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**Prevalence of antibodies against Kaposi's sarcoma associated herpes virus (KSHV)
complement inhibitory protein (KCP) in KSHV-related diseases and their correlation with
clinical parameters.**

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Short communication

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Abstract

Kaposi's sarcoma-associated herpes virus (KSHV) encodes its own inhibitor of the complement system, designated KSHV complement control protein (KCP). Previously, we detected anti-KCP antibodies in a small group of 22 patients suffering from Kaposi's sarcoma (KS) and KSHV-related lymphoproliferative diseases (Vaccine, 25:8102-9). Anti-KCP antibodies were more prevalent in individuals suffering from KSHV-related lymphomas than KS and also in those with high titer of antibodies against lytic KSHV antigens. Herein we analyze anti-KCP antibodies in 175 individuals originating from three different groups from northern Sweden or Italy, which included patients suffering from classical or HIV-associated KS, Multicentric Castleman's Disease, KSHV-associated solid lymphoma, pleural effusion lymphoma and healthy individuals with detectable KSHV immune response. Our current study confirmed previous observations concerning antibody prevalence but we also analyzed correlations between anti-KCP antibodies and classical KS evolution, clinical stage and viral load in body fluids. Furthermore, we show that patient's anti-KCP antibodies are able to decrease the ability of KCP to inhibit complement. This fact combined with results of statistical analysis suggests that KCP inactivation by specific antibodies may influence progression of classical KS.

Keywords: KSHV, complement, antibody, cancer

Abbreviated title: Anti-KCP antibodies in KSHV-related diseases.

1. Introduction

Kaposi's sarcoma-associated herpes virus (KSHV) complement control protein (KCP) acts as an endogenous complement inhibitor expressed from the viral genome. KCP is encoded by ORF4 within the KSHV genome and consists of four complement control protein (CCP) domains, which are typical for proteins controlling the complement system [1]. We have shown previously that KCP interferes with initial stages of complement cascade activation by enhancing dissociation of C3 convertases and acting as cofactor to serine proteinase factor I in degradation of activated complement factors C3b and C4b (reviewed in [2]). Being not only a surface antigen but also a viral protector against the host immune response, KCP emerges as a unique potential target for immunotherapy. Although the role of KCP in pathogenesis has not been examined yet due to the lack of an animal model of infection, one potentially important question is whether infected individuals produce specific antibodies directed against KCP protein and whether this fact has any further consequences for the disease progression. While analyzing sera from 22 patients suffering from classical or HIV-associated Kaposi's sarcoma (KS) and KSHV-related lymphoproliferative diseases such as primary effusion lymphoma (PEL) or solid KSHV-related lymphoma (SLY) we found anti-KCP antibodies in seven of the patients [3]. Due to limited number of patients and limited amounts of sera we could perform neither a reliable statistical analysis for individual diseases nor functional assays. Despite these limitations, we observed that anti-KCP antibodies appear more frequently in patients suffering from KSHV-related lymphomas than classical or HIV-associated KS and mostly in those with high titer of KSHV anti-lytic antibodies. The latter is in agreement with the fact that KCP belongs to the group of lytic proteins of KSHV [1]. We also analyzed immune responses against the VCA antigen of Epstein-Barr virus, which is expected to be present in 90% of adults [4] and we did not observe any difference

between VCA response in HIV positive and HIV negative patients. Therefore, the lower percentage of anti-KCP antibodies in HIV positive KS patients did not stem from a general inability of antibody production due to immunosuppression. Finally, we showed that anti-KCP antibodies increase complement deposition on the surface of cells expressing KCP. However, it was not possible to determine if the observed effect was due to functional neutralization of KCP or additional deposition of antibodies and ensuing complement activation on the cell surface.

Remaining questions, which can only be answered based on the study of larger numbers of patients are: i) whether previously observed higher prevalence of anti-KCP antibodies in KSHV-related lymphomas patients is reproducible, ii) are there any differences in prevalence of anti-KCP antibodies between particular KSHV-related diseases, iii) are anti-KCP antibodies able to protect individuals already exposed to KSHV from disease, iv) if the clinical stage or disease progression is reflected by presence of anti-KCP antibodies and v) if anti-KCP antibodies are capable of blocking the function of KCP. To investigate these issues, we analyzed three groups of patients suffering from KSHV-related diseases or healthy individuals who only showed detectable immune response against KSHV antigens. Blood samples of the two patients' groups were collected in Italy, a region where KS occurs with higher frequency compared to the rest of Europe [5]. Additionally, the second Italian group included patients suffering only from classical KS, who have been classified as slow or fast progressing [6]. The third group included a population-based sample of healthy individuals from northern Sweden, who were positive for anti-lytic or anti-latent KSHV antigens [7]. Since the incidence of classical KS in northern Sweden is estimated to be four-fold lower than in Italy [8], we wanted to check whether these individuals have elevated levels of anti-KCP antibodies compared to previously studied groups of

Italian patients. This could suggest possible role of anti-KCP response in protection from KS development.

2. Methods

2.1 Patients

The first group of patients suffering from KSHV-related diseases included 9 patients with Multicentric Castleman's Disease (MCD), among them 8 individuals were HIV-positive and 1 HIV-negative, 5 HIV-positive patients with PEL, 46 patients with HIV-associated KS (HIV+KS), 11 patients with classical KS (HIV-KS) and 5 patients with SLY among which 3 were HIV-positive. This group of patients was diagnosed and followed-up at the Department of Oncology & AIDS, Centro di Riferimento Oncologico in Aviano, Italy, during the period of 1997–2007. Diagnostic criteria for these patients are described in [9].

The second group included 35 patients suffering from classical KS, partially described in the study of *Mancuso et al.* [6]. These individuals were stratified into subgroups of patients with fast (23) or slow (12) progression of KS. Moreover, clinical stage and viral load in body fluids were assessed at the point of blood collection. In total, there were 12 patients with clinical stage I, 10 with clinical stage II, 9 with stage III and 4 with stage IV. Other available clinical data were anti-latent and anti-lytic KSHV antibody titer estimated by immunofluorescence assay. All the methods used to obtain clinical data are described in the original study [6].

The third group of patients was chosen from 520 individuals from Västerbotten county in northern Sweden, previously classified for the KSHV seroprevalence [7]. Out of a total of 520 serum samples available we chose 75, which were positive for anti-lytic or anti-latent KSHV antigens. This group is subsequently called healthy controls.

Since data obtained from groups I and III were used to compare a prevalence of anti-KCP antibodies, in particular KSHV-related diseases and controls, we examined if patients suffering from particular disease and healthy controls were matched concerning their age and gender (Table 1). While analyzing the demography of group II, which contains only individuals suffering from classical KS, we observed no correlation of the level of anti-KCP antibody with either age or gender. This group was used further to analyze potential links between anti-KCP antibodies and clinical parameters of classical KS. Also, no correlations between age and anti-KCP antibody level were observed in all patients grouped together as well as in each disease group separately or healthy controls.

Table 1 Demographic and clinical characterization of the patients with different KSHV-related diseases enrolled in the comparative study.

	MCD	PEL	SLY	KS HIV +	KS HIV-	healthy controls	
Age	46	39	43	42	66	49	
median	(36-79)	(27-47)	(34-75)	(25-68)	(30-84)	(29-60)	
(range)	9	5	5	45	11	75	
<i>n (number of individuals)</i>	KSHV-related lymphomas				#		
	43						
	(27-79)			KS			
	19			44			
				(25-84)			
				56			
	Diagnosed for KSHV-related diseases						
	43						
	(25-84)						
	75						
Gender							
M/F	8/1	4/1	4/1	45/0	9/2	39/36	

The age of KS HIV- patients differed significantly from the age of individuals in PEL, KS HIV+ or healthy control subgroup as well as KSHV-related lymphomas subgroup (P at least < 0.05, according to Kruskal-Wallis test with Dunn's multiple comparison test).

2.2 Detection of anti-KCP antibodies in patients' sera

Anti-KCP antibodies were detected as described in [3]. Briefly, CHO cells transfected with cDNA coding for KCP cloned into pKevin or empty vector (mock) were incubated with analyzed, heat-inactivated patient serum at the dilution 1:50, followed by detection of bound IgG with specific antibodies (Dako Cytomation) and FITC-conjugated secondary antibody (Dako Cytomation). Cells were analyzed by flow cytometry (Partec CyFlow Space) and the results were expressed as mean fluorescence intensity (MFI) ratios between KCP and mock-transfected cells. Flow cytometry-based method is superior to ELISA, since it analyzes KCP in its physiological conformation on the cell surface and excludes the problem of possible antigen masking/reorientation due to immobilization on the plate surface (discussed in [3]). The performance of the assay is characterized by the values of 8.89% for intra-assay coefficient of variation (CV) and 11.08% for inter-assay CV.

2.3 Purification of natural KCP antibodies from patient's serum

Total immunoglobulin fraction was purified from the serum of the patient who showed the highest level of anti-KCP antibodies in order to test if these antibodies could block function of KCP. Ten ml of this serum as well as normal human serum (control) were incubated with 0.3 % tributyl phosphate (Schariab) and 1 % Tween 20 (Sigma Aldrich) for 1 h at 37° C. Then the samples were loaded onto 1 ml protein G column (GE Healthcare), washed with 10 column volumes of PBS and eluted with 0.1 M glycine pH 2.5 into tubes containing 1 M Tris pH 8.0 for neutralization. Fractions containing human immunoglobulins were pooled, dialysed against PBS and finally concentrated to equal concentration of 30 mg/ml.

2.4 C3b/C4b degradation assay

Factor I degrades activated complement factors C3b and C4b only in the presence of appropriate cofactor such as KCP [10]. To assess the ability of patient's anti-KCP antibodies to neutralize the cofactor activity of KCP, we incubated purified KCP (0.05 µg/ml), FI (0.02 µg/ml for C3b and 0.06 µg/ml for C4b), trace amounts of ¹²⁵I-labelled C3b or C4b and purified immunoglobulin fraction from patient or control serum (3 mg/ml) for 1.5h at 37 °C. Afterwards, we used densitometry (phosphorimager FLA3000 with MultiGauge software (FujiFilm) to analyze the amount of residual α-chains of C3b or C4b molecules normalized to their uncleaved subunits (β or γ chains, respectively). The methods for purification of FI and KCP as well as methods of radiolabelling of C3b and C4b molecules were described in the original publication [1].

2.5 Ethical consideration

Samples from healthy controls and patients were obtained with the informed consent and the study was approved by the ethics committees and followed the Declaration of Helsinki.

3. Results and discussion

3.1 Demographic distribution of individuals from groups I and III.

Classical KS patients were significantly older than patients with PEL (P<0.05), HIV+KS (P<0.01) and healthy control subgroup (P<0.01), according to Kruskal-Wallis test. These differences remained even when MCD, PEL and SLY patients were pooled as a KSHV-related lymphoma subgroup but were not statistically significant when KS patients were considered as

uniform subgroup. Regarding gender, women were underrepresented to a varying degree in all patient groups but not in healthy controls. Therefore, we included additional analyses of anti-KCP antibodies presence restricted to men from groups I and III. More advanced age of classical KS patients in relation to other KSHV-related diseases as well as higher incidence of KSHV infection in men than woman are phenomena generally recognized for these diseases [11-13].

3.2 Anti KCP antibodies occur more frequently in patients with KSHV-related lymphomas than in patients with KS.

We analyzed the prevalence of anti-KCP antibodies in the first group, which included 75 patients with different KSHV-related diseases. Additionally, we included 75 healthy controls, positive for antibodies against KSHV antigens but who were not diagnosed with any KSHV-related diseases. Results had normal distribution in the MCD, PEL and SLY subgroup but were not distributed normally in KS and healthy control subgroups, therefore nonparametric statistical methods were used to compare all the subgroups. Medians (95% confidence intervals CI) of MFI ratio between KCP/mock-transfected cells were: 3.73 (1.19-7.95) for MCD, 1.49 (0.46-4.10) for PEL, 1.06 (1.03-1.34) for HIV+KS, 0.91 (0.07-3.16) for HIV-KS, 1.38 (0.62-2.91) for SLY and 1.02 (0.00-1.16) for the healthy group (Fig.1a). Among these subgroups, only MCD differed significantly from HIV+KS ($p<0.05$), HIV-KS ($p<0.01$) and healthy group ($p<0.01$), according to Kruskal-Wallis with Dunn's multiple comparison test. It is important to note, that in the HIV-KS subgroup there is only one out-of-range value of 8.55, which prevented observation of the statistically significant difference ($p<0.05$) between HIV-KS and PEL or HIV-KS and SLY groups. When MCD, PEL and SLY were grouped together as KSHV-related lymphomas, there was a statistically significant difference between KSHV-related lymphomas and both HIV+KS,

HIV-KS and healthy group ($p < 0.001$) (Fig. 1b). The same level of significance was reached when HIV+KS and HIV-KS were combined into one KS subgroup (Fig. 1c). When all patients with diagnosed KSHV-related disease were compared to healthy group, the Mann-Whitney two-tailed test returned the value of 0.0548, which was close to the level of significance and 95% CI of 1.28-2.16 (diagnosed group) vs. 1.006-1.16 (healthy group), respectively (Fig. 1d). Similar statistical analyses performed exclusively on male subjects returned the same statistically relevant differences except for the MCD and HIV-KS pair (Fig. 1a). Medians (95% confidence intervals CI) of MFI ratio between KCP/mock-transfected cells were: 3.02 (0.91-7.86) for MCD, 1.48 (-0.48-4.71) for PEL, 1.06 (1.03-1.34) for HIV+KS, 1.04 (-0.08-3.78) for HIV-KS, 1.31 (0.71-2.06) for SLY and 1.04 (0.99-1.09) for the healthy group. These results confirm our previous observation that anti-KCP antibody prevalence is higher in patients with KSHV-related lymphomas than in those suffering from KS. It may reflect the results of previous *in vitro* studies: KCP expression is abundant on PEL cell lines and can be further increased following stimulation of the cells [1], while only 2% of experimentally infected endothelial cells (KS lesions arise from endothelium [14]) expressing latency-associated KSHV nuclear antigen expressed KCP and this could not be increased by treatment with sodium butyrate or phorbol esters [15]. Explanation of such observations can stem from the fact, that KCP is expressed in the lytic cycle of KSHV and its appearance should be correlated with other viral lytic proteins. While studying individuals from groups I and III, we found that anti-KCP antibodies were detected more frequently in subjects with high ($> 1:500$) titer of anti-lytic KSHV antibodies ($P < 0.0001$). To this end, KSHV-related lymphoma patients had significantly higher titer of anti-lytic KSHV antibodies than KS patients ($P < 0.05$). Furthermore, we aimed to check if anti-KCP antibodies may protect individuals already immunized with KSHV from developing virus-related disease. However, our

control group from northern Sweden did not have elevated but even decreased levels of anti-KCP responses compared to all diagnosed patients. Thus, we conclude, that a humoral response to KCP antigen is not a factor protecting from disease development, but is rather elevated at the time of active disease.

3.3 Anti-KCP antibodies correlate with anti-lytic KSHV antibodies in classical KS but not with clinical stage, disease progression and viral load in body fluids.

We analyzed anti-KCP antibodies in patients with classical (HIV-) KS. Patients from this group were additionally classified into subgroups depending on slow (A) or fast (B) disease progression [6], clinical stage (I-IV, according to criteria described in [16]) and viral load in blood, serum and saliva [6].

Disease progression did not correlate with the presence of anti-KCP antibodies (Fig. 2a). Values in these two populations were not distributed normally and medians were 1.375 (0.6459-3.542) and 1.57 (0.907-4.041) for slow and fast progressing KS, respectively. Mann-Whitney U test returned the value of 0.3571 (not significant). Progression in clinical stage of KS resulted in higher median values of anti-KCP antibody prevalence (1.33; 1.38; 1.57 and 2.945 for stages 1-4, respectively). However, a Kruskal-Wallis test showed a general P value of 0.059. It is likely that inclusion of more stage IV patients would result in statistical significance (Fig. 2b). Next we correlated the anti-lytic and anti-latent antibody titers with anti-KCP presence. It is important to note, that this group of patients had much higher titers of lytic and latent KSHV antibodies, comparing to those from our pilot study [3] and the healthy group [7]. In the first study, 54.5 % of patients had a titer of anti-lytic KSHV antibodies lower than 1:640 and 50 % of patients had similar titers for anti-latent antibodies. In the healthy group these numbers were 96 % and 98.6

%, respectively. The numbers of patients from actual group II with a titration value lower than 1:1000 were 31.4 % and 22.8 % for lytic and latent KSHV antigens, respectively, but there were also individuals with titers such high as 1:10000 or more (data not shown). When analyzing nominal values of both anti-KCP and titers of anti-viral antibodies, linear regression did not show any significant correlation between the level of anti-KCP antibodies and anti-latent KSHV antibodies (r coefficient = -0.057, P value = 0.745). Instead, highly significant correlation with anti-lytic KSHV antibodies (r = 0.667, P < 0.0001) was detected but this result was heavily influenced by extremely high level of anti-KCP antibodies and relatively high anti-lytic KSHV antibodies titer in one patient. To perform a more reliable statistical analysis as well as to compare with previous studies, we classified patients into two groups regarding their lytic or latent anti-KSHV antibody levels: low (titer \leq 1:500) and high (titer > 1:500), respectively. Assuming these categories, there was a statistically significant difference in KCP antibody levels between low and high anti-lytic KSHV antibody carriers (P =0.0271) according to Mann-Whitney two-tailed test, and no difference related to anti-latent KSHV antibody carriers (P =0.529). Further analysis showed no correlation with blood viral load (r = -0.061, P = 0.7252), serum viral load (r = -0.0309, P = 0.86) and saliva viral load (r = -0.091, P = 0.6028). Previous study of this patient group showed higher titer of anti-lytic KSHV antibodies in fast (type B) vs slow KS progressors [6]. Additionally, a higher viral load was observed in blood and serum in patients with fast evolution of the disease while the opposite was found for viral saliva load [6]. Comparing our current results of anti-KCP antibodies with these findings one can conclude that KCP-specific antibodies, although strongly correlated with anti-lytic KSHV antibodies, are not enriched in the group of fast progressors of KS. Also, they do not preferentially appear in those patients with higher blood and serum viral load. This shows that an anti-KCP immune response

does not simply reflect disease progression with concomitant increase of lytic KSHV antigens, as these are present in the same degree in slow-progressing patients. On the contrary, it is possible that at least some individuals with high anti-lytic KSHV antibody titer were turned to slow KS progressors because of the presence of KCP-specific antibody.

3.4 anti-KCP antibodies isolated from patient serum block KCP function.

Next we tested whether antibodies against KCP may block the complement inhibitory function of KCP. We obtained substantial amount of serum from the patient who had the highest titer of anti-KCP antibodies in order to purify the immunoglobulin fraction. Purified immunoglobulins were then used as an inhibitor of KCP in C3b and C4b degradation assays. In both experiments we observed strong inhibition of KCP cofactor activity, which could not be observed with immunoglobulins isolated from healthy control (Fig. 3).

Taken together, we showed that prevalence of anti-KCP antibodies in classical KS patients does not follow the prediction based on other correlations described for the same group, i.e. since fast progression of KS correlated with high anti-lytic KSHV antibody titer, and high anti-KCP antibody levels correlated with high anti-lytic KSHV antibody titer, we expected to find higher anti-KCP antibody level in fast KS progressors. Our opposite finding suggests a specific role of anti-KCP antibodies in regulation of KS progression and such a role may be based on blocking of the KCP complement inhibitory function by specific antibodies developed by patients.

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Figure captions :

Fig. 1

Anti-KCP antibodies in patients suffering from various KSHV-related diseases and in healthy individuals with detectable anti-KSHV immune response evaluated for all individuals (left panels) or men only (right panels). a) individuals were divided into MCD, PEL, KS HIV+, KS HIV- and SLY patient groups as well as healthy control group b) the same evaluation as in "a" but MCD, PEL and SLY patients were grouped as "lymphomas" c) comparison between grouped KSHV-related lymphoma patients and all KS patients d) comparison between KSHV-related disease diagnosed patients and healthy controls. * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$, according to Kruskal-Wallis test.

Fig. 2

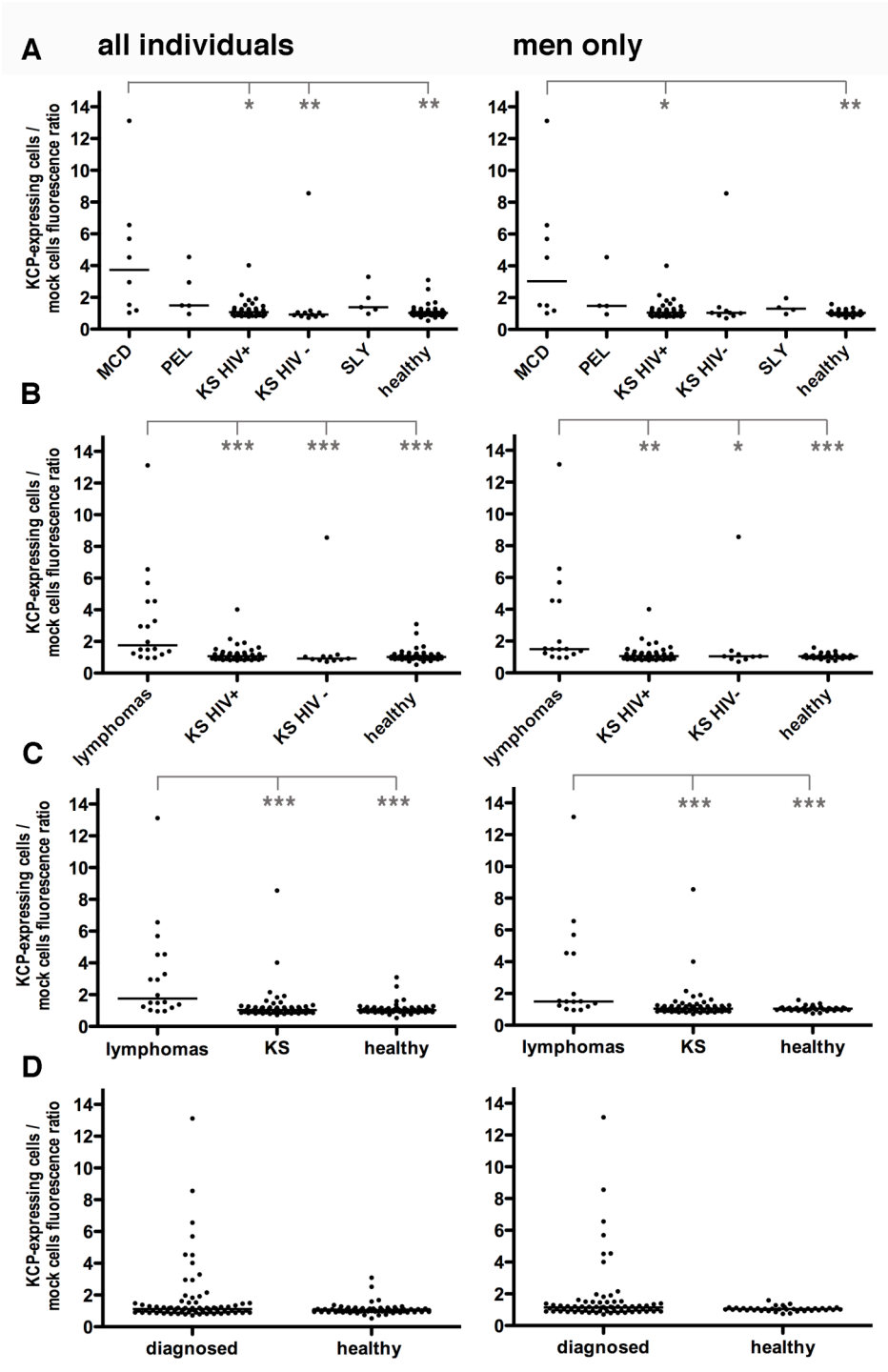
Evaluation of anti-KCP antibodies in classical KS (HIV-KS) patients stratified depending on: a) slow (type A) of fast (type B) KS progression and b) clinical stage of KS.

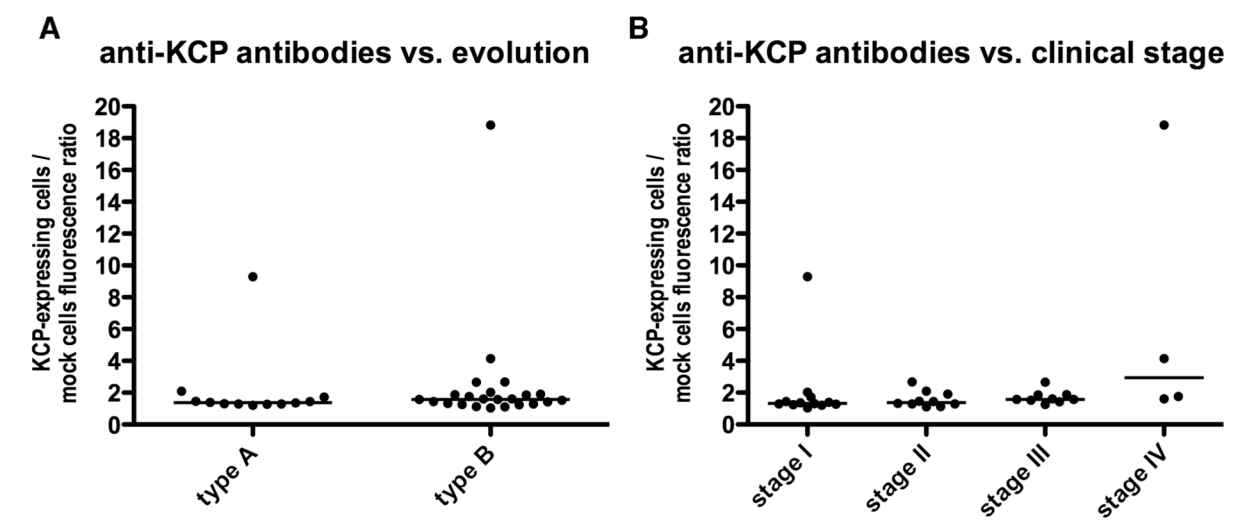
Fig. 3

Representative radiograms (out of three independent experiments) of degradation assay of complement proteins C3b (a) and C4b (b). Immunoglobulin fraction purified from serum of

the patient with the highest titer of anti-KCP antibodies was used to block KCP cofactor function. 1- negative control (no KCP), 2 - positive control (no immunoglobulin), 3 - control immunoglobulins from normal human serum, 4 - immunoglobulin fraction from anti-KCP sufficient patient. Bottom panel shows the average values of α -chain cleavage represented as the ratio to residual β (C3b) or γ chain (C4b), measured by densitometric analysis. ** - $P < 0.01$, according to ANOVA assay.

Vaccine, Okroj et al. Figure 1





Vaccine, Okroj et al. Figure 3

