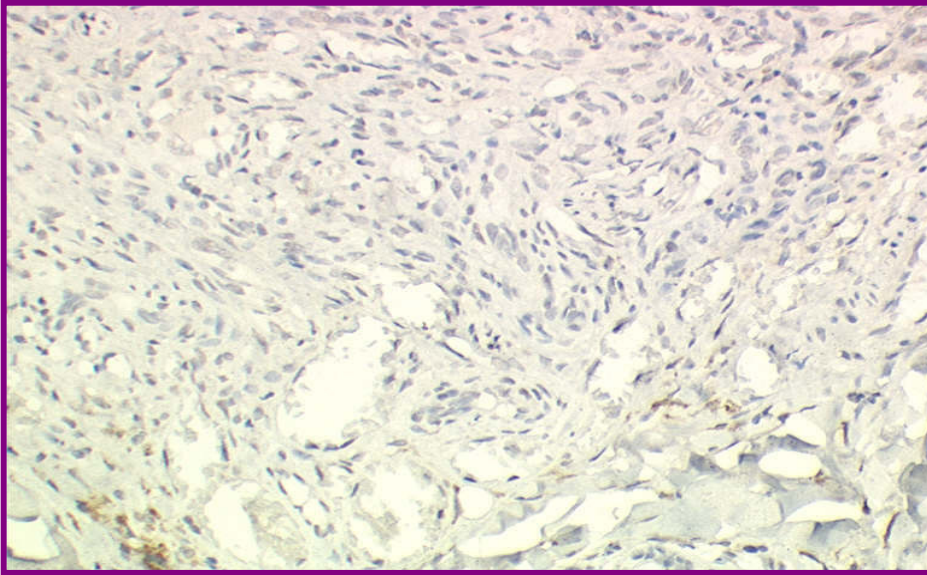


Figure 8.4 Positive staining of mutant p53 in a nodular lesion (x400)

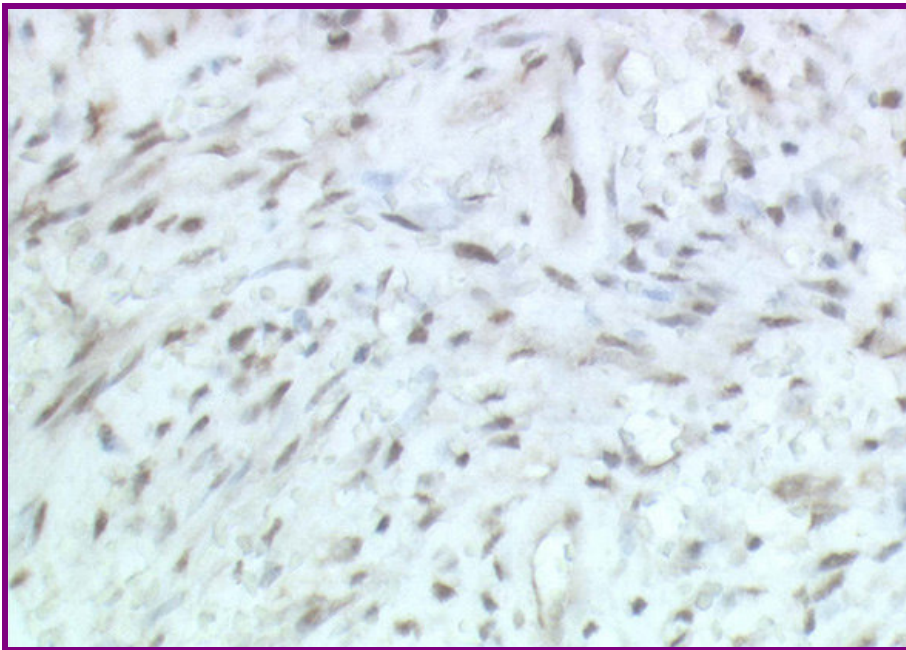


Table 8.1: Cyclin G1 expression according to clinical parameters

	G1 positive	G1 negative	p value
Overall	24 (75%)	8 (25%)	
HIV status			
Positive	17 (77.2%)	5 (22.8%)	
Negative	9 (90%)	1 (10%)	0.013
HHV8 status			
Positive	20 (80%)	5 (20%)	
Negative	5 (71.4%)	2 (28.6%)	0.14
Histology			
Patch	5 (55.6%)	4(44.4%)	
Plaque	6 (85.7%)	1 (14.3%)	
Nodular	18 (90%)	2 (10%)	<0.05
Site			
Cutaneous	5 (16.7%)	25 (83.3%)	
Non-cutaneous	2 (33.3%)	4 (66.7%)	<0.05
Gender			
Male	6 (20.7%)	23 (79.3%)	
Female	1 (14.3%)	6 (85.7%)	0.24

Table 8.2: Mutant p53 expression according to clinical parameters

	Mutant p53 positive	Mutant p53 negative	p value
Overall	45.5%	55.5%	
HIV status			
Positive	9 (36%)	16 (64%)	
Negative	6 (75%)	2 (25%)	0.056
HHV8 status			
Positive	12 (50%)	12 (50%)	
Negative	3 (42.8%)	4 (57.2%)	0.85
Histology			
Patch	1 (10%)	9 (90%)	
Plaque	2 (33.3%)	4 (66.7%)	
Nodular	12 (70.5%)	5 (29.5%)	<0.005
Site			
Cutaneous	12 (48%)	13 (52%)	
Non-cutaneous	3 (37.5%)	5 (62.5%)	0.78
Gender			
Male	13 (44.8%)	16 (55.2%)	
Female	2 (50%)	2 (50%)	ns

8.4 Discussion

Cyclin G1 was originally identified as a novel member of the cyclin family with homology to c-src (Tamura, Kanaoka, Jinno, Nagata, Ogiso, Shimizu, Hayakawa, Nojima and Okayama 1993). It is part of the G cyclin family, which also includes cyclin G2 and cyclin I. Cyclin G2 was isolated as a homologue of cyclin G1 (Bates, Rowan et al. 1996; Horne, Goolsby et al. 1996) and cyclin I has a high sequence homology to cyclin G1 and cyclin G2 (Nakamura, Akazawa et al. 1995; Bates, Rowan et al. 1996). Cyclin G1, a proto-oncogene, is one of the target genes of transcription factor p53. The p53 tumour suppressor gene is involved in diverse cellular processes, including regulation of the cell cycle, apoptosis, senescence, DNA repair, cell differentiation and angiogenesis. These activities are mediated through a variety of biochemical functions, such as transcriptional activation, DNA annealing, and exonuclease activity, which require a large set of target gene products and interacting proteins (Levine 1997; Amundson, Myers and Fornace 1998; Bates and Vousden 1999). The increase in p53 protein levels, which occurs in response to stress, can result in the transcription of target genes that mediate the various functions associated with the p53 gene.

This study showed a strong correlation between cyclin G1 and mutant p53 expression with classical lesions and the more advanced histological subtypes of KS. The rate of cyclin G1 expression in the different clinical subtypes of KS has not been reported previously. The results indicated that cyclin G1 and mutation in

p53 play a role in the progression from the patch to the more advanced histological stage. Cyclin G1 is normally expressed in terminally differentiated tissues such as the brain, cardiac muscle and skeletal muscle (Tamura, Kanaoka et al. 1993; Horne, Donaldson, Goolsby, Tran, Mulheisen, Hell and Wahl 1997). The overall expression profile of cyclin G1 is not associated with cell proliferation but with maintaining the quiescent state of differentiated cells. All the skin cases in the study showed positive staining at the normal skin epithelium and this is consistent with the normal expression in differentiated tissue. Increase in cyclin G1 expression has been demonstrated with progressive tumour growth in other malignancies. In colon cancer cells, overexpression of cyclin G1 was associated with accelerated cell growth (Smith, Kontny et al. 1997). In an animal model of osteosarcoma, cyclin G1 antisense inhibited tumour growth. In-situ analysis of the cell cycle kinetics showed an accumulation of tumour cells in the G1 phase and a reduced proportion of cells in the S and G2/M phases (Chen, Zhu, Hung, Skotzko, Hinton, Tolo, Hall, Anderson and Gordon 1997).

The function of cyclin G1 is to serve as a negative regulator of p53 by activating Mdm2 through dephosphorylation. Cyclin G1 null cells had markedly higher levels of p53 protein when compared with wild type cells (Okamoto, Li, Jensen, Zhang, Taya, Thorgeirsson and Prives 2002). It has also been shown that cyclin G1 null mice had fewer and smaller carcinogenic tumours (Okamoto, Li et al. 2002). The higher level of expression of cyclin G1 in the more advanced stage of KS might be

due to down regulation of p53 by cyclin G1 and hence the development of the more advanced disease.

The findings of higher level of mutant p53 in the histologically more advanced stage of KS is consistent with previous studies (Noel, De Thier, Simonart, Andre, Hermans, Van Vooren and Heenen 1997; Pillay, Chetty and Reddy 1999). Using IPX staining, only nodular stage lesions showed positive staining with p53 (54.16%) and there was no positive staining in the patch and plaque stages in 73 cases (Pillay, Chetty et al. 1999). However, the HIV status of these cases was not reported. It is known that mutants of p53 can inhibit apoptosis and render the tumour cells immortal (Zambetti and Levine 1993). The loss of p53-dependent apoptosis is believed to be critical in carcinogenesis (Dei Tos, Doglioni, Laurino, Barbareschi and Fletcher 1993). The findings of higher frequency of mutant p53 expression in nodular lesions in this thesis indicate that mutation in p53 might be responsible for the progression from the early patch stage to the more advanced nodular stage. The usefulness of p53 as a prognostic indicator in KS has not been examined. This is because KS is a relatively uncommon disease and the patients often have multiple complications from HIV infection.

Despite its important role as a tumour suppressor gene, p53 remains a controversial prognostic indicator. In head and neck squamous cell carcinoma, 16 studies using immunohistochemistry for p53 protein have found that p53 protein expression is a useful prognostic value of survival (Sauter, Ridge, Gordon and

Eisenberg 1992; Field, Pavelic, Spandidos, Stambrook, Jones and Gluckman 1993; Bradford, Zhu, Wolf, Poore, Fisher, Beals, McClatchey and Carey 1995; Sauter, Ridge, Litwin and Langer 1995; Shin, Lee, Lippman, Lee, Tu, Choi, Heyne, Shin, Ro, Goepfert, Hong and Hittelman 1996; Caminero, Nunez, Suarez, Ablanado, Riera and Dominguez 1996; Gasparini, Bevilacqua, Bonoldi, Testolin, Galassi, Verderio, Boracchi, Guglielmi and Pezzella 1995; Hirvikoski, Kumpulainen, Virtaniemi, Johansson, Haapasalo, Marin, Halonen, Helin, Raitiola, Pukander, Kellokumpu-Lehtinen and Kosma 1997; Michalides, van Veelen, Kristel, Hart, Loftus, Hilgers and Balm 1997; Jin, Kayser, Kemp, Ordonez, Tucker, Clayman, Goepfert, Luna, Batsakis and El-Naggar 1998; Rowley, Roland, Helliwell, Caslin, Kinsella and Jones 1998; Kaur, Srivastava and Ralhan 1998; Narayana, Vaughan, Gunaratne, Kathuria, Walter and Reddy 1998; Raybaud-Diogene, Fortin, Morency, Roy, Monteil and Tetu 1997; Chiang, Huang, Wang, Liu, Kuo, Hahn and Kuo 1999; Ito, Fujieda, Tsuzuki, Sunaga, Fan, Sugimoto, Fukuda and Saito 1999). However, 11 studies have found no correlation between p53 protein expression and survival (Dolcetti, Doglioni, Maestro, Gasparotto, Barzan, Pastore, Romanelli and Boiocchi 1992; Dursun, Sak, Akyol, Dursun, Ereku, Demireller and Akiner 1995; Frank, Bur, Garb, Kay, Ware, Sismanis and Neifeld 1994; Wilson, Richman, Dische, Saunders, Robinson, Daley and Ross 1995; Kokoska, Piccirillo, el-Mofty, Emami, Haughey and Schoinick 1996; Mineta, Borg, Dictor, Wahlberg, Akervall and Wennerberg 1998; Pai, Rochon, Clark, Black and Shenouda 1998; Pruneri, Pignataro, Carboni, Ronchetti, Cesana, Ottaviani, Neri and Buffa 1998; Saunders, MacKenzie, Shipman, Fransen, Gilbert and Jordan 1999; Taylor, Koch, Zahurak,

Shah, Sidransky and Westra 1999; Grabenbauer, Muhlfriedel, Rodel, Niedobitek, Hornung, Rodel, Martus, Iro, Kirchner, Steininger, Sauer, Weidenbecher and Distel 2000). In non-small cell lung cancer, the correlation between p53 protein expression and survival is also not clear. Immunohistochemistry staining of p53 was performed on tumours from 29 cases of primary lung cancer with brain metastasis and on 29 control cases of primary lung cancer without brain metastasis (Bubb, Komaki, Hachiya, Milas, Ro, Langford, Sawaya, Putnam, Allen, Cox, McDonnell, Brock, Hong, Roth and Milas 2002). Each control case was matched by age, gender and histology to a patient with brain metastasis. It was observed that low expression of p53 in the primary tumours was associated with poorer overall survival ($p=0.04$) and these patients had a higher rate of non-brain metastasis ($p=0.02$). The patients with brain metastases were particularly prone to developing non-brain distant metastases if the percentage of p53 positive cells in brain metastasis was low ($p = 0.01$). There was a positive correlation in the expression p53 ($p < 0.001$) between lung primaries and brain metastases. On the other hand, IPX staining of p53 on 85 cases of stage 1 non-small cell lung cancer showed that patients whose tumours were p53 positive had the worse outcome, with a survival rate of only 20% (Han, Landreneau, Santucci, Tung, Macherey, Shackney, Sturgis, Raab and Silverman 2002). Patients with p53 negative tumours had a survival of 50%. Multivariate analysis showed that p53 overexpression was an independent predictor of poor survival.

In conclusion, high rates of cyclin G1 and mutant p53 expression can be detected in KS. In particular, increased expression of cyclin G1 and mutant p53 may be important in the progression from early patch stage to the late nodular stage, and in the development of KS in HIV negative patients. The results suggest that the mutation along the p53 apoptotic pathway is important in the pathogenesis of classical KS, and other factors unique to HIV infection might be responsible for the rapid progression of AIDS-related KS.

Chapter 9: p57 and p27 expression in KS

Chapter 9: p57 and p27 expression in Kaposi's sarcoma

9.1 Introduction

p27KIP1 is a cyclin dependent kinase inhibitor that plays an important role in the control of cellular progression from G1 to S phase of the cell cycle. The gene is localised to chromosome 12p13 (Ponce-Castaneda, Lee, Latres, Polyak, Lacombe, Montgomery, Mathew, Krauter, Sheinfeld, Massague and et al. 1995) and the p27KIP1 protein is found to negatively regulate G1 to S phase progression of the cell cycle by inhibiting G1 cyclin-cyclin dependent kinase protein complexes (Slingerland and Pagano 2000).

p27 is expressed in quiescent, non-proliferating cells and the cellular level decreases upon entry into the cell cycle (Polyak, Kato, Solomon, Sherr, Massague, Roberts and Koff 1994; Loda, Cukor, Tam, Lavin, Fiorentino, Draetta, Jessup and Pagano 1997). Reduced or loss of p27KIP1 expression occurs in tumour development or progression and correlates with poor prognosis in a number of human tumours (Mori, Murakami-Mori, Jewett, Nakamura and Bonavida 1996; Cordon-Cardo, Koff, Drobnjak, Capodieci, Osman, Millard, Gaudin, Fazzari, Zhang, Massague and Scher 1998; Newcomb, Sosnow, Demopoulos, Zeleniuch-Jacquotte, Sorich and Speyer 1999; Palmqvist, Stenling, Oberg and Landberg 1999). However, mutation of the p27 gene is rare or absent in human tumours (Kawamata, Nakamura, Miki, Sato, Isobe, Furusawa, Hirose and Oshimi 1998),

which means that immunohistochemical detection of the p27 protein if performed reliably, can be used to accurately assess expression of the gene.

The p57KIP2 protein is also a cyclin-dependent kinase inhibitor and has an inhibitory effect on the cell cycle and tumour suppressor function. Reduced p57 level is found to result in loss of control of the cell cycle and increased cell proliferation (Zhang, Wong, DePinho, Harper and Elledge 1998).

This chapter of the thesis presents the findings of p27KIP1 and p57 protein expressions in KS lesions and its relationship with HHV8 status and clinico-pathological features.

9.2 Methods

Immunohistochemistry was performed as previously described in Chapter 2 and Appendix 1. Antibodies against human p27 at 1:50 dilution and p57 at 1:50 dilution were used. The primary antibody was substituted with PBS in sections used as negative controls. The sections were counterstained with Harris Haematoxylin. Assessment of immunohistochemical staining for p27 was made according to that described by Zagzag et al. (Zagzag, Blanco, Friedlander, Miller and Newcomb 2003). Cells were classified as p27 positive when there was distinct nuclear staining of $\geq 5\%$ cells. Lesions with value of $< 5\%$ nuclei positivity were considered as negative. Cases with $\geq 5\%$ and $< 50\%$ cells positive were considered as having

low level of p27 expression. Lesions with $\geq 50\%$ cells positive were classified as having high level of p27 expression.

For p57 expression, a lesion is considered as positive when there is any lesional cell showing distinct nuclear staining for the antibody (Castrillon, Sun, Weremowicz, Fisher, Crum and Genest 2001). Histologically normal tissues located away from the primary lesions were used as normal non-neoplastic controls.

9.3 Results

A high level of p27KIP1 expression was detected in only 17% (7/41) of all KS cases (Table 9.1). The majority of cases showed low (37%; 15/41 cases) or negative (46%; 19/41 cases) p27KIP1 expression. A significant correlation was found between histological stage and level of p27KIP1 expression. The early patch stage KS lesions (33%; 4/12 cases) were more likely to show a high level of p27KIP1 expression than the more advanced plaque and nodular stage (10%; 3/29 cases) ($p < 0.05$). However, there was no correlation found between p27KIP1 expression and the following clinico-pathological factors: HHV8 status, HIV status, age, gender, multiplicity, recurrence and site of lesions (Table 9.2).

Overall p57 was detected in 63% (15/24) of the cases. Positive staining occurred in the nucleus and any cytoplasmic staining was disregarded as of any significance. No significant correlation was found between histological stage and level of p57KIP2 expression ($p = 0.58$). About 56% (5/9) cases of early patch stage KS lesions, 80% (4/5) cases of plaque stage and 60% (6/10) cases nodular stage KS lesions were positive for p57. There was no significant correlation between p57 expression and HHV8 status, HIV status, age, gender, multiplicity, recurrence and site of lesions.

Table 9.1 p27 expression in different stages of Kaposi's sarcoma

Histological subtypes	No.	P27 expression			Chi-square	p value
		High	Low	Negative		
Patch	12	4 (33.3%)	2 (16.7%)	6 (50%)	3.9	0.047
Plaque	9	1 (11.2%)	4 (44.4%)	4 (44.4%)		
Nodular	20	2 (10%)	4 (45%)	4 (45%)		

Table 9.2 p27 expression and correlation with clinico-pathological features

Parameter	Total no.	High	P27 level Low	Negative	Chi- square	p value
HIV status						
Positive	31	4 (12.9%)	13 (41.9%)	14 (45.2%)		
Negative	11	3 (27.3%)	2 (18.2%)	6 (54.5%)	1.2	0.27
HHV8 status						
Positive	29	5 (17.2%)	10 (34.5%)	14 (48.3%)		
Negative	9	1 (11.1%)	6 (66.7%)	2 (22.2%)	0.19	0.66
Age						
<60	35	5 (14.3%)	12 (34.3%)	18 (51.4%)		
≥60	6	1 (16.7%)	4 (66.7%)	1 (16.7%)	0.023	0.92
Gender						
Male	33	5 (15.2%)	14 (42.2%)	14 (42.2%)		
Female	6	1 (16.7%)	2 (33.3%)	2 (33.3%)	0.009	0.92
Multiplicity of lesions						
Single	23	5 (21.8%)	9 (39.1%)	9 (39.1%)		
Multiple	16	1 (6.3%)	7 (43.7%)	8 (50%)	1.74	0.19
Recurrence						
Absent	30	6 (20%)	12 (40%)	12 (40%)		
Present	9	0 (0%)	4 (44.4%)	5 (55.6%)	2.1	0.14
Site						
Cutaneous	31	5 (16.7%)	13 (41.9%)	13 (41.9%)		
Non-cutaneous	10	1 (10%)	3 (30%)	6 (60%)	0.23	0.63

9.3 Discussion

The results in part of the thesis show that reduced expression of p27KIP1 is more likely to be found in the advanced stage KS lesions, suggesting that reduced p27KIP1 expression is associated with progression of KS. The results confirm those of another study of 49 cases of KS (Fernandez-Figueras, Puig et al. 2000), but the latter not only showed reduced p27KIP1 expression in occurred in the higher stage KS lesions but also with extracutaneous involvement by the tumour. However, the current study did not show correlation between reduced p27KIP1 expression and other features of tumour aggression such as multiplicity, recurrence or extracutaneous involvement of the KS lesions. Despite these discrepancies, the evidence in the current literature and the results of the current study support the tumour suppressing role of p27KIP1 and its effect on progression of KS.

Reduced expression of p27 has previously been correlated with poorer clinical outcomes in some malignancies, including colorectal, breast and hepatocellular carcinomas. In colorectal carcinoma, low level of p27 was associated with poorer survival (Palmqvist, Stenling et al. 1999). In that study, IPX staining of p27 was performed in 89 colorectal cancer specimens. Tumours with <50% p27 staining was a strong predictor of poorer survival both in univariate and multivariate analyses. In younger patients with breast cancer, low level of p27 expression in the primary tumour correlated with poorer survival (Porter, Malone, Heagerty,

Alexander, Gatti, Firpo, Daling and Roberts 1997). It was also shown that the level of p27 was controlled by post-transcriptional mechanism rather than mutation of the gene. In hepatocellular carcinoma, the labeling index of p27 protein was significantly lower in tumours with a higher T stage and in tumours with multiple nodules (Tannapfel, Grund, Katalinic, Uhlmann, Kockerling, Haugwitz, Wasner, Hauss, Engeland and Wittekind 2000).

The failure in the current study to demonstrate a strong correlation between p27KIP1 expression and features of tumour aggression may be related to the small number of cases studied, particularly in the high p27KIP1 expression group. With only 7 cases in the latter group, it would be difficult to extrapolate any statistically meaningful results. The tissue blocks of the cases used in this thesis were cutting out, particularly when the studies of p27KIP1 and p57 were made towards the end of the candidature and this accounted for the smaller number of cases available for examination.

In addition, the methodology used in the assessment of p27KIP1 expression in the current study is different to that used by another similar study on KS by Fernandez-Figueras et al. (Fernandez-Figueras, Puig et al. 2000). The latter assessed p27KIP1 expression by counting the number of positive cells divided by the total number of cells counted. At least 500 tumour cells, and/or 20 fields if there were less than 500 cells present, were assessed. Cases were classified as having high expression of p27KIP1 when there were 70% or more tumour cells positive for

p27KIP1, and low expression when less than 70% cells positive. However, there are two major deficiencies with that method of assessment. The first is on the selective nature of the areas of the tumour lesions assessed. There was no mention of which parts of the lesion were assessed. On the other hand, we assessed the lesions in their entirety to derive the score. Secondly, the method used by Fernandez-Figueras et al. assessed a minimum of 20 fields but no mention was made of the actual size of the fields or the brand of microscope used. Field diameters of different microscope brands are variable in size. In view of these potential problems, we therefore did not follow the method used by Fernandez-Figueras et al. for the misgiving of having difficulty to reproduce of any the result obtained. Instead, we opted for the method used by Zagzag et al. (Zagzag, Blanco et al. 2003), which is a method originally used by Newcomb et al. (Newcomb, Sosnow et al. 1999). This method is more easily reproducible since it takes into consideration of the expression pattern of the cross-section of the entire lesion and it has been successfully used and documented previously in the literature.

The mechanism in which p27KIP1 is involved in the development and progression of KS appears to be related to the modulation of the levels of this protein by the HHV8 in KS (Mann, Child, Swanton, Laman and Jones 1999). The v-cyclin produced by HHV8 forms complexes with CDK6 that can phosphorylate the p27KIP1 protein resulting in its destabilisation and triggering of its degradation via the ubiquitin-proteasome pathway (Pagano, Tam, Theodoras, Beer-Romero, Del

Sal, Chau, Yew, Draetta and Rolfe 1995). The subsequent reduction of p27KIP1 levels enables the HHV8 infected KS lesional cells to enter the S phase of the cell cycle.

p57 expression can be detected in 60% of the KS cases studied in this thesis and there was no statistically significant correlation with histological stage. The difference in the rate of progression of classical and AIDS-related KS does not seem to be related to the differential loss of p57 function. Moreover, the level of p57 expression does not appear to significantly relate to features of tumour aggression such as multiplicity, recurrence or extracutaneous involvement of the KS lesions.

The p57 gene, a tumour suppressor gene, has been localised to chromosome 11p15.5 (Matsuoka, Edwards, Bai, Parker, Zhang, Baldini, Harper and Elledge 1995). It is part of the CIP/KIP family, which also includes p21 and p27. p57 binds to the CDK-cyclin complexes and is able to inhibit multiple G1 kinases. Frequent allelic loss has been demonstrated in breast carcinoma, hepatocellular carcinoma and bile duct carcinoma (Winqvist, Mannermaa, Alavaikko, Blanco, Taskinen, Kiviniemi, Newsham and Cavenee 1993; Ito, Takeda, Sasaki, Sakon, Yamada, Ishiguro, Imaoka, Tsujimoto, Monden and Matsuura 2002; Nakai, Masaki, Shiratori, Ohgi, Morishita, Kurokohchi, Watanabe and Kuriyama 2002).

Germ line mutations in the p57 gene have been detected in some patients with Beckwith-Wiedemann syndrome, a familial cancer prone syndrome associated with hyperplastic growth in numerous tissues and a thousand-fold increase in the risk of childhood cancer (Hatada, Ohashi, Fukushima, Kaneko, Inoue, Komoto, Okada, Ohishi, Nabetani, Morisaki, Nakayama, Niikawa and Mukai 1996). Genetically engineered mice that lack the p57 gene have a variety of development defects consistent with the Beckwith-Wiedemann syndrome (Zhang, Liegeois, Wong, Finegold, Hou, Thompson, Silverman, Harper, DePinho and Elledge 1997). On the other hand, overexpression of transfected p57 has been shown to lead to G1 phase arrest in cell (Lee, Reynisdottir and Massague 1995; Matsuoka, Edwards et al. 1995). All these indicate lines a role for p57 in the control of cellular proliferation and differentiation in some conditions.

In conclusion, reduced expression of p27KIP1 is found more frequently in the advanced stage KS lesions than in early stage lesions. In view of the effect of the v-cyclin produced by the HHV8 on p27KIP1, reduced p27KIP1 expression appears to be significant in the progression of KS. On the other hand, p57 can be detected in 60% of the KS cases examined and reduction of its level does not appear to be significant in tumour progression based on histological stage. Hence, the difference in the rate of progression of classical and AIDS-related KS is not related to the differential loss of p57 function.

Chapter 10: Concluding Remarks

Chapter 10: Concluding remarks

10.1 Introduction

The epidemiology and natural history of KS have gone through major changes over the past two decades. The tumour was uncommon in the past until an aggressive form of the disease emerged during the AIDS epidemic in the 1980s. With the successful containment of HIV infection in many parts of the world, KS has become much less common in developed countries over the past decade. While the discovery of HHV8 as a direct cause of KS has provided information on the initiation of the disease, the exact mechanisms underlying the differences in the presentation and progression of the disease between HIV and non-HIV infected patients are not clearly understood. Moreover, there is a small proportion of patients with KS who apparently have no evidence of HHV8 infection and conversely, there are many in the population who have a positive HHV8 serology but with no evidence of KS. It seems that the KS story does not end with the discovery of HHV8 either. In addition, with the diversity of the rate of progression, KS serves as a unique model for the study of factors related to tumour progression.

The initial study cohort in this thesis was of 43 cases but some cases did not have enough material for IPX staining towards the end of the candidature. Therefore the number of cases available for IPX staining was reduced. The number of patients in the study cohort was relatively small compared with studies on other malignancies. This is because KS is a relatively uncommon entity and the diagnosis of KS is

often based on history and clinical appearance rather than histological examination. However, the number of patients included in the thesis is comparable with other publications on KS cell biology.

This thesis examined the role of cell cycle proteins in the progression of two main clinical subtypes of KS: classical and AIDS-related. Table 10.1 shows the summary of findings in this thesis. Classical clinical subtype and nodular histological subtype were independent predictors of cyclin D1, cyclin A, cyclin G1 and mutant p53 expression. The findings indicate that the progression of KS in HIV negative patients may be through defects in the Rb/cyclin D1/p16 growth control pathway and the p53-apoptotic pathway.

A total of 43 cases were studied for the expression of the different cell cycle proteins and the correlation between different cell cycle protein expressions was examined. Of these cases, 12 were positive for cyclin D1 expression. Correlation of cyclin D1 and of other cell cycle protein expressions was examined. Cyclin D1 positivity correlated significantly with the expression of cyclin A, cyclin G1 and p16 (Table 10.2). Significantly more cyclin D1 positive lesions were cyclin A positive (81.8% vs 18.2%, $p=0.04$) and they were all of the nodular subtype. Of the 12 cyclin D1 positive cases, 10 were also p16 positive (83.3%) and only one was p16 negative (16.7%, $p=0.04$). All the cases were of the nodular subtype. All the cyclin D1 positive cases were also cyclin G1 positive. Nine of the cyclin D1/G1 positive lesions were of the nodular subtype and only one was of the patch subtype. Ten of

the 12 cyclin D1 positive lesions were also positive for p16 and they were all of nodular subtype. However, there was no relationship between cyclin D1 expression and mutant p53 expression.

The levels of the different cell cycle protein expression were examined in the HHV8 positive and HHV8 negative cases (Table 10.3). Only the levels of Rb expression were significantly different between HHV8 positive and HHV8 negative cases (83.3% vs 42.8%, $p=0.03$). There was no significant difference in the levels of expression of cyclin D1, cyclin A, cyclin G1, mutant p53, and Ki-67 between the HHV8 positive and HHV8 negative lesions. The results suggest that HHV8 negative lesions progressed through a deficient Rb pathway, whereas the role of RB in the progression of HHV8 positive lesions may not be as important as in the HHV8 negative cases. This is not unexpected finding given that one of the postulated mechanisms of action of the HHV8 virus is via the v-cyclin it produces. The v-cyclin produced by HHV8 acts through the Rb pathway much the same as cyclin D1 and one would have expected that HHV8 positive cases are more likely to be positive for the Rb protein since Rb expression increases as a compensatory mechanism with increase cyclin D1 level to prevent further progression of the cell cycle.

In addition, the interaction between other viral proteins and regulation of the cell cycle transition has been reported. The E6 protein of the human papilloma virus

has been shown to have the ability to deregulate the G1/S phase transition (Malanchi, Caldeira et al. 2002). The E6 viral protein acted upstream of Rb and its mechanism in promoting cell cycle progression is dependent on Rb phosphorylation.

10.2 Conclusions and significance of the findings in this thesis

The issues raised by the hypotheses and aims of this thesis are addressed in the following section based on the findings and conclusions arrived at this thesis:

1. Documentation of the clinico-pathological characteristics and the incidence of HHV8 infection in a cohort of Australian patients that include both classical and AIDS-related KS cases.

There were 29/37 (78%) cases found to have HHV8 infection in a cohort of patients with KS studied in this thesis using IPX staining with a robust antibody. This figure is somewhat lower than the reported figure of about 95% in the literature (Chang, Cesarman et al. 1994). There are two plausible explanations for this discrepancy. Firstly, the antibody used may not be as sensitive as suggested by the manufacturer. However, all of the staining procedures carried out in this thesis had accompanying positive and negative controls. The control cases had consistently produced the unequivocal and expected positive and negative staining results. It is therefore reasonable to assume that the antibody and technique used in these studies are sufficiently robust to produce accurate results. If that is the case, then

the only other possible explanation for a negative result would be the loss of the HHV8 antigen during the fixation and processing of some of the archival tissues in which the virus was not detected. Unfortunately, there is no possible way to prove or disprove this hypothesis.

Alternatively, the lower incidence rate of HHV8 infection in the study cohort is a true reflection of the incidence in the study cohort. If this was the case, the immediate question that arises would be: how do the non-HHV8 and HHV8 positive cases relate, or not relate, to each other in the mechanisms of development and progression of KS.

Moreover, the findings have also given credence to one of the hypotheses postulated at the beginning of this thesis: that there might be a small subgroup of cases in which development of KS lesions is not related to HHV8 infection at all. As noted earlier, the existence of this subgroup of HHV8 negative KS cases bears weight when one takes into account that fact that the virus is not seen in parts of Africa where KS is endemic. Clearly, the contention for an alternative pathway of KS development apart from infection by HHV8 becomes more cogent. This is discussed further in the latter section of this chapter.

2. Investigation of the expression of cell cycle proteins of the Rb/cyclin D1/p16 pathway and mitotic cyclins in the progression of KS lesions.

The experiments in this thesis revealed that Rb protein overexpression occurred in the majority (76%) of KS lesions. The rate of Rb expression did not differ significantly among the histological subtypes (patch=76.5%, plaque=66.6% and nodular=80%). Similarly, about two thirds of the cases showed positive staining for p16. Of note was the progressive increase of p16 level with the more advanced histological stages. The difference between the patch stage and nodular stages was statistically significant (50% vs 77.8%, $p=0.04$).

On the other hand, cyclin D1 overexpression was detected in 29% (12/41) of all KS cases and there was a strong correlation between cyclin D1 overexpression and the pathological stage, with cyclin D1 overexpression more prevalent in classical and nodular stage lesions (0% in the patch stage, 13% in the plaque stage, 50% in the nodular stage; $p=0.0017$).

Of the mitotic cyclins investigated in this thesis, cyclin A and p34^{cdc2} expressions were demonstrated in the KS lesions. p34^{cdc2} expression was found in 44% of the KS cases with the highest rate in the patch stage (64%) followed by the nodular stage (47%) and then the plaque stage (18%). In contrast, cyclin A expression was found in the majority of the KS lesions (68%), with the 79% positivity in the

nodular stage (79%) whereas only 36.4% of the patch stage lesions and 45.5% of the plaque stage were positive.

The aforementioned findings highlighted and confirmed the significance of the role of the Rb/cyclin D1/p16 regulatory pathway in the progression of the different stages of KS. Since it was found that the majority of the KS lesions express Rb protein irrespective of the histological stage and Rb is a known tumour suppressor and cell cycle arrest checkpoint protein, this suggests that a defective or reduced expression of the protein may not be important in the development or progression of the tumour. Similarly, p16 acts as a tumour suppressor and it has an inhibitory effect on cyclin D1. Since p16 expression was low in early stage KS but high in the more advanced lesions, it seems that defective p16 occurs early in the development of KS. Although p34^{cdc2} expression was found in slightly less than half of the KS cases, it appears to be more important in the early stage of KS development since the patch stage lesions were found to be positive in the majority of cases. On the other hand, the more advanced stage nodular lesions were more likely to be positive for cyclin D1 and cyclin A, and this implies that these genes are important in the stage progression of KS.

Expression of the cyclin dependent kinase inhibitor, p27KIP1 appears to be more likely to be reduced in the advanced stage KS lesions than in the early patch stage lesions. This finding suggests that reduced p27KIP1 expression is associated with progression of KS and confirms its significant role in the regulation of the cell cycle

in KS. In addition, reduction in p27KIP1 expression may also be related to the effect of the v-cyclin produced by the HHV8, which is known to complex with CDK6 that can phosphorylate and facilitate proteasome-mediated degradation of p27KIP1. In contrast, p57 can be detected in as high as 60% of the KS cases and the reduction of its level does not appear to be significant in tumour progression, suggesting that loss of or reduction in p57KIP2 expression may not be important in the development and/or progression of KS.

3. Demonstration of the frequency of KS lesions that display expression of the proteins involved in the p53-apoptotic pathway

About 46% of KS lesions were found to express mutant p53. Mutant p53 expression was more common in more advanced stage nodular KS than in early patch stage lesions. The pattern of cyclin G1 expression parallels that of mutant p53. The majority of the KS lesions investigated in this thesis show cyclin G1 expression (30/37 cases; 81%), with a significant correlation between an increasing level of cyclin G1 expression and advanced histological stage.

The findings suggest that cyclin G1 and mutant p53, which are proteins that can directly or indirectly regulate the p53 pathway of the cell cycle, are important in the development of advanced stages of KS. These results are consistent with the known functions of both cyclin G1 and p53 in the regulation of the cell cycle and cell growth. Since cyclin G1 is important in promoting cell progression from the G2

to M phase of the cell cycle (Kimura, Ikawa et al. 2001), it is not surprising that the more advanced stage nodular KS, which are in a more proliferative state, have higher levels of cyclin G1. It is significant that mutant p53 proteins are expressed more in advanced stage nodular KS despite cyclin G1 acting as a negative regulator for p53. In fact, the latter further validate and support the findings in this thesis in relation to p53 expression in KS: it was found that virtually all of the KS cases investigated showed very low or absent wild-type p53 expression. The antibody stain that detects both wild-type and mutant p53 proteins shows almost the same level of expression in the KS lesions as that seen in the antibody stain that only detects mutant p53 protein, implying that the p53 proteins expressed by the KS lesions were mutant proteins. This suggests that KS progression can occur through a pathway that is an alternative to the Rb/cyclin D1/p21 cell cycling pathway. KS lesions that express the mutant p53 protein could have developed and progressed through the loss of the normal p53 tumour suppressing function or through the loss of normal p53 dependent apoptotic pathway.

4. Demonstration of the differential expression of cell cycling and related proteins in classical and AIDS-related cases

One of the hypotheses at the beginning of the thesis was that the difference in the progression rate of AIDS-related and classical KS might be explained by the divergence in the expression of cell cycle regulatory proteins or possibly an

alternative route of molecular progression which is related to the Rb/cyclin D1/p16 cell cycling pathway, that is the p53-apoptotic pathway.

Of the nine different representative proteins that regulate different pathways of the cell cycle investigated in this thesis, the proteins that were found to have a statistically significant association with classical KS cases were cyclin D1, cyclin A, cyclin G1 and mutant p53. No association was found with any of the proteins investigated in relation to HIV status, suggesting that HIV probably has no direct interaction with these cell cycling regulatory proteins in the initiation or progression of KS. On the other hand, the correlation of cyclin D1, cyclin A, cyclin G and mutant p53 with classical KS cases suggests that certain components of Rb/cyclin D1/p16 and the p53-apoptotic pathways are important in the progression of classical KS. Other unidentified factors associated with HIV infection might also be responsible for the rapid progression of AIDS-related KS.

5. Assessment of the differences in the expression of various cell cycling regulatory proteins in HHV8 positive and HHV8 negative cases

The rate of Rb protein expression was the only difference detected according to HHV8 status. The majority of HHV8 positive cases (83.3%) were Rb positive compared with 57.2% of the HHV8 negative cases. This suggests that the HHV8 negative lesions can progress through a deficient Rb pathway possibly via phosphorylation of the Rb protein by the viral cyclin produced by HHV8. However,

there were as many as half of the HHV8 negative cases also showed overexpression of cyclin D1 (Table 10.3), and this finding suggests that many of these tumours, which were not initiated by HHV8, can develop and progress along a similar cell cycling route that involves the Rb/cyclin D1/p16 pathway. In other words, the HHV8 negative cases can also progress through a route that is similar to the Rb/cyclin D1/p16 pathway imparted by HHV8.

In addition, up to 50% (4/8 cases) of the cases which were HHV8 negative were also cyclin D1 negative. Therefore, it is clear that some HHV8 negative KS lesions can progress through a different molecular route apart from the cyclin D1 pathway.

6. In vitro demonstration of a molecular route that bypasses the cyclin D1 pathway in HHV8 negative KS lesions

One of the early findings in the pathogenesis of KS, in relation to the molecular genetics of HHV8, was the significant homology of HHV8 to cyclin D1 (Cesarman, Chang et al. 1995). The open reading frame (ORF 72) of the virus has a sequence similar to cyclin D proteins and HHV8-cyclin latently infected cells can overcome Rb mediated cell cycle arrest (Chang and Moore 1996), which can contribute to KS development. Since HHV8 has been regarded as the main initiating factor in the development of KS (Moore and Chang 1995), it had been initially assumed that most KS lesions should demonstrate cyclin D1 overexpression, particularly in the nodular lesions. However, as has been clearly demonstrated in Chapter 6 of this thesis, only a small proportion of (12/41 cases; 29%) KS lesions was found to

demonstrate cyclin D1 overexpression. In spite of this, most of the latter (11/12 cases; 92%) were advanced stage nodular lesions and only half (11/22 cases; 50%) of the nodular KS showed cyclin D1 overexpression even though the HHV8 positivity rate for the nodular lesions was 74% (14/19 cases were available for testing).

In addition, cyclin D1 overexpression was detected in 78% of the classical nodular lesions and 31% of the AIDS-related nodular lesions ($p=0.03$), but up to 50% (4/8 cases) of the cases which were HHV8 negative were also cyclin D1 negative. Therefore, it is clear that some HHV8 negative KS lesions can progress through a different molecular route apart from the cyclin D1 pathway.

This curious finding of the thesis was further validated by in vitro experiments performed towards the end of the candidature. A HHV8 negative KS cell line that had the morphological features of an advanced stage nodular KS was shown to be negative for cyclin D1 protein expression. Further studies were not performed to elucidate the exact pathway the HHV8 negative KS cells progress through as this would be beyond the scope of this thesis. However, the obvious area for future study in KS in relation to the aforementioned findings will be by investigation of the p53-apoptosis pathway.

10.3 Summary of the findings in this thesis

This thesis has confirmed many of the findings in relation to the cellular biology of KS documented in the current literature. In addition, it has shed some light on the mechanism of progression in the two main clinical subtypes of KS that are not yet documented in the literature, particularly in relation to possible interacting factors with concurrent HIV/HHV8 infections and in a small subset of KS cases that have no evidence of HHV8 infection. The conclusions from this thesis that have an impact on the current understanding of KS cell biology are summarised in the following:

1. The incidence of KS has been dramatically reduced over the last decade. Distinct differences in the clinical and histopathological features of KS lesions were found in patients with classical and AIDS-related KS. These differences may be indicative of the possibility of different pathogenetic mechanisms, which occur in HIV positive and HIV negative patients in the progression of these lesions.
2. The majority of the cohort of Australian KS lesions studied was HHV8 positive.
3. The sequential progression of the KS lesion observed on the histology can be regarded as a reasonable model of tumour progression since it was demonstrated that the rate of Ki-67 positivity, which is generally regarded as a

reliable indicator of cellular proliferation, was higher with more advanced stage nodular lesions.

4. The Rb/cyclin D1/p16 cell cycling regulatory pathway appears to be important in the progression of the different stages of KS. Expression of the p34^{cdc2} protein and a deficiency in p16 expression seem important in the initial development of the, whereas cyclin D1 and cyclin A are more important in the progression of KS. The majority of HHV8 positive cases were Rb positive suggesting that these lesions were initiated by the virus and have progressed along the Rb/cyclin D1/p16 pathway. However, increased levels of v-cyclin produced by the virus result in increase in Rb expression in an attempt to prevent further progression of the KS cells through the cell cycle.
5. Expression of the proteins involved in the p53 pathway of the cell cycle such as cyclin G1 and mutant p53, were found to be important in the advanced stage development of KS. On the other hand, expression of the CDK inhibitor, p27KIP1, a protein that normally negatively regulates cyclin G1, was reduced in nodular KS. These findings suggest some KS may progress through a route that is alternative to that of the Rb/cyclin D1/p16 pathway of the cell cycle.
6. There was a differential expression of cell cycle proteins between classical and AIDS-related KS cases in that classical KS cases were more likely to display cyclin D1, cyclin A, cyclin G1 and mutant p53 expression. This suggests that

certain components of both Rb/p16/cyclin D1 and the p53 pathways appear to be important in this type of KS. HIV does not appear to have a direct interaction with these cell cycle proteins in the initiation or progression of KS.

7. The only difference in the cell cycle protein expression according to HHV8 status was a significantly higher rate of Rb protein expression in HHV8 positive lesions.
8. There are some KS lesions that do not have HHV8 infection and might progress through a molecular route that does not involve the cyclin D1 pathway.

These conclusions are encapsulated in the schematic representation of the initiation and progression of KS as outlined below (Figure 10.1).

Table 10.1 Summary of findings

Cell cycle protein	Known function	HIV status	Histological subtypes
Cyclin D1 (29%)	Regulate G1 phase progression	Higher in HIV-	Higher in nodular stage
p16 (67%)	Tumour suppressor gene	No difference	No difference
Rb (76%)	Tumour suppressor gene	No difference	No difference
Cyclin A (68%)	Marker of proliferation	Higher in HIV-	Higher in nodular stage
Cyclin G1 (81%)	Target of p53	Higher in HIV-	Higher in nodular stage
Mutant p53 (46%)	Tumour suppressor gene	Higher in HIV-	Higher in nodular stage
p57 (67%)	Tumour suppressor gene	No difference	No difference
p34^{cdc2} (44%)	Marker of proliferation	No difference	Higher in nodular stage
p27 (54%)	Marker of proliferation	No difference	Higher in patch stage

Table 10.2: Correlation of cell cycle regulators in the cyclin D1 positive cases

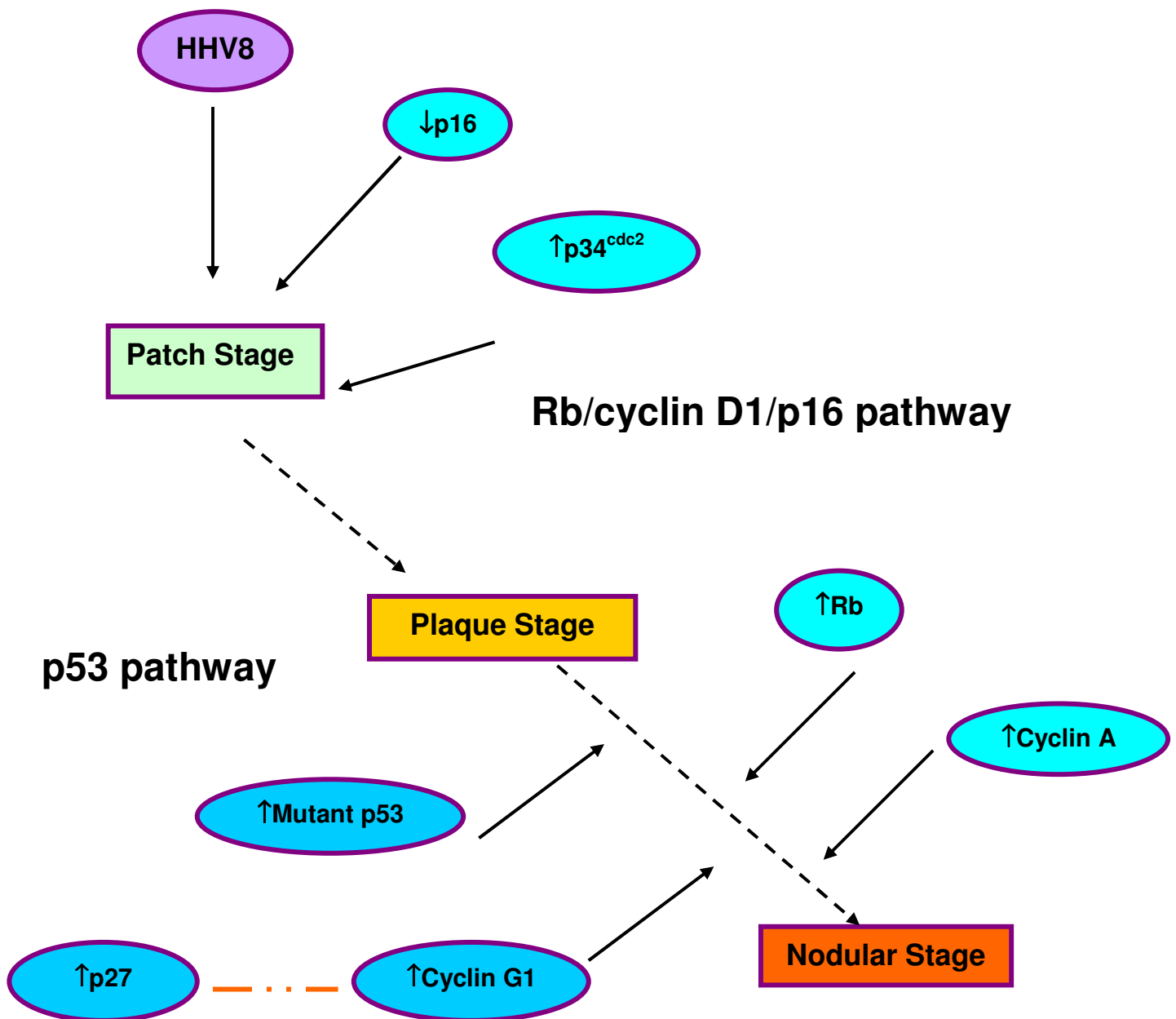
Cyclin A positive	9 (75%)		All nodular
Cyclin A negative	2 (25%)	p=0.04	2 cases missing
Cyclin G1 positive	10 (83.3%)		9 nodular, 1 patch
Cyclin G1 negative	0 (0%)	p=0.03	2 cases missing
p16 positive	10 (83.3%)		All nodular
p16 negative	1 (16.7%)	p=0.04	1 case missing
Rb positive	8 (66.7%)		
Rb negative	3 (25%)	P=0.056	1 case missing
Mutant p53 positive	7 (58.3%)		
Mutant p53 negative	4 (41.7%)	p=0.32	1 case missing

Table 10.3 Correlation between cell cycle protein expression and HHV8 status

	HHV8 positive	HHV8 negative	p value
Cyclin D1 positive	8 (33.3%)	4 (50%)	ns
Cyclin D1 negative	16 (66.7%)	4 (50%)	
Cyclin A positive	15 (65.2%)	5 (62.5%)	ns
Cyclin A negative	8 (34.8%)	3 (37.5%)	
Cyclin G1 positive	16 (80%)	4 (80%)	ns
Cyclin G1 negative	4 (20%)	1 (20%)	
Mutant p53 positive	12 (50%)	3 (42.8%)	ns
Mutant p53 negative	12 (50%)	4 (57.2%)	
Rb positive	20 (83.3%)	3 (42.8%)	p=0.03
Rb negative	4 (16.7%)	4 (57.2%)	
Ki-67 score 1	12 (57.1%)	3 (42.8%)	ns
Ki-67 score 2	5 (23.8%)	2 (28.6%)	
Ki-67 score 3	4 (19%)	2 (28.6%)	

Fig.10.1 Summary of the key events in the pathogenesis of Kaposi's sarcoma

—————► indicates stimulatory effect; -----► indicates stage to stage progression of KS; — . . indicates inhibitory effect



Appendix 1 Protocol for immunohistochemistry

PREPARATION OF REAGENTS

Silane coated slides for Immunoperoxidase

1. Place glass slides in racks.
2. Wash slides for 3 minutes in acetone.
3. Place in 2% APTS in acetone for 3 minutes
4. Rinse in distilled water for 3 minutes
5. Dry slides in 90°C oven
6. Once dried store in paper boxes.

Endogenous peroxidase block

Methanol 90mls

30% hydrogen peroxidase 10mls

Protein Block

Tris buffer 100mls

Skim milk powder 2g

TRIS BUFFER

TRIS (Sigma) 6g

Normal Saline

(for irrigation) 1000mls

Adjust to pH 7.6 with 10N HCL

DAKO Antibody Diluent

Used to prepare antibody working dilutions.

Contains Tris-HCL buffer containing carrier protein and 0.015M sodium azide

DAKO LSAB+ Kit

Contains:

1. Secondary or Link antibody consists of biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate buffered saline (PBS), containing carrier protein and 0.015M sodium azide.
2. Streptavidin Peroxidase (label) which consists of streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and anti-microbial agents.

DAKO Liquid DAB+

DAB Chromogen: 3,3'-diaminobenzidine in chromogen solution.

Buffered substrate: Imidazole-HCL buffer pH 7.5 containing hydrogen peroxide and an anti-microbial agent.s

1 drop of DAB chromogen to 1 ml of substrate solution.

Specimen is covered with DAB solution for 5 minutes and then washed in water.

METHOD

Dewax sections in xylene and take to 95% ethanol

Place in 3% hydrogen peroxide (endogenous peroxidase block) 10mins

Wash slides in water

Place in TRIS buffer	5mins
Place slides in non-specific block (1% Skim milk)	15mins
Rinse briefly in water	
Apply diluted antibody	60mins
1. Wash in 2 changes of TRIS buffer	5mins each
2. Apply biotinylated link	15mins
3. Wash in 2 changes of TRIS buffer	5mins each
4. Apply streptavidin label	15mins
5. Wash in 2 changes of TRIS buffer	5mins each
6. Apply DAB reagent	5mins
7. Wash slides in water	
8. Counterstain in Harris' Haematoxylin	60secs
9. Differentiate in 1% acid alcohol	5 dips
10. Blue in Scott's blueing solution	1min
11. Dehydrate, clear and mount.	

Appendix 2: Raw data of clinicopathological parameters

No	Biopsy date	Age	Sex	HIV status	RT dose	Extent of KS	Duration of response (months)	Anti-retroviral	Concurrent infection	Other treatment	CD4 count	Other AIDS illness
1	2/4/91	46	M	+		skin						
2	2/11/91	35	M	+		skin						
3	2/27/91	65	F	-		leg, foot				surgical excision	84, 88, 91, 93, 97	
4	4/3/91	37	M	+	8Gy/1#	Sole of foot	9					
5	6/19/91	25	M	+		toe	1					
6	11/18/91	56	M	?, high risk homosexual		chest, trunk						
7	12/20/91	32	M	+	24Gy/8#	Anal lesion	2					
8	5/29/92	72	M	-, mediterranean		upper limb, lower legs				liquid nitrogen		
9	3/16/93	26	M	+, homosexual		skin	3		PCP			HIV induced pancytopenia, seizure
10	3/22/93	43	M	+		skin						
11	4/8/93	29	M	+	12Gy/3#, 20Gy/10#	Penis, scrotum, wrist, skin						
12	4/16/93	33	M	+, liver transplant		skin, inguinal LN, pharynx, legs		3TC, cyclosporin, prednisone, bactrim	hepatitis B	vinblastine, bleomycin	600	
13	9/10/93	37	M	+	30Gy/10#, 10Gy/4#, 20Gy/10#	Lower legs (regional LN), forearm, thigh		acyclovir, zidovudine	cerebral toxoplasmosis		<10	
14	10/13/93	32	M	+	10Gy/4#, 20Gy/10#	upper arm, hard palate						
15	11/3/93	57	M	-, renal transplant, Mediterranean		legs						
16	1/13/94	62	F	-, Mediterranean		hand						
17	2/8/94	33	F	+		skin						
18	2/16/94	70	M	-	25Gy/10#, 25Gy/10#	foot, knee				bleomycin, vincristine		

No	Biopsy date	Age	Sex	HIV status	RT dose	Extent of KS	Duration of response (months)	Anti-retroviral	Concurrent infection	Other treatment	CD4 count	Other AIDS illness
19	2/17/94	30	M	+		skin	6					PCP
20	2/24/94	33	M	+	8 Gy/1#, 10 Gy/4#, 7.5Gy/3#	chest wall, face, arm, small intestine	6, cutaneous KS 92		MAC, candidiasis, CMA in CIT			
21	6/17/94	35	M	+		Oesophageal-gastric junction no bx	5	ddi, ayclovir, bactrim	pop	bleomycin, vincristine, partial response in skin		
22	6/28/94	28	M	+	15gy/5#, 12 Gy/4#, 9 gy/3#, 25Gy/10#	face, arm, palate, eyelids	5		PCP	bleomycin, vincristine	140	
23	8/24/94	43	M	+, homosexual		face, legs, arm						
24	10/17/94	71	M	-, mediterranean		heel				surgical excision		
25	11/24/94	36	M	+, homosexual		skin	9		PCP			
26	11/28/94	37	M	+, homosexual		posterior pharynx	8	azt		chemo	30	oral candidiasis, peripheral neuropathy, lymphadenopathy
27	12/12/94	41	M	+, IVDU		legs			PCP			
28	1/12/95	33	M	+		leg	5	azt, trimethoprim, acyclovir, pentamidine	CMV retinitis, pneumocystis pn, MAC, genital herpes		30	cov polyradiculopathy
29	3/2/95	33	M	+, heterosexual	10Gy/4#	oral, alveolar margin, lungs	9	acyclovir, fluconazole, bactrim, azt, ddc	herpes zoster, thrush, maic stool, cmv pn		46	dementia
30	4/13/95	35	M	+, homosexual	6Gy/6#	non resolving pneumonia	1			PCP, cryptococcal		
31	11/15/95	41	M	+, homosexual	15Gy/5#	face eyelid, nose, small bowel	10	acyclovir, ddi,		bleomycin, vincristine, PR	<50	CMV retinitis
32	2/12/96	32	M	+	18 Gy/10#	nasopharynx, cervical Ln (bx-ve)	3	acyclovir				
33	2/22/96	35	F	+		skin						
34	3/11/96	82	M	-		skin						
35	6/27/96	50	M	+		skin of eyelid						

No	Biopsy date	Age	Sex	HIV status	RT dose	Extent of KS	Duration of response (months)	Anti-retroviral	Concurrent infection	Other treatment	CD4 count	Other AIDS illness
36	3/10/96 3/10/97	54	M	+, homosexual		small intestine	5	lamivudine		intralesional alpha interferon, 6 million units, 3/wk	460	
37	6/12/98	71	F	-, Italian		skin of elbow						
38	9/9/98	48	M	+		skin						
39	9/10/98	77	M	-, mediterranean		skin						
40	70		F	-ve		skin						
41	8/29/90	41	M	+, homosexual		leg, groin lymph node						
42	11/8/90	29	M	+, homosexual		skin of abdomen						
43	9/15/97	50	M	+, Homosexual		groin lymph node			PCP, Mac			

Appendix 3: Raw data of immunohistochemistry results

No.	Type	Sex	Age	Site	HIV	No. of	Recurrent	HHV8	Ki67	cyclin D1	cyclin G1	p34cdc2	Cyclin A	p16 (nucleus)	p16 (cytoplasm)	mp53	Rb	p57 (cytoplasm)	p57 (nucleus)	p27	CycyclinB E
1	Nodular	M	46	skin	+	M	Yes	-	30%		1+, 60%	2+, 30%N	2+, 5%	2-3, >90%	3, 100%	1, 70%	3, <5%	1, 70%	-	1, 30%	-
2	Nodular	M	35	lung	+	S	N	-	20%	3	1+, <5%	-	1+, <5%	1, <5%	1, >90%	1, 30%	2, 10%	Background staining	Background	-	-
3	Nodular	F	65	skin	-	M	Yes	3, 80%	20%	6	3+, 90%		3+, 20%	2, 70%	2, >90%	2, 90%	3, 100%	2, 80%	-	-	-
4	Patch	M	37	skin	+	S	N	1, 10%	1%		1+, 5%	1+10%N;30%C	-	3, 70%	2, 70%	1, 1%	3, 70%	-	1, 5%	1, <5%	-
5	Nodular	M	25	skin	+	S	N	3, 60%	20%	2	2+, 90%		1+, 20%	2, 50%	1, 90%	1, 70%	3, 100%	1, 80%	-	1, 30%	-
6	Patch	M	56	skin	+	M	N	2, 60%	5%	2	1+, 5%	2+, 50%N; 50%C	2+, 5%	-	1, 60%	1, 5%	3, 90%	-	1, 20%	1, 10%	-
7	Nodular	M	32	skin	+	S	Yes	3, 25%		4	3, 80%	-	2+, 5%	3, 90%	1, 90%	1, 40%	1, 3%	1, 90%	-	-	-
8	Nodular	M	72	skin	-	M	Yes	3, 60%	30%	3	1+, 40%	2+, 30%N; 50%C	3+, 20%	1-2, 60%	2-3, >90%	40%	1, 3%	-	-	1, 10%	-
9	Patch	M	26	skin	+	S	N	3, 40%	-	0		-	-	-	1, 40%	1, 1%	3, 70%	-	-	-	-
10	Patch	M	43	skin	+	S	N	3, 40%	5%	1	1+, 70%	1+10%N;30%C	1+, 10%	1, 40%	3, 100%	1, 1%	3, 40%	1, 90%	-	-	-
11	Patch	M	29	skin	+	M	N	1, 10%	5%	1	1+, <5%	-	1+, 5%	-	-	1, 5%	3, 70%	-	-	1, <5%	-
12	Nodular	M	33	tonsil	+	M	Yes	1, 20%	10%	5		-	2+, 5%	2, >90%	1, 90%	1, 10%	3, 20%	Background staining	-	-	-
13	Nodular	M	37	skin	+	M	N	3, 80%	70%	2	2+, 80%	1+30%N; 70%C	2+, 5%	2, 70%	1-2, 90%	70%	1, 3%	-	-	1, <5%	-
14	Nodular	M	32	lung	+	M	N	3, 25%	20%	2	3+, 100%	2+, 30%N; 70%C	1+, 5%	1, 10%	1, 20%	1, 3%	3, 90%	-	-	1, 20%	-
15	Nodular	M	57	skin	-	M	N	3, 25%		2	2+, 50%	-	1+, 5%	1, <5%	1, <5%	40%	1, 5%	-	-	1, <5%	-
16	Plaque	F	62	skin	-	S	N	-	20%	4		-	1+, 5%	1, <5%	1, <5%	-	1, 5%	-	-	-	-
17	Patch	F	33	skin	+	S	N	-		3		1+, 10%N	-	1, 30%	-	1, 1%	3, 20%	-	2, 70%	2, 70%	-
18	Nodular	M	70	skin	-	S	Yes	3, 60%	10%	4	3+, 100%	1+<5%N;30%C	2+, 5%	1, 10%	1, 80%	20%	3, 90%	1, 60%	2, 10%	1, 20%	-
19	Patch	M	30	skin	+	S	N	-	1%	0		1+, 20%N	-	-	1, 60%	-	-	-	2, 30%	-	-
20	Plaque	M	33	duode num	+	M	N	1, <5%	10%	2	1+, <10%	-	-	1, 20%	1, 60%	1, 40%	3, 90%	-	2, 50%	2, 40%	-
21	Plaque	M	35	duode num	+	S	N	2, 20%	5%	0		-	-	1, <5%	1, 5%	1, 5%	3, 10%	-	1, <5%	1, <5%	-
22	Patch	M	28	skin	+	M	N	2, 20%	1%	0	3+, 70%	1+ 20%N; 20%C	1+, 5%	1, 20%	1, 50%	-	3, 50%	1, 70%	-	1, 50%	-

No.	Type	Sex	Age	Site	HIV	No. of	Recurrent	HHV8	Ki67	cyclin D1	cyclin G1	p34cdc2	Cyclin A	p16 (nucleus)	p16 (cytoplasm)	mp53	Rb	p57 (cytoplasm)	p57 (nucleus)	p27	CyccyclinB E
23	Patch	M	43	skin	+	M	N	3, 40%	10%	3	3+, 90%	1+5%N	-	1, <5%	2, 60%	1, 30%	3, 90%	-	-	2, 60%	-
24	Nodular	M	71	skin	-	S	N	1, 10%	30%	4	3+, 60%	-	Necrotic tissue only	NTL	NTL	-	-	NTL	NTL	1, 10%	NT
25	Nodular	M	36	skin	+	S	N	NTL	NTL	2	3+, 100%	-	NTL	NTL	NTL	NTL	NTL	NTL	NTL	NTL	-
26	Nodular	M	37	tongue	+	S	N	3, >80%	20%	2	3+, 100%	-	-	1, <5%	1, <5%	1, 5%	3, 80%	1, <5%	1, <5%	2, 70%	-
27	Plaque	M	41	skin	+	S	N	-	1%	3	3+, 70%	1+, 30%N	2+, 10%	NTL	NTL	-	3, 70%	Background staining	Background staining	-	-
28	Plaque	M	33	skin	+	S	N	2, 5%	10%	0	3+, 100%	1+5%N;30%C	3+, 5%	3, 80%	1, 80%	1, 40%	3, 90%	Background staining	Background staining	1, 20%	-
29	Plaque	M	33	bronchus	+	M	N	-	NTL	0	-	-	-	-	background staining	NTL	NTL	-	1, <5%	-	-
30	Nodular	M	35	oesophagus	+	S	N	-	5%	5	2+, 5%	1+, 5%N	2+, 5%	3, >90%	2, >90%	1, 5%	3, 30%	1, 70%	-	3, 30%	-
31	Plaque	M	41	small bowel	+	M	Yes	-	NTL	0	-	-	-	-	-	NTL	NTL	-	1, 10%	-	-
32	Nodular	M	32	nasopharynx	+	M	Yes	3, 60%	30%	4	3+, 100%	1+10%N;30%C	3+, 20%	2, 50%	2, 80%	1, 90%	3, 100%	-	-	-	-
33	Plaque	F	35	skin	+	S	N	1, 10%	5%	3	3+, 100%	1+, 5%N;20%C	1+, 1%	3, 90%	3, 90%	1, 5%	3, 30%	Background staining	Background staining	2, <5%	-
34	Plaque	M	82	skin	-	S	N	-	20%	4	2+, 50%	1+, 30%N	-	2, >90%	2, >90%	1, 90%	3, 100%	-	1, <5%	2, 70%	-
35	Nodular	M	50	skin	+	S	N	-	1%	2	2+, 70%	-	-	1, 10%	1, 10%	-	1, 5%	1, 90%	-	1, 30%	-
36	Patch	M	54	small bowel	+	S	N	3, 25%	1%	2	2+, 70%	-	-	1, 10%	1, 10%	-	1, 5%	-	1, 30%	1, 20%	-
37	Patch	M	48	skin	+	S	N	2, 40%	1%	3	2+, 60%	1+, 10%N	-	2, 30%	2, 80%	-	2, 20%	-	-	1, 70%	-
38	Nodular	F	71	skin	-	M	Yes	1, 5%	40%	5	3+, 90%	2+, 30%, 1+30%	3+, 25%	3, 100%	1, 100%	1, 30%	2, 40%	1, >90%	-	1, 10%	-
39	Plaque	M	77	skin	-	S	N	1, 5%	20%	0	3+, 90%	-	2+, 10%	3, 70%	1, >90%	1, 40%	3, 90%	2, 90%	-	2, 30%	-
40	Nodular	F	70	skin	-	S	N	3, 25%	25%	4	1+, 50%	-	1+, 5%	-	-	-	2, 5%	1, 60%	1, 10%	1, 30%	-
41	Nodular	M	41	skin	+	M	N	3, 25%	20%	2	1+, 50%	1+<5%N;10%C	1+, 5%	2, 50%	2, 80%	-	1, <5%	Background staining	Background staining	-	-
42	Plaque	M	29	skin	+	S	N	2, 20%	10%	3	2+, 90%	1+10%N;10%C	1+, 5%	3, 70%	1, 70%	-	3, 20%	-	1, 30%	1, 10%	-
43	Patch	M	50	skin	+	S	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NTL= no tissue left

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