

**SHORT THESIS FOR THE DEGREE OF PHILOSOPHY (PhD)**

**Characterization of Human Malignancies Caused by Human  
Herpesvirus 8**

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**UNIVERSITY OF DEBRECEN**

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# Characterization of Human Malignancies Caused by Human Herpesvirus 8

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(Microbiology programme)

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The Examination takes place at the Department of Pharmacology (Library), Faculty of Pharmacy, University of Debrecen, on Thursday Jun 19, 2014, at 11 a.m.

Head of the **Defense Committee:** Árpád Tószaki DP, PhD, DsC  
Reviewers: Katalin Burián MD, PhD  
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Members of the **Defense Committee:** Ferenc Somogyvári PhD  
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on Thursday Jun 19, 2014, at 1 p.m.

## **INTRODUCTION**

### **Human herpesvirus type 8**

Early studies suggested an infectious agent for KS and herpesvirus-like particles in tissue culture of KS specimens from different geographic regions were observed more than 30 years ago and a relation to the human cytomegalovirus (HCMV) was proposed. However, in 1994 a new virus initially called Kaposi's sarcoma-associated herpesvirus (KSHV) was characterized in an AIDS-associated KS lesion through isolation of DNA fragments of open reading frames (ORFs) 26 and 75 using representational difference analysis (RDA). Subsequently, KSHV was demonstrated in all clinico-epidemiological forms and stages of KS and identified as human herpes virus type 8 (HHV-8). It was soon found to be also associated with some rare types of lymphomas in AIDS patients, namely primary effusion lymphoma (PEL) or body-cavity-based-lymphoma (BCBL) and the plasmablastic variant of multicentric Castleman's disease (MCD). Currently, KSHV/HHV-8 is regarded as the primary pathogenic factor for KS development.

### **HHV-8 Epidemiology**

The HHV-8 genome consists of one single continuous long unique region (LUR) of 140 kb, which is flanked on both sides by terminal repeats of several 801 bp-long repeat subunits. The great majority of the LUR of HHV-8 is highly conserved, while both ends show high variability. The molecular analysis of the high genetic variability of the K1 gene can be exploited for epidemiological studies. K1 is located at the left end of the genome and encodes a highly variable membrane glycoprotein of 298 amino acids (aa). The amino acid sequence of K1 varied from 0.4% to 44%, with the changes concentrated to the two hypervariable regions, VR1 and VR2. Seven major molecular subtypes (designated A, B, C, D, E, F and Z) of HHV-8 have been classified on the basis of K1 sequence analysis, that appear, at least for some subtypes, to be linked to the geographical origin of the samples. The distribution of HHV-8 isolates varies with geography and ethnicity. Compared with other human herpesviruses, HHV-8 appears to be infrequent in the general population of North America and Northern and Western Europe, but is more prevalent in several Mediterranean countries, in particular in Italy and Greece, and it is widespread in many parts of Africa. Among individuals of Western countries at increased risk of HIV-infection, HHV-8 is more prevalent

among homosexual men than among patients with hemophilia, injecting drug users and those who contracted HIV heterosexually. Subtypes A and C have been found in Europe, the USA, Middle East and Asia. Subtype B is found mainly in Africa and French Guiana. Subtype D was first reported in Taiwan, in some Pacific islands and Australia, and subtype E has been found among Amerindian population of the Brazilian and Ecuadorian Amazon regions. Subtype Z has been found in a small cohort of Zambian children and a new subtype F has been identified in Uganda.

### **Kaposi's sarcoma**

Kaposi's sarcoma (KS) first described by the Hungarian dermatologist Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin" is a multicentric, angioproliferative, maculo-papular eruption most often presenting primarily in the skin but also in mucosal membranes, viscerae and lymphnodes as a characteristic purplish lesion which blanches to pressure and progresses with woody oedema and skin hyperpigmentation to florid, ulcerative lesions. Previously a rare disease, it has become a major epidemiological concern globally and in particular sub-Saharan Africa including Tanzania mostly because of its association with the HIV infection. Four different clinico-epidemiological KS forms with similar histopathology are now recognized including: 1) AIDS-related KS: The most aggressive and rapidly growing KS form with early dissemination in the skin and viscera. AIDS-related KS is the most frequent tumor in human immunodeficiency virus type I (HIV-1) infection where it is frequent among homo-bisexual men and intra-venous (IV) drug users in Western countries but most often seen among heterosexuals in developing countries. 2) Classical or sporadic KS: Originally described as a slow growing, indolent tumor mostly seen in the extremities of elderly Caucasian males of eastern Europe and Mediterranean origin. 3) Endemic/ African KS: Predominant in eastern and central Africa before the AIDS epidemic and clinically similar to classical KS, but also seen in children in a more fulminant and fatal form. The childhood endemic KS is mostly lymphoglandular with or without skin involvement. 4) Iatrogenic or post-transplantation KS: seen in immunosuppressed patients, e.g. transplant patients receiving immunosuppressive drugs, emphasizing the importance of immune disturbance as a co-factor in the pathogenesis of iatrogenic KS.

## **Body-cavity based lymphoma**

Primary effusion lymphoma (PEL), or alternatively: body cavity based lymphoma (BCBL) is a non-Hodgkin's lymphoma (NHL) of B-cell origin that develops predominantly in the serous body cavities. The lymphoma cells, although lacking many conventional B-cell markers, carry immunoglobulin gene rearrangement and express syndecans, suggesting pre-plasma cell origin. At the clinico-pathological level, PEL is characterized by liquid growth in the serous body cavities associated with spreading along the serous membranes without infiltrative or destructive growth patterns. Morphologically, PEL bridges immunoblastic and anaplastic features and frequently displays a certain degree of plasmacell differentiation. In all known cases, the monoclonal B-cell population is infected with HHV-8. Half of the lymphomas are dually infected with HHV-8 and Epstein-Barr virus (EBV). In the context of AIDS, most cases are associated with other KSHV/HHV8-related diseases such as Kaposi's sarcoma or multicentric Castleman's disease (MCD). As PEL typically lacks a solid component, its diagnosis rests on the cytological examination of body fluid. Phenotypically, expression of the CD45 antigen (>90% of cases) confirms the lymphoid derivation of PEL cells, which exhibit an indeterminate immunophenotype, as they usually lack expression of B- and T-cell associated antigens (the majority of cases reported). There are, however, cases in the literature that had a B-cell or T-cell phenotype respectively. The prognosis of PEL is poor, as the median survival in the previously published series does not exceed 3 months.

## **The way of the future: The drug sensitivity assay**

*In vitro* sensitivity assays are promising tools to predict the individual outcome of different chemotherapy regimens. The long-awaited era of personalized genetic medicine may finally be arriving for people with cancer. Some cancer centres are preparing to screen all patients for genetic glitches associated with the disease, and scientists are starting to use detailed information about patients' tumour genomes to decide which treatments might benefit them most. Treatments that target specific mutant genes have been available for certain cancers since 2001, and a handful of studies have pinpointed mutations that reveal which tumours will respond best. For instance, mutations in the epidermal-growth-factor receptor (EGFR) gene affect how well patients with lung cancer respond to drugs that target the EGFR protein. Every person has their own unique variation of the human genome. Although most of the

variation between individuals has no effect on health, our state of health stems from this variation in combination with influences from our environment. Modern advances in Personalized Medicine rely on technology that confirms a patient's fundamental biology, DNA, RNA or protein, which ultimately leads to confirming disease. For example, personalized medicine techniques such as genome sequencing can reveal mutations in the DNA code that lead to diseases ranging from cystic fibrosis to cancer.

## **AIMS OF THE STUDY**

In the first part of our studies I had an opportunity to work Laszlo Szekely and Henriette Skribek's Group at Karolinska Institutet, Stockholm, where we were using a specific in vitro fluorescence drug sensitivity method to study the anti-tumor therapeutic issues.

- Determination of the body cavity based lymphoma cell lines in vitro drug sensitivity.
- Compare the drug sensitivity pattern of all available body cavity based lymphoma-derived cell lines from different origin in order to find new drugs which are still not used in chemotherapy treatment protocols of body-cavity based lymphoma.

In the second part of my study I investigated DNA samples from paraffin-embedded, formaldehyde-fixed biopsies carried out Kaposi's sarcoma.

- Determination of HHV-8 genotypes in Hungarian patients with Kaposi's sarcoma.
- Create a HHV-8 K1 gene partial sequences database, then using these for the phylogenetic analysis of the strains.

## **MATERIALS AND METHODS**

### *Determination of the drug sensitivity of the body cavity B-cell lymphoma cell lines*

#### **Cell lines and culture conditions**

The following primary effusion (body cavity based) lymphoma-derived cell lines were used in the present study. CRO-AP/2, CRO-AP/5, CRO-AP/6, BC-2, BC-3 were established from pleural effusion, CRO-AP/3, HBL-6, BC-3, BCBL-1, JSC-1 were established from ascites fluid and BCP-1 from peripheral blood. Body-cavity cell lines were cultured in IMDM (Sigma), supplemented with 20% heat-inactivated (at 56<sup>0</sup> C for 45 min) fetal calf serum (FCS, Sigma), 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2mM L-glutamine (Sigma). Cell lines were grown at 37<sup>0</sup> C in the presence of 5% CO<sub>2</sub>. Cultures were fed twice weekly with the above-mentioned medium; maintained at ca. 0.5 x 10<sup>6</sup> cells/ml. All cell lines were examined daily in their culture vessels under an inverted microscope. Absence of mycoplasma contamination was routinely assessed using staining with Hoechst 33258.

#### ***In vitro* drug sensitivity assay**

*In vitro* drug resistance of body-cavity cell lines were assessed using a 3-day cell culture on microtiter plates. 27 drugs were tested, each at 4 different concentrations in triplicates on 384 well plates. Each well was loaded with 30 µl cell suspension containing 9000 cells. After three days of incubation the living and dead cells were differentially stained using fluorescent VitalDye (Biomarker Hungary). The precise number of living and dead cells was determined using a custom built-automated laser confocal fluorescent microscope (a modified Perkin-Elmer UltraView LCI) at the Karolinska Institute core Visualization Facility (KIVIF). The images were captured using the computer program QuantCapture 4.0. Image correction and counting of living and dead cells was carried out by the program QuantCount 5.0. All programs were created by using the symbol based graphical programming environment OpenLab Automaton (Improvisation). The 15 control wells, that were used to determine the control cell survival (CCS), contained cells with only culture medium and 50 nl DMSO without drugs. 5 wells contained cells with culture medium alone. Comparing the two types of control wells no toxic effect of DMSO could be seen. Mean cell survival (MCS) was determined from the average of cell survival of all 11 body-cavity cell lines.

## Drugs

For the *in vitro* drug sensitivity test 27 drugs were used. All the drugs were dissolved in 50% dimethyl sulfoxide (DMSO) – 50% phosphate buffered saline (PBS) and were printed on the 384 well plates using high-density array replicator metal pins with 50 nl replica volumes in a Biomek 2000 fluid dispenser robot (Beckman). The same robot was used to generate the drug masterplates containing the triplicates of four different drug dilutions (1x, 5x, 25x, 125x) using a single tip automatic pipettor dispenser head. The starting concentration of the dilution series for the individual drugs was initially determined based on the solubility of the different agents. The drug plates that were used in this study were also tested on a large number of *in vitro* tumor cell lines and cells from primary tumor samples. In these assays we could show that it was possible to find sensitive cell lines for each individual drug, demonstrating that all the drugs on the plate were active. To calculate the relationship between the *in vitro* drug concentrations and the *in vivo* ones, we used area under curve (AUC; area under the plasma, concentration curve versus time) values of the individual drugs. For this comparison Quotient of Area Under Curve values (QAUC<sup>72hr</sup>) were determined by the following formula:

$$\textit{in vitro} \text{ used concentration} \times 72 \text{ hours } (\mu\text{g}\cdot\text{hr}/\text{ml}) / \textit{in vivo} \text{ AUC}^{72\text{hr}} (\mu\text{g}\cdot\text{h}/\text{ml})$$

The *in vivo* AUC<sup>72hr</sup> corresponds to the area under curve value achieved in patients under a 72 hours period. The *in vivo* AUC<sup>72hr</sup> was established from the clinical dose and half-time using the standard trapezoidal rule calculation. A QAUC<sup>72hr</sup> value higher than 1 indicates that the *in vitro* drug concentration is higher than the one used in the clinical practice. If this value is 1, it means that the *in vitro* concentration corresponds to the clinically achieved *in vivo* concentration.

### *Phylogenetic analysis of HHV-8 strains in Hungary*

## Patient samples

A total of 36 paraffin-embedded biopsies were tested from 17 patients. (We have more samples from the same patients, which were taken different times.) 21 specimens from 6 patients were described by Juhasz et al. The remaining specimens from 11 patients were also paraffin-embedded, formaldehyde-fixed biopsies of ‘classic’ (HIV-negative) KS. The samples



were collected for routine diagnostics between 1995 and 2004 at the Department of Dermatology, University of Debrecen.

### **DNA isolation**

At least three 20 µm sections were obtained from each of the nine formalin fixed and paraffin-embedded KS tissue samples. The sections were deparaffinized with xylol for 3 x 5 min, washed 5 times with ethanol and air-dried. The deparaffinized samples were incubated with 200 µl lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 8.0, 1% SDS, 25 mmol/l EDTA, pH 8.0 and 200 µg/ml Proteinase K) at 55°C for one hour, followed by heat inactivation of Proteinase K at 95°C for 10 min. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). After centrifugation, the water phase was transferred to a new tube with 1 ml isopropanol and kept at -20°C overnight. The pellet was washed in 70 % ethanol and resuspended in 30 µl TE.

### **Polymerase chain reaction**

The K1 sequence of HHV-8 is notoriously difficult to amplify due to its high sequence heterogeneity. We have optimized the temperature and cycling conditions of PCR amplification of the K1 sequences. For all experiments stringent precautions against PCR contamination were taken. The DNA extraction process was validated by amplifying the β - globin gene using the PCO3 and PCO4 primers. The presence of HHV-8 was detected by amplification of the conserved ORF26 gene. The ORF26 primers amplified a 172 bp-long amplicon. (All patient samples contained the virus.)

To amplify the K1 region, we used K1-N (nt 105 to 121) - K1-C (nt 971 to 955) outer primers in combination with nK1-1 (nt 204 to 223) - nK1-2A (nt 442 to 423) and nK1-1 (nt 204 to 223) - nK1-2B (nt 603 to 584) primer pairs for the nested round. The genomic positions correspond to genome of BC-1 strain. Briefly, a 25 µl reaction volume containing 12 µl JumpStart REDTaq ReadyMix Reaction Mix (Sigma), 0.5 µl primers and 100-500 ng DNA in 12 µl. Thermocycling conditions were set at 95°C denaturation for 3 min, followed by 30 cycles of 95°C for 30 sec, and 1 min at 58°C at the appropriate annealing temperature, 72°C

for 1 min, and 4 min extension at 72°C. Simultaneously, each PCR run contained blank (distilled water instead of DNA templates) and positive controls (HHV-8 DNA isolated from KS patients infected HHV-8). The reaction products were checked on 1.5% agarose gels, observed by a gel imaging system.

## **Sequencing**

Sequencing reaction of the nested PCR products of the ORF K1 PCR was performed using the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). Sequencing products were detected by ABI Prism 310 Genetic Analyzer automated sequencer (Applied Biosystems). To ensure that the observed mutations were not the result of errors in PCR, sequencing was performed on at least two distinct PCR products generated from each DNA sample, and both strands of each product were independently sequenced.

## **Phylogenetic analysis**

With phylogeny.fr, we created a phylogenetic tree of the partial K1 amino acid sequences. 19 reference sequences from GenBank and published reports were included in the analysis to aid subtype identification. All sequences were first aligned in ClustalW. Radial and unrooted linear phylogenetic trees were drawn by the program DRAWTREE.

## **RESULTS**

### *Determination of the drug sensitivity of the body cavity B-cell lymphoma cell lines*

#### **The *in vitro* drug sensitivity assay**

We have tested the drug sensitivity patterns of the body cavity lymphoma lines in short term, *in vitro* survival assays. Each cell line was tested against 27 different drugs, in triplicates, at four different concentrations. The assay was carried out on 384 well plates. After 3 days of

incubation each individual well of the test plates was photographed using a custom developed, automated extended field confocal microscope. Living and dead cells were differentially stained using viability dependent fluorescent dyes. Each individual living or dead cell was identified counted and their fluorescence intensity distribution was recorded using automated image analytic and quantitation programs. For each well the percentage of surviving cells was calculated by comparing the number of living cells in the given well to the average of living cells in the untreated control wells.

### **Heat map of the cluster analysis**

In order to identify possible co-segregation of the sensitivity patterns of the individual drugs as well as to systematically compare all the lines with each other, we have carried out unsupervised two-dimensional hierarchical clustering of the simplified drug sensitivity data using the Cluster 3.0 program for MacOS X. The results were visualized using the program TreeView. The sensitivity to the drug was represented on a 5 step scale where every step represents less than 50% viability at the four different drug dilutions. (Resistant - if more than 50% survival at the highest concentration, maximum sensitivity - if less than 50% survival at the lowest concentration.)

### **Pharmacokinetic comparison**

Absolute drug sensitivity values have relatively little clinical relevance if they are not correlated with clinically achievable *in vivo* concentrations. In order to analyze the data in relation to the pharmacokinetic behavior of the given drugs we have plotted the mean survival values as the function of the Quotient of the Area Under Curve (QAUC<sup>72hr</sup>) values of the particular drugs. The QAUC<sup>72hr</sup> values of a drug were created by dividing the calculated *in vitro* AUC<sup>72hr</sup> values by the *in vivo* achievable AUC values (which were calculated from clinical dose and half-time). Plotting the mean cell survival for each individual drug against a common QAUC<sup>72hr</sup> axis shows that Daunorubicin, Epirubicin, Paclitaxel and Vinorelbine were the most effective drugs (low survival at low QAUC<sup>72hr</sup> values). Moreover most body cavity lymphoma lines were sensitive to these drugs. Importantly, Doxorubicin, the only anthracyclin drug that is currently included in chemotherapy protocols against body cavity

lymphomas showed a rather heterogeneous effect. Two of the eleven lines were highly sensitive for Doxorubicin whereas two were completely resistant at the maximum drug concentration that we could reach in the current assay (QAUC=0.11). The two lines (JSC-1 and BC-3) were, in general, the least sensitive for chemotherapeutic drugs, however were still sensitive for Daunorubicin, Epirubicin and Vinorelbine. Dactinomycin showed the highest killing efficiency in the present *in vitro* assay. The calculation of the QAUC<sup>72hr</sup> value for the corresponding Dactinomycin concentration however revealed that the concentration that was required for the high killing effect is higher than the levels that are realistically achievable in a patient. When treating the body-cavity lymphoma cells with Carboplatin at low QAUC<sup>72hr</sup> values, a relative increase in the number of surviving cells was observed as compared to non-treated controls. The survival was above 100% in case of all the 11 lines suggesting that low dose Carboplatin protected from spontaneous cell death.

#### *Phylogenetic analysis of HHV-8 strains in Hungary*

##### **Orf26 PCR**

The diagnosis of the patients with KS was based on the clinical and histopathological characterizations of skin lesions. Out of the 36 paraffin-embedded tissue specimens the orf26 regions was detected in 36 (100%) by nested PCR.

##### **Orf-K1 PCR**

Of the 36 orf26 positive samples, orf-K1 region was detected in 22 (61%) by semi-nested PCR. The orf-K1 PCR bands were suitable for sequencing and sequence analysis from 12 biopsies. PCR amplification of the remaining 10 biopsies did not result in K1 amplicons of proper quantity or quality for bi-directional sequence analysis. The lengths of the partial sequences of HHV-8 isolates from this study (GenBank accession numbers KF829938-KF829947) ranged from 281 to 358 nucleotides. There was a common 167 bp region (nt 169 to 336) revealing nucleotide sequence from each isolate. This common region was used to analyze the phylogenetic relationship.

## Phylogenetic studies

Into the phylogenetic analysis, we inserted other known Hungarian sequences. We used two different sets of sequences for the phylogenetic studies. Phylogenetic trees were generated from multiple alignments of the ORF-K1 gene using 19 different sequence prototypes of the four major KSHV ORF-K1 genotypes available in Genbank: B1 (K1-431KAP, AF133040), B2, (UgD1, AF130292), D1 (TKS10, AF133043), D2 (ZKS3, AF133044), E (Tupi2, AF220293), C (K1-43Berr, AF178810), C (K1-8Dem, AF178780), C1 (ASM72, AF133041), C3 (BC-2, AF133042), A5 (K1-7/Ngo, AF178779), A3 (BCBL-1, U86667), A3 (K1/E36, AY204662), A2 (K1-E41, AY204657), A2 (K1/E12, AY204663), A2 (BC-1, U75698), A2 (K1-E23, AY204668), A1 (BCBL-R, AF133038), A1 (K1-E25, AY204654), A1 (K1-77/47, AF201850). The first one involved all K1 subtype nucleotide sequences, while the second set, based on amino acid, comprised all the corresponding subtypes A sequences. Regarding the orf-K1, all of the tested strains belonged to the subtype A. Specifically, six were related to the A1 subgroup, four to the A2 subgroup and three to the A3 subgroup. The isolates Hun30a, Hun30b, Hun31b, Hun32, Hun33 and Hun34a exhibited 100% identity with the K1/E25 and K1-77/47 prototype, while the isolates Hun31a 96,5%, Hun34b, Hun35 94,8% and Hun36 93% identity with the K1/E41 prototype on an amino acid level. The isolates Hun14a and Hun13 exhibited 100% and Hun14b 93% amino acid identity with the A3 prototype BCBL-1.

## DISCUSSION

*Related findings of determination of the drug sensitivity of the body cavity B-cell lymphoma cell lines*

*In vitro* growing cell lines are the closest model systems available today for studying the biological features of body cavity lymphomas. The cell lines that were used in the present study represent a variety of different origin. The investigation included cell lines established from ascites fluid, pleural effusion or from the peripheral blood of PEL patients. Despite their different origin, the body-cavity lymphoma lines showed a remarkably similar sensitivity pattern for a number of drugs. Only one cell line was highly resistant for most of the drugs (JSC1) whereas two cell lines (BC1 and BCBL-1) showed increased overall sensitivity to

most of the drugs. The presented data suggests that, for a number of cytostatic drugs the body cavity lymphoma cell lines share a common cytotoxic drug sensitivity profile. These profiles show no obvious correlation with the biological or clinical features of the lymphomas. Clustering of the drug sensitivity data revealed that the profiles are independent of the EBV status, anatomical localization of the lesion, the age the patient or the rapidity of the progression of the disease. The two cell lines (BCP-1 and BC-3) that arose from HIV negative patients showed relatively low drug sensitivity.

The current treatment alternative is a combination of Methotrexate with CHOP (Cyclophosphamide, Doxorubicin, Prednisolone, Vincristine)-regimes. The present data showed that the cell lines exhibit varying sensitivity to Methotrexate and Vincristine and are completely resistant to Prednisolone. Cyclophosphamide or Ifosphamide were not tested on the body cavity lymphoma lines, because both of these compounds are prodrugs that have to be converted into active metabolites by the liver *in vivo*.

It has been reported that the proteasome inhibitor Bortezomib induces apoptosis of the cell lines BCBL-1 and BCP-1 *in vitro*. In this study only BCBL-1 showed detectable sensitivity to Bortezomib and only at the highest concentration whereas all the other lines were resistant.

In the present study body cavity lymphoma lines showed considerable sensitivity for anti-microtubule drugs and anthracyclins. Importantly all lines were sensitive to Epirubicin and Vinorelbine even at low QAUC<sup>72hr</sup> values. Epirubicin required tenfold lower concentration than the *in vivo* achievable concentration to kill more than 80% of the cells for most of the lines. Epirubicin is primarily used against breast and ovarian cancer, gastric cancer, lung cancer and lymphomas, but has not yet been tested against body cavity lymphomas.

In summary, the analysis of drug sensitivity profiles of the available body cavity lymphoma lines against 27 commonly used drugs revealed considerable heterogeneity in drug response. Four drugs, namely, Daunorubicin, Epirubicin, Paclitaxel and Vinorelbine showed uniformly high efficiency on the cell lines. These drugs are not yet included in the current chemotherapy protocols of body cavity lymphomas. The heterogeneity of drug response also suggests that optimal care of the lymphoma patients would include the determination of drug sensitivity patterns of the primary tumor samples and that these patients would benefit from assay guided individualized therapy.

### *Related findings of phylogenetic analysis of HHV-8 strains in Hungary*

Hayward et al. hypothesized that HHV-8 is an evolutionary old human virus distributed worldwide along broad ethnic and geographic lines and acts as a marker for ancient human migration. The first expansionary migration was into Sub-Saharan Africa starting 100,000 years ago (B subgroup), then into South Asia and Australia beginning 60,000 years ago (D subgroup). Finally, two major branches migrated into Europe and North Asia about 35,000 years ago (A and C subgroups).

HHV-8 DNA is present in all KS tumor samples and in the peripheral blood mononuclear cells (PBMC). Serological evidence obtained by LANA immunofluorescent antibody assay also indicates that the infection is widespread in the world. The prevalence of the virus is variable, lower in Northern Europe and the USA (0-3% seropositivity), higher in Southern Italy (5 to 20% seropositivity) and very high in Central and Southern Africa (40 to 60 % seropositivity), where endemic and classic KS have the highest incidence rates, reaching up to 1.0 and 10 per 100,000 person years. Similarly, KS patients and male homosexual AIDS patients, human immunodeficiency virus (HIV)-positive intravenous drug users and hemophiliacs have extremely high seropositivity rates of around 85%. The prevalence in Hungary is 2,3 - 3,5% among blood donors, and 31,5% among the Hungarian HIV-positive patients.

Since limited data is available on HHV-8 molecular epidemiology in Hungary, the aim of the present work was to investigate the distribution of HHV8 DNA sequences among hospitalized individuals in Hungary. We analyzed the genetic variation and polymorphism of a genomic region of HHV8 (ORF K1) located at the left-hand side of the viral genome of 42 KSHV strains originating from Hungarian patients with Kaposi-sarcoma.

Among our patients 1 of the 9 was a female, consistent with previous evidence that KS is more frequent in males than in females.

From one patient (Hun8), different variants of HHV-8 could be isolated from serum and PBMC. We have two different samples from another 3 patients, which were taken in different times. The elapsed time between the samplings is minimum 3 years. At 2 patients could we identified different K1 strains. The differences are 3% on nucleotide levels and it causes 5

amino acid changes). It could be occur to detect more variants of the HHV-8 virus at the same patient are likely common to see a patient more variants of the virus, even concurrently.

Thirteen samples could be amplified and clustered to subtype A. This genotype is considered to be originated from Europe and North Asia. The C subtype is very common in this region as well, but we could not identify it among the isolates, where the K1 section was successfully amplified.

The main outcome of this study will give useful data for molecular epidemiological studies in Hungary. It is necessary to continue the surveillance of cancers such as Kaposi's sarcoma for designing more effective diagnostic and treatment procedures. Nevertheless, the available samples within the studied group were limited in number, so we cannot make a general claim about the association of a specific viral genotype with clinical presentation and further studies are ongoing to clarify this issue.

## **Novel results**

1. Body cavity based lymphoma (BCBL) cell lines revealed to share common *in vitro* sensitivity patterns against a series of 27 cytostatic drugs.
2. BCBL cell lines proved to be sensitive particularly to anthracyclins and cytostatic drugs acting on microtubules.. *In vitro* drug sensitivity data provide implications on developing the present empirical chemotherapy
3. *Kaposi sarcoma* biopsies were used to identify HHV-8 subtypes identified based on sequencing orf-K1 gene. Sequencing revealed the occurrence of subtype A, more specifically subtypes A1 and A2 in *Kaposi sarcoma* biopsies. Two patients carried double orf-K1 variants.
4. *Kaposi sarcoma* biopsies harbored a subgroup of variants presenting themselves in this geographic region.



## **SUMMARY**

The human herpesvirus 8 (HHV-8) is the etiologic agent in Kaposi's sarcoma which was discovered 20 years ago. It was found to be associated with some rare types of lymphoma in AIDS, namely body-cavity based lymphoma and the plasmablastic variant of multicentric Castleman's disease and some cases was shown in bone marrow samples of myeloma multiplex and benign monoclonal gammopathy disease. The survival of patients with malignant disease can be increased in the future using a more individualized therapy. Using of effective drugs determined by in vitro drug sensitivity test might result in a better clinical outcome.

As body cavity cell lines (BCBLs) are well established in vitro models for body-cavity based lymphoma we have assessed 11 BCBLs for cytotoxic drug sensitivity. The precise number of living and dead cells was determined using a custom made automated laser confocal fluorescent microscope. Independently from their origin, BCBLs showed very similar patterns against 27 frequently used cytostatic drugs. BCBLs were highly sensitive for epirubicin, daunorubicin, paclitaxel and vinorelbine.

The prognosis of PEL is poor, as the median survival in the previously published series does not exceed 6 months. Despite the improvement in therapeutical strategies during the last few years, there is no evidence of a cure for BCBL patients with conventional systemic chemotherapy addressed to aggressive NHL. Our data suggest that inclusion of the above drug into BCBL chemotherapy protocols may be justified.

Based on the sequence variation in the open reading frame (orf) K1, HHV-8 is now classified into subtypes A, B, C, D, E, F and Z. We attempted to develop a typing system based on amplification and sequencing of the K1 region. A total of 36 paraffin-embedded biopsies were tested in 17 patients. Based on the K1 region we identified subtype A within subtype A1 and A2. We determined different K1 strains in two specimens.

The main outcome is that it provides useful data for molecular epidemiological studies in Hungary and the found genotype is considered to originate from Europe.

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Candidate: Ríta Ötvös

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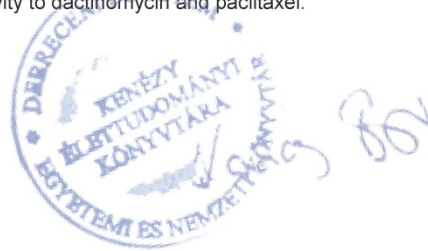
### List of publications related to the dissertation

1. **Ötvös, R.**, Juhász, A., Szalai, E., Újvári, D., Ötvös, K., Szabó, K., Remenyik, É., Székely, L., Gergely, L., Kónya, J.: Molecular Typing of Human Herpesvirus 8 Isolates from Patients with Kaposi's Sarcoma in Hungary.  
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**Total IF of journals (all publications): 21.313**

**Total IF of journals (publications related to the dissertation): 4.724**

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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