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Research Paper

Plasma Viral miRNAs Indicate a High Prevalence of Occult Viral Infections

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ABSTRACT

Prevalence of Kaposi sarcoma-associated herpesvirus (KSHV/HHV-8) varies greatly in different populations. We hypothesized that the actual prevalence of KSHV/HHV8 infection in humans is underestimated by the currently available serological tests. We analyzed four independent patient cohorts with post-surgical or post-chemotherapy sepsis, chronic lymphocytic leukemia and post-surgical patients with abdominal surgical interventions. Levels of specific KSHV-encoded miRNAs were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and KSHV/HHV-8 IgG were measured by immunoassay. We also measured specific miRNAs from Epstein Barr Virus (EBV), a virus closely related to KSHV/HHV-8, and determined the EBV serological status by ELISA for Epstein-Barr nuclear antigen 1 (EBNA-1) IgG. Finally, we identified the viral miRNAs by in situ hybridization (ISH) in bone marrow cells. In training/validation settings using independent multi-institutional cohorts of 300 plasma samples, we identified in 78.50% of the samples detectable expression of at least one of the three tested KSHV-miRNAs by RT-qPCR, while only 27.57% of samples were found to be seropositive for KSHV/HHV-8 IgG ($P < 0.001$). The prevalence of KSHV infection based on miRNAs qPCR is significantly higher than the prevalence determined by seropositivity, and this is more obvious for immuno-depressed patients. Plasma viral miRNAs quantification proved that EBV infection is ubiquitous. Measurement of viral miRNAs by qPCR has the potential to become the "gold" standard method to detect certain viral infections in clinical practice.

1. Introduction

Early detection of common (e.g., herpesvirus) and new emerging viruses (e.g., Zika virus) infections is extremely important for control of disease transmission, prompt initiation of treatment and prevention of infection-related complications. Human herpesvirus 8 (HHV-8; KSHV, Kaposi sarcoma-associated herpesvirus) is a member of the gamma-herpes virus family that evolved to maintain life-long latent infections in the human host (Levy, 1997; Luppi and Torelli, 1996; Mesri et al., 2010). KSHV is a causative factor for Kaposi sarcoma, primary effusion lymphoma, and some subtypes of multicentric Castleman disease (Ganem, 2010). Unlike the other members of the gamma-herpes virus family, wide variation is seen in the seroprevalence of KSHV, which is generally high in African and Mediterranean regions (20%–80%), and low in non-endemic areas such as the United States and Northern Europe (1.5%–7%) (Rohner et al., 2014; Stiller et al., 2014). These geographical variations in KSHV/HHV-8 seroprevalence and the incidence of classic

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Kaposi sarcoma (KS) remain largely unexplained. Most cases of KS reported outside endemic areas are in immunosuppressed patients (e.g., HIV-infected patients, and post-transplant recipients) (Osmond et al., 2002).

In the last two decades, modern techniques (e.g., real-time PCR or RT-qPCR, ELISA) have improved identification of viral infections. However, there is no agreement on a standard assay to detect the presence of KSHV infection and thus estimate its prevalence (Bhutani et al., 2015). Although the current method of choice is to detect antibodies produced in the patients after being infected by KSHV, the seroprevalence of KSHV varies greatly geographically and the true prevalence of KSHV infection may be underestimated (Mesri et al., 2010; Stiller et al., 2014).

MicroRNAs (miRNAs) are short non-coding RNAs (ncRNAs) that post-transcriptionally regulate gene expression, thereby affecting multiple cellular processes, and miRNAs can serve as biomarkers for prognosis of different diseases including malignancies (Ambros, 2003; Calin et al., 2002; Calin et al., 2005; Fabbri et al., 2011). Previous studies led to the discovery of virally encoded miRNAs that play important roles in regulating the latent-lytic switch of gamma-Herpesviruses infections (Mesri et al., 2010; Zhu et al., 2013). Viral miRNAs can modulate both viral and host cellular gene expression during infections without generating antigenic viral proteins that can be detected by the host immune system (Skalsky and Cullen, 2010). Changes in cellular and viral miRNAs expression levels in the circulation (plasma or serum) showed specific patterns in various diseases (e.g., malignancies, sepsis, atherosclerosis) (Boss et al., 2011; Fabris and Calin, 2016; Ferrajoli et al., 2015; Giza et al., 2016; Herman et al., 2015; Tudor et al., 2014; Zhang et al., 2012). These miRNAs remain in circulation in a stable form, being highly resistant to acute changes in pH, endogenous RNase activity, and variations in temperature (Shah and Calin, 2013). Furthermore, several mature miRNAs, derived from 12 precursor miRNAs in the latency locus of the KSHV genome, play important roles in KSHV-induced cell transformation (Cai et al., 2005; Samols et al., 2005). Moreover, we previously reported that higher plasma levels of KSHV miRNAs are associated with a worse clinical outcome in patients with sepsis (Tudor et al., 2014). We hypothesized that, since viral DNA/messenger RNA/proteins cannot be detected in all infected individuals, detection of miRNAs encoded by viruses may represent a more sensitive assay to determine the true prevalence of certain viral infections, including latent KSHV infection. Therefore, we compared the results of measurement of plasma miRNA by RT-qPCR to serological testing for KSHV in four groups of Caucasian patients from US and Romania to determine the relative effectiveness of the two methods to measure and detect evidence of occult KSHV infection.

2. Methods

2.1. Patients and Samples

We used 300 plasma samples from a total of 214 Caucasian patients from four independent patient cohorts whose characteristics have been previously described (Ferrajoli et al., 2015; Ferrajoli et al., 2013; Tudor et al., 2014) (Fig. 1 and Table S1). We initially used a training cohort containing 33 patients with sepsis from Fundeni Clinical Hospital (FCH), Bucharest, Romania as previously reported in Tudor et al. (2014). We used a validation cohort containing 43 patients with sepsis from The University of Texas MD Anderson Cancer Center (UT-MDACC), Houston, Texas, US (Tudor et al., 2014). Two additional independent patient cohorts were used: 43 patients that underwent abdominal surgery (with collected samples at three time points: one day before surgery, and after surgery day 1 and day 7) from FCH (Tudor et al., 2014); and 95 patients with chronic lymphocytic leukemia (CLL) from UT-MDACC as previously reported (Ferrajoli et al., 2013; Ferrajoli et al., 2015). A schematic representation of the main plasma samples cohorts and workflow is shown in Fig. 1. Bone morrow (BM) samples from 8 independent patients with CLL from UT-MDACC were used for miRNA *In Situ Hybridization* (ISH; Supplementary Methods). We selected only Caucasian patients because they have the lowest reported incidence of HHV-8 infection and also to have a homogenous population for the study and exclude bias related to race as a study variable. All clinical data and blood samples were obtained from participants who had given written informed consent, according to protocols approved by the FCH Ethics Committee and UT-MDACC Institution Review Board. For all the patients the age, gender, diagnosis, lymphocytes count, white blood cells (WBC) count, absolute neutrophils count (ANC), platelet count (PLT) and survival status were known and used for the study (Tables S1 and S2).

2.2. RNA Extraction and Expression Analyses

Total plasma RNAs extraction and normalization (by addition of fixed amount of 10 fmol per 100 μl of plasma of each *C. elegans* miRNAs cel-miR-39-3p and cel-miR-54-3p), reverse transcription and expression analyses (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b, KSHV-miR-K12-12*, EBV-miR-BART4 and EBV-miR-BHRF1-1) were performed as previously described (See Supplementary Tables S1 and S3) (Bustin et al., 2009; Ferrajoli et al., 2015; Ferrajoli et al., 2013; Muller et al., 2014; Schwarzenbach et al., 2015; Tudor et al., 2014). Briefly, total plasma RNA was reverse transcribed and amplified using the TaqMan®

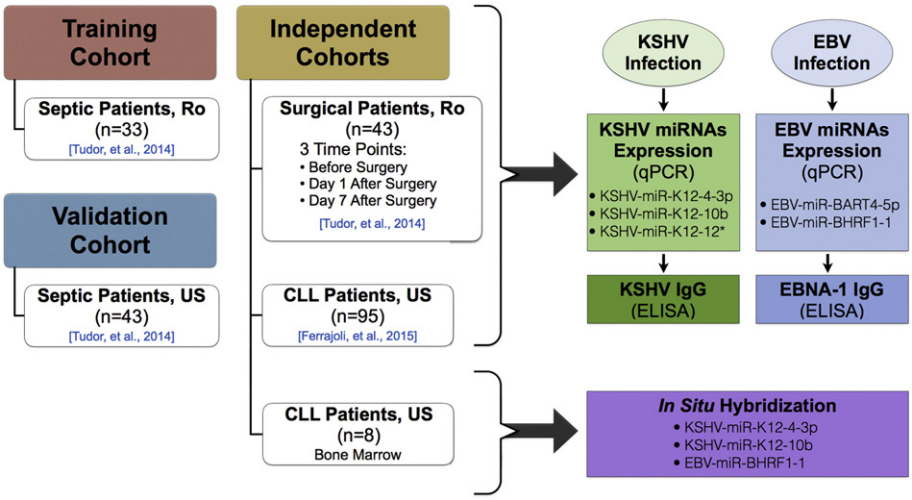

Fig. 1. Schematic representation of the set of patient plasma samples used for the present study. Workflow of the plasma sample collection from four independent patient cohorts and a set of bone marrow samples, and the processing steps followed in the study. Ro: Fundeni Clinical Hospital (FCH), Romania; US: The University of Texas MD Anderson Cancer Center (UT-MDACC), United States.

miRNA Reverse Kit (Applied Biosystems) with primers/probes specific for each miRNA described above using SsoFast™ Probes SuperMix (Bio-Rad Laboratories, Hercules, CA) as previously described (Bustin et al., 2009; Tudor et al., 2014; Ferrajoli et al., 2015). Each amplification was performed in triplicates, Ct values beyond the upper limit of the measuring system were imputed as 40, and the expression levels were considered to be positive for Ct values ≤ 35, according to the MIQE recommendations (Bustin et al., 2009). To confirm this threshold, we used two miRNAs from zebrafish (dre-miR-456 and dre-miR-458) that share no homology to the human genome and also are never ingested in the humans' food (Supplementary Table S4). Supplementary Table S1 contains a summary of all raw profiling data obtained by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and ELISA. In order to identify the detection limits of the method, we selected a mimic viral miRNA for each virus (KSHV-miR-K12-12* and EBV-miR-BART4) and the cellular miRNA hsa-miR-16-5p to perform a RT-qPCR based standard curve of known copy numbers (0, 100, 250, 500, 10^3, 10^4, 5×10^4, 10^5, 10^6, 10^9, 10^{12} copy numbers) to correlate the miRNA copy numbers with corresponding Ct values (Supplementary Fig. S9). Plasma total RNA samples from CD-1® IGS mice were used as negative controls for the presence of the viral miRNAs (Supplementary Table S5).

2.3. Enzyme-Linked Immuno-Sorbent Assay (ELISA)

KSHV/HHV8 IgG ELISA assay (Advanced Biotechnologies, Inc., Eldersburg, MD) was performed according to manufacturer's instructions to evaluate the KSHV/HHV-8 serological status. Epstein-Barr nuclear antigen 1 (EBNA-1) IgG ELISA assay (Diamedix, ERBA Diagnostics, Inc., Miami FL) was performed according to manufacturer's instructions to evaluate the EBV serological status as previously described (Ferrajoli et al., 2015). For reproducibility testing, we measured in two independent days 28 samples randomly selected (Fig. S1).

2.4. miRNA In Situ Hybridization (ISH)

miRNA-ISH for two KSHV/HHV8 miRNAs (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b) and one EBV miRNA (EBV-miR-BHRF1-1) were performed in 8 BM biopsy specimens of patients with CLL as previously described (Ferrajoli et al., 2015). Briefly, double digoxigenin-labeled locked nucleic acid probes (LNA; Exiqon, Vedbaek, Denmark) antisense to the above miRNAs (Fig. 5 and Supplementary Figs. S4 and S8) were hybridized on tissue sections for 3 h at 55 °C. Detection was accomplished with anti-DIG alkaline phosphate Fab fragment followed by nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color development (Ventana, Roche, Basel, CH). U6 probe reactivity was used as positive control, and the scrambled-miRNA probe (Exiqon Vedbaek, Denmark) was used as negative control for staining.

2.5. Statistical Analysis

Chi-square test or Fisher's exact test was used to assess the efficacy of different measurement methods to determine viral infection in different cohorts. To find the relationship between white blood cells (WBC), lymphocytes (LYM) and other clinical parameters and plasma presence/absence of a viral miRNA we first employed a Shapiro-Wilk test and verify that the data doesn't follow a normal distribution. Accordingly, we apply the nonparametric test Mann-Whitney-Wilcoxon to assess the relationship between the presence or absence of viral miRNA and WBC or LYM levels. The nonparametric Kruskal-Wallis test (together with Dunn's post hoc test) was further applied to assess the degree of association between WBC, LYM levels and presence of none, one, two or three viral miRNAs detected. The Fisher's exact test, Shapiro-Wilk test, Mann-Whitney-Wilcoxon nonparametric test, Kruskal-Wallis test, and Dunn's post hoc test analyses were performed in R (version 3.0.1) (http://www.r-project.org/). Chi-square test, Pearson correlation and Spearman correlation analyses were performed in GraphPad Prism (version 6.0h). Statistical significance was defined as a P value less 0.05 ($P < 0.05$).

3. Results

3.1. The Selection of KSHV miRNAs to Measure and the Samples Selection

We investigated the prevalence of KSHV/HHV-8 infection in 300 plasma samples from 214 Caucasian adults from Europe and US based on RT-qPCR measurements of viral miRNAs that are non-orthologous (without complete sequence match) with any human miRNA or any human genome sequence or transcript (Fig. 1). We quantified three miRNAs specific for KSHV viral infection latent phase (KSHV-miR-K12-4-3p) and lytic phase (KSHV-miR-K12-10b and KSHV-miR-K12-12*), respectively, selected according to a microarray screening of expressed cellular and viral miRNAs in human mononuclear cells (Tudor et al., 2014). In the same samples, we further compared the results obtained with an assay commonly used in clinical practice, the KSHV/HHV-8 IgG measured by ELISA, as a measure for seropositivity (Goedert et al., 1997; Spira et al., 2000).

We considered as "positive" the samples where the mean Ct values of the duplicates was ≤35, which is in accordance to the MIQE recommendations (Bustin et al., 2009). As we quantified miRNAs that are not produced by the human genome and therefore should normally not exist in human cells and body fluids, we further confirmed this threshold by testing the levels of two additional non-human miRNAs from zebrafish (dre-miR-456 and dre-miR-458). We selected this specie, as it is never used in the human diet; we obtained for dre-miR-456 a mean Ct value of 38.77 (S.D. of ± 1.31) while for dre-miR-458 we did not identified amplification till 40 cycles (Table S4). The 35 cycles Ct threshold is therefore below two standard deviation (S.D.) of average Ct value of the mean of triplicates for any of these two zebra fish miRNAs in any of the 5 human plasma samples tested for non-specific amplification (Supplementary Table S4). To test the species-specificity for the detection of viral infection, samples from CD-1® IGS mice were used as negative controls for the presence of the viral miRNAs, as EBV and KSHV infect humans, and not mice (Supplementary Table S5). Our results showed no amplification of the viral miRNAs in mouse samples, except for ebv-miR-bart4, that has 100% similarity (with no gaps) for 18 nucleotides in miRNA sequence detected by the TaqMan specific probe with the mouse Nef2 transcript variant 5. Thus, the ebv-miR-bar4 results could be, therefore, considered as false-positive in the mice samples (Supplementary Table S5). These data further support the used threshold of ≤35 cycles. We used human hsa-miR-16, a well expressed miRNA in plasma (Ferrajoli et al., 2013), as a control for RNA quality, and selected only samples with mean Ct < 30 for this transcript for inclusion in this study. We further assessed the presence (Ct ≤ 35) or absence (Ct > 35) of KSHV miRNAs as binary result (Yes/No) in a set of multi-institutional samples collected from patients from two countries (US and Romania).

3.2. The Prevalence of KSHV Infection by Levels of Viral miRNAs is Significantly Higher Than by KSHV IgG ELISA

In the training cohort of patients ($n = 33$ patients with sepsis; Fig. 1) we found that all patients had detectable expression of at least one KSHV-miRNAs (27.27% with one, 39.39% with two and 33.33% with three miRNAs detected), whereas only 36.36% of the patients were found to be seropositive for KSHV/HHV-8 IgG ($P < 0.001$; Fig. 2A, Fig. S2A, Table S6). As a reproducibility test for the ELISA assay, we randomly selected a set of 28 samples and performed the assay in two independent days and obtained highly significant positive correlations (Pearson correlation of 0.952, $P < 0.001$ and a Spearman correlation of 0.861, $P < 0.001$) (Fig. S1). We then performed the same analyses in a validation cohort ($n = 43$ patients with sepsis, Fig. 1) and we found similar results: all patients had detectable expression of KSHV-miRNAs (27.91% with

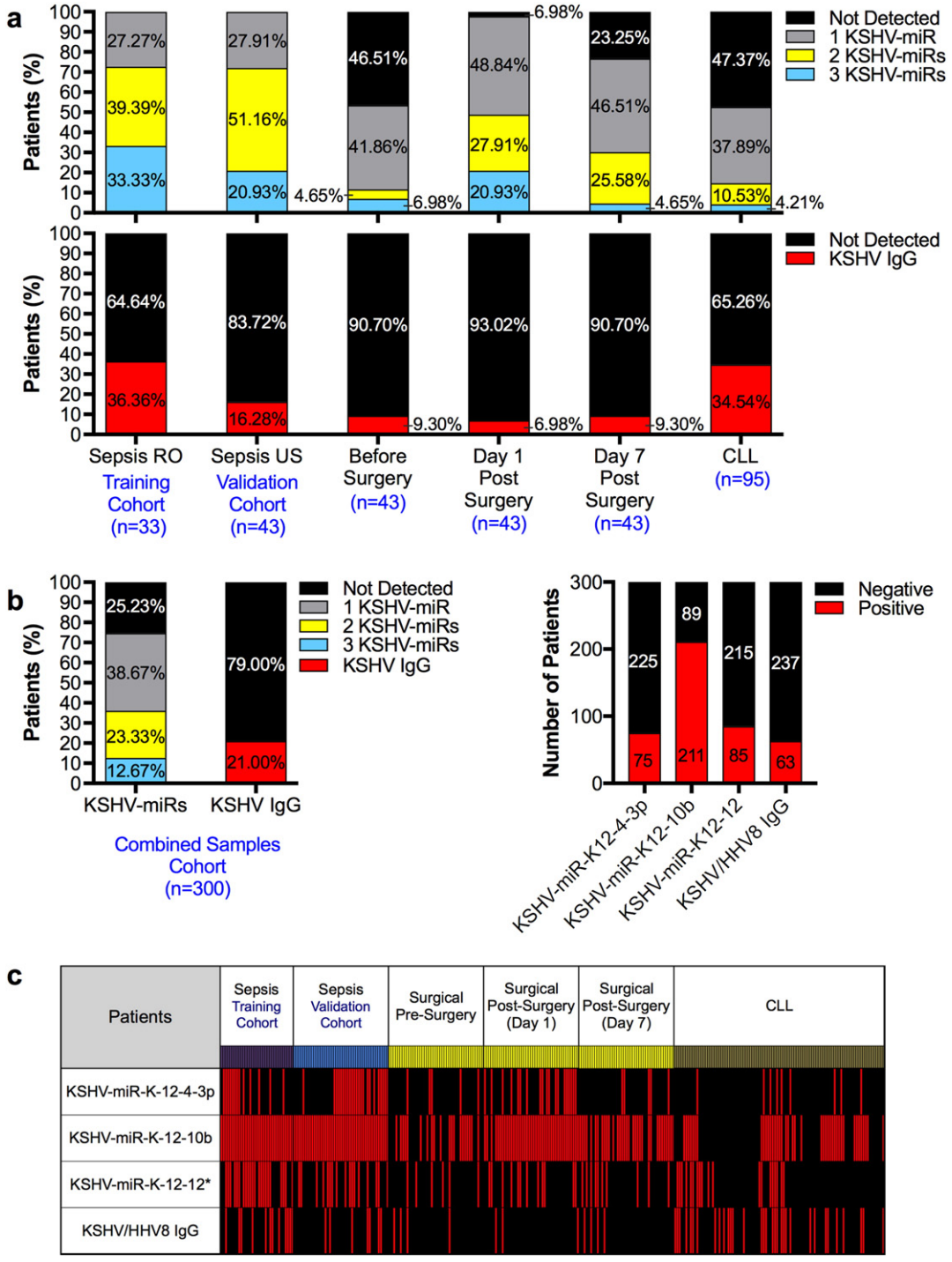

Fig. 2. KSHV miRNAs vs. KSHV/HHV-8 IgG as plasma biomarkers for KSHV infection. a. Percentage of patients positive (Ct value ≤ 35) for the plasma expression of one, two or three KSHV-miRNAs (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b and KSHV-miR-K12-12*) (upper panels). Percentage of patients positive for the plasma KSHV/HHV8 IgG (lower panels). $P < 0.001$ as analyzed with Chi-square test. See individual analysis of the patient cohorts in Table S6 and Table S7. b. Percentage of patients positive (Ct value ≤ 35) for the plasma expression of one, two or three KSHV-miRNAs (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b and KSHV-miR-K12-12*) and percentage of patients positive for the plasma KSHV/HHV8 IgG (left panel). Number of patients (300 plasma samples of 214 patients from four independent cohorts, including 43 surgical patients with samples before surgery, day1 post-surgery and day7 post-surgery) positive (Ct value ≤ 35) for each of the plasma KSHV-miRNAs (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b and KSHV-miR-K12-12*) and HHV-8/HHV8 IgG right panel. See statistical analysis details in Table S8. c. Detection of the plasma KSHV-miRNAs (KSHV-miR-K12-4-3p, KSHV-miR-K12-10b and KSHV-miR-K12-12*) and HHV-8/HHV8 IgG in 300 plasma samples of 214 patients from four independent cohorts (including 43 surgical patients with samples before surgery, day1 post-surgery and day7 post-surgery). Red color = positive for expression (Ct value ≤ 35), black color = negative for expression, and grey color = not determined. See statistical analysis details in Table S8.

one, 51.16% with two and 20.93% with three miRNAs detected), while only 16.28% of the patients were found to be seropositive for KSHV/HHV-8 IgG ($P < 0.001$; Fig. 2A, Fig. S2A, Table S6). Among the individual miRNAs, KSHV-miR-K12-10b was detected in all of patients from both training and validation cohorts ($P < 0.001$; Table S7).

We performed the same measurements in two other independent cohorts, one composed of 43 patients that underwent abdominal surgery for various malignant or non-malignant diseases ($P < 0.001$; Fig. 2A, Fig. S2B, Table S6), and a second consisting of 95 patients with CLL ($P < 0.001$; Fig. 2A, Fig. S2C, Table S6), also showing increased

KSHV prevalence by the detection of the viral miRNAs compared to the measurements of seropositivity for KSHV/HHV-8 IgG. For the surgical patients' cohort, we obtained sequential samples collected one day before surgery, one day post-surgery and one week post-surgery. In surgical cohort we observed detectable expression of at least one KSHV miRNA in 53.48% of the patients (before surgery, $P < 0.001$), 97.67% of the patients (day 1 post-surgery, $P < 0.001$) and 76.74% of the patients (day 7 post-surgery, $P < 0.001$). Only 16.28% of these patients were seropositive for KSHV/HHV-8 IgG (Fig. 2A, Fig. S2B, Table S6). In the independent cohort of CLL patients (Fig. 1), we found that 52.63% of the patients had detectable expression of KSHV-miRNAs (37.89% with one, 10.53% with two and 4.21% with three miRNAs detected), while only 34.74% of the patients were found to be seropositive for KSHV/HHV-8 IgG ($P < 0.001$; Fig. 2A, Fig. S2C, Table S6).

Analysis of all 214 patients from the four independent cohorts showed detectable expression of at least one KSHV-miRNA in 78.50% of the patients (36.45% with one, 26.64% with two and 15.42% with three miRNAs detected), while only 27.57% of the patients were found to be seropositive for KSHV/HHV-8 IgG ($P < 0.001$; Fig. 2B, C, Fig. S3). Analysis of individual KSHV-miRNAs showed that KSHV-miR-K12-10b was most common, detected in 73.83% of the patients ($P < 0.001$; Table S8).

3.3. The Presence of Plasma KSHV miRNAs Correlates with Low WBC and Low Lymphocytes Counts

We further check for correlation between plasma KSHV miRNAs and clinical parameters to strength the translational significance of our findings. The data revealed the presence of viral KSHV-miRNAs in the vast majority of cases with abnormal low WBC count and low lymphocyte count where the ELISA fails to identify the presence of IgG against the KSHV (Fig. 3, Fig. S4, Tables 1 and 2). More precisely, the viral miRNAs were detected in 19 cases out of the 21 cases with abnormal low WBC count (WBC < 4000 cell/μl; 90.48%), and in 26 cases out of the 27 cases with abnormal low lymphocyte count (LYM < 1000 cell/μl; 92.59%). Compared to these results, the detection power of KSHV IgG antigen by ELISA was significantly lower as it was found to be positive in only 3 among the 21 cases with abnormal low WBC count (14.29%; Fisher test $P < 0.001$; Table 1), and in only 5 among the 27 cases with abnormal low lymphocytes count (18.52%; Fisher test $P < 0.001$; Table 2). When analyzed the patients with high WBC or high LYM defined as over the 75 percentile according to data in Fig. 3, we identified that the viral miRNAs were detected in 39 cases out of the 74 cases with either abnormal high WBC count (WBC > 34,075 cell/μl; 52.70%) or with abnormal high lymphocyte count (LYM > 25,422 cell/μl; 52.70%). Compared to these results, the detection power of KSHV IgG antigen by ELISA was

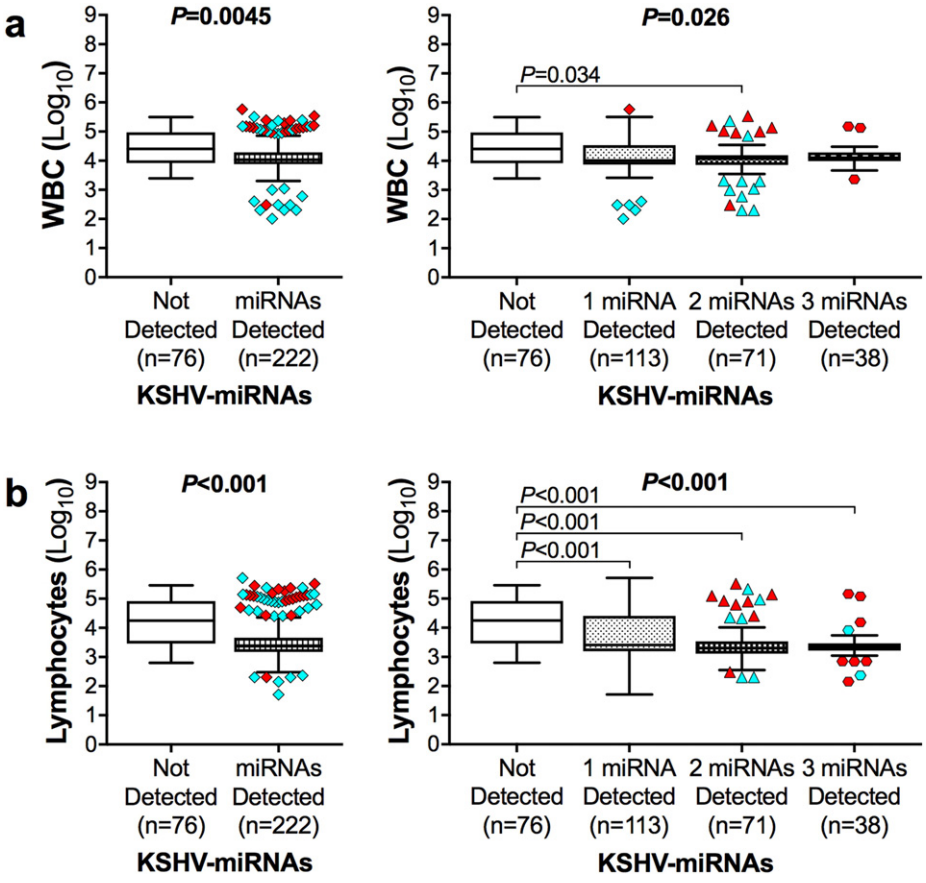

Fig. 3. KSHV-miRNAs prevalence correlated to white blood cells (WBC) and lymphocytes count. a. White blood cells (WBC) count in patients from all independent cohorts, with KSHV-miRNAs not detected ($n = 76$) versus patients with KSHV-miRNAs detected ($n = 222$) ($P = 0.0045$; left panel). WBC count in patients from all independent cohorts, with KSHV-miRNAs not detected ($n = 76$) and in patients with one KSHV-miRNAs detected ($n = 113$), two KSHV-miRNAs detected ($n = 71$), and three KSHV-miRNAs detected ($n = 38$) ($P = 0.026$ as analyzed with Kruskal-Wallis test; right panel). Outliers in cyan color represent samples with KSHV-miRNAs detected, but no KSHV IgG detected, while outliers in red color represent samples with both KSHV-miRNAs and KSHV IgG detected. b. Lymphocytes count in patients from all independent cohorts, with KSHV-miRNAs not detected ($n = 76$) and in patients with KSHV-miRNAs detected ($n = 222$) ($P < 0.001$; left panel). Lymphocytes count in patients from all independent cohorts, with KSHV-miRNAs not detected ($n = 76$) and in patients with one KSHV-miRNAs detected ($n = 113$), two KSHV-miRNAs detected ($n = 71$), and three KSHV-miRNAs detected ($n = 38$) ($P < 0.001$ as analyzed with Kruskal-Wallis test; right panel). Outliers in cyan color represent samples with KSHV-miRNAs detected, but no KSHV IgG detected, while outliers in red color represent samples with both KSHV-miRNAs and KSHV IgG detected.

Table 1
Correlation between KSHV-miRNAs, EBV-miRNAs and ELISA detection in patients with white blood cells (WBC) count < 4000 cell/μl.

Group	Sample	KSHV miRNAs Detected (# of miRs)	KSHV IgG Detected	EBV miRNAs Detected (# of miRs)	EBV IgG Detected	SEX	AGE	WBC (cells/μl)
Sepsis	10US1	Yes (1)	No	Yes (1)	Yes	M	50	100
Sepsis	19US	Yes (1)	No	Yes (1)	Yes	F	66	200
Sepsis	20US	Yes (2)	No	Yes (1)	Yes	M	72	200
Sepsis	33US	Yes (2)	No	Yes (2)	Yes	M	62	200
Sepsis	13US	Yes (1)	No	Yes (1)	No	M	59	300
Sepsis	57US	Yes (2)	Yes	Yes (1)	Yes	F	59	300
Sepsis	60US	Yes (1)	No	No	Yes	M	69	300
Sepsis	12US	Yes (1)	No	Yes (1)	Yes	F	80	400
Sepsis	63US	Yes (2)	No	Yes (1)	Yes	F	55	600
Sepsis	6US	Yes (2)	No	Yes (2)	Yes	M	34	1000
Sepsis	24US	Yes (2)	No	Yes (1)	Yes	F	58	1100
Sepsis	25US1	Yes (2)	No	Yes (1)	Yes	F	64	2000
Sepsis	47US	Yes (2)	No	Yes (1)	Yes	F	52	2000
Sepsis	44US	Yes (2)	No	Yes (2)	Yes	M	79	2100
Sepsis	67US	Yes (3)	Yes	Yes (2)	Yes	F	52	2300
SS43	94	No	No	Yes (1)	Yes	M	27	2500
Sepsis	27RS	Yes (1)	Yes	Yes (2)	No	F	63	2600
Sepsis	15US	Yes (1)	No	Yes (1)	Yes	F	50	3000
SS43	19	Yes (1)	No	Yes (1)	Yes	F	51	3290
Sepsis	22US	Yes (2)	No	Yes (1)	Yes	F	61	3500
SS43	21	No	No	Yes (1)	No	F	51	3550

Blue color = plasma samples in which only KSHV-miRNAs, but no KSHV IgG were detected; Red color = plasma samples in which both KSHV-miRNAs and KSHV IgG were detected; Black color = plasma samples in which none KSHV-miRNAs and KSHV IgG were detected; Light orange background = positive detection; and Light blue background = no detection.

lower but did not reach significance, as it was found to be positive in only 27 among the 74 cases with either high WBC or high lymphocyte count (36.49%; Fisher test $P = 0.069$; Fig. 3 and Table S1). Of note, only 25% of the patients with all 3-tested KSHV-miRNAs positive were also positive by ELISA (9 out of 36 cases).

3.4. EBV miRNAs Detection Identifies Ubiquitous Expression

To test if the finding of significantly higher identification by RT-qPCR is specific for the KSHV/HHV-8 or more general, we selected another member of the gamma-herpesvirus family, the Epstein-Barr virus (EBV) that is reported to infect humans more frequently than KSHV (Young et al., 2016). We also performed RT-qPCR to detect the presence of viral miRNAs (selected after an initial screen by small RNAs deep sequencing as previously described) (Ferrajoli et al., 2015), and compared to EBV seropositivity (defined as detection of EBNA-1 IgG by ELISA) to assess viral infection prevalence (Fig. 4A, Figs. S5 and S6, Tables S9–S11). Analysis of all four independent cohorts together shows detectable expression of at least one EBV-miRNAs in 98.99% of the patients, while 95.97% of the patients tested positive for EBNA-1 IgG ($P = 0.04$, Fig. 4B, C). The data also revealed that significantly more cases with abnormal low lymphocyte count (LYM < 1000 cell/μl; 26 out of 27 cases, 92.59%; Fisher test $P = 0.005$) were positive for the viral EBV-miRNAs compared to EBV IgG antigen positivity by ELISA (17 out of 27 cases, 62.96%; Table 2). When analyzed the patients with high WBC or high LYM defined as over the 75 percentile, we identified that the viral miRNAs were detected in 100% of the 74 cases with either leukopenia or lymphocytopenia, compared to the detection power of EBV IgG antigen by ELISA (66 out of 74 cases, 89.19%; Fisher test $P = 0.006$; Table S1). Of note, 168 out of 178 cases (94.38%) with all 2-tested EBV-miRNAs positive were also positive by ELISA.

3.5. Multiple Validation Methods Confirmed the RT-qPCR Findings

As these data were obtained by using commercially designed and tested assays of qRT-PCR, we then validated our finding by performing In Situ Hybridization (ISH) with a double goal: first, to detect the KSHV/HHV-8 or EBV viral miRNAs by an independent technique using another source, the bone marrow specimens from CLL patients. ISH is based on the detection of the viral miRNA sequences by complementarity with a locked nucleic acid (LNA) probe (Ferrajoli et al., 2015; Nishimura et al., 2013; Silahtaroglu et al., 2004). The second goal was to identify the types of cells harboring the viral miRNAs, being known that BM is a tissue composed by multiple categories of blood cells. Our results showed detection of the viral miRNAs in all 8 BM specimens available in both malignant B cells and megakaryocytes (Fig. 5, Figs. S4 and S7), further confirming the ubiquitous presence of KSHV and EBV infections in humans lymphocytes and other types of cells from blood or other tissues could harbor the KSHV.

In order to further validate our findings, we performed a double blinded experiment using randomly selected samples from the patient cohorts. The results validated the initial RT-qPCR assessment and showed statistically significant correlations between the two independent measurements by different scientists (Supplementary Fig. S8 and Table S12). Despite the wide Ct value range (Ct of 36 to 40) defining the absence of miRNA, we found for all of the miRNAs tested statistical significant correlation between original RT-qPCR results and the blinded experiments performed from the initial step of plasma RNA extraction (Supplementary Fig. S8).

Moreover, the detection limit for the viral miRNAs was similar with the one for the ubiquitous human miR-16: RT-qPCR of the standard curve of known copy numbers (0, 100, 250, 500, 10^3, 10^4, 5×10^4, 10^5, 10^6, 10^9, 10^{12} copy number) of mimic viral miRNA for each virus

Table 2
Correlation between KSHV-miRNAs, EBV-miRNAs and ELISA detection in patients with lymphocytes (LYM) count < 1000 cell/μl.

Group	Sample	KSHV miRNAs Detected (# of miRs)	KSHV IgG Detected	EBV miRNAs Detected (# of miRs)	EBV IgG Detected	SEX	AGE	LYM (cells/μl)
Sepsis	10US1	Yes (1)	No	Yes (1)	Yes	M	50	50
Sepsis	67US	Yes (3)	Yes	Yes (2)	Yes	F	52	140
Sepsis	19US	Yes (1)	No	Yes (1)	Yes	F	66	200
Sepsis	20US	Yes (2)	No	Yes (1)	Yes	M	72	200
Sepsis	33US	Yes (2)	No	Yes (2)	Yes	M	62	200
Sepsis	46US1	Yes (3)	No	Yes (1)	Yes	M	63	230
Sepsis	13US	Yes (1)	No	Yes (1)	No	M	59	300
Sepsis	57US	Yes (2)	Yes	Yes (1)	Yes	F	59	300
Sepsis	60US	Yes (1)	No	No	Yes	M	69	300
Sepsis	6US	Yes (2)	No	Yes (2)	Yes	M	34	350
Sepsis	12US	Yes (1)	No	Yes (1)	Yes	F	80	360
Sepsis	27RS	Yes (1)	Yes	Yes (2)	No	F	63	364
Sepsis	63US	Yes (2)	No	Yes (1)	Yes	F	55	390
Sepsis	31RS1	Yes (1)	Yes	Yes (2)	No	M	65	570
Sepsis	28RS1	Yes (2)	No	Yes (2)	No	M	36	574
SS43	94	No	No	Yes (1)	Yes	M	27	630
Sepsis	20RS1	Yes (2)	No	Yes (2)	No	M	56	661
SS43	123	Yes (3)	No	Yes (2)	No	M	48	690
Sepsis	11RS	Yes (3)	No	Yes (2)	Yes	M	61	700
Sepsis	72US	Yes (3)	No	Yes (2)	Yes	M	60	700
Sepsis	11US	Yes (1)	No	Yes (1)	Yes	M	30	710
SS43	93	No	No	Yes (1)	No	F	48	810
Sepsis	47US	Yes (2)	No	Yes (1)	Yes	F	52	880
Sepsis	56	Yes (1)	No	Yes (1)	No	M	63	900
Sepsis	24US	Yes (2)	No	Yes (1)	Yes	F	58	900
Sepsis	33RS	Yes (2)	Yes	Yes (2)	No	M	60	950
Sepsis	77	Yes (2)	No	Yes (2)	No	M	76	980

Blue color = plasma samples in which only KSHV-miRNAs, but no KSHV IgG were detected; Red color = plasma samples in which both KSHV-miRNAs and KSHV IgG were detected; Black color = plasma samples in which none KSHV-miRNAs and KSHV IgG were detected; Light orange background = positive detection; and Light blue background = no detection.

(KSHV-miR-K12–12* and EBV-miR-BART4) showed that >1000 copies of each viral miRNAs tested, as well as of hsa-miR-16, are necessary to be detected by RT-qPCR at Ct values ≤35 (Supplementary Fig. S9).

4. Discussion

Our data prove that the prevalence of latent preexisting or newly acquired viral infections with viruses such as KSHV/HHV8 is underestimated by the prevalence of seropositivity. A limitation of the serological detection is low sensitivity that can lead to false negative results (Frances et al., 2009; Schatz et al., 2001). The viral miRNAs selection in our study was not random, but instead based on the expression identified by an array profiling followed by functional studies showing direct targeting of Toll-like receptors, that in turns mediate the antiviral immune responses by recognizing virus infection (Tudor et al., 2014). The KSHV miRNAs have no homology with the human genome, and therefore could not generate "false-positive" results from non-specific annealing of the RT probes to the human genome transcripts. Furthermore, each of the five tested viral miRNAs have specific and unique sequences of six nucleotides at 3–prime of the molecule that are used for assay selectivity for the TaqMan miRNA probes (see Supplementary Table S3), and therefore the cross-amplification did not occurs and their quantitative detection can reliably measure KSHV/EBV infection prevalence. Furthermore, we identified the presence of viral miRNAs by two different types of techniques, the RT-qPCR and miRNA ISH, further reducing the possibility of false negative results. Because miRNAs are stable molecules with a degradation half-life longer than viral antigens, they can serve as useful biomarkers in blood samples stored for a long time (Shah and Calin, 2013).

In our previous study on septic patient cohorts also used here, we reported that the sepsis patients had high levels of two KSHV miRNAs (KSHV-miR-K12-10b and KSHV-miR-K12–12*) compared with non-sepsis controls and increases one day after open surgery (Tudor et al., 2014). We further showed in another study, that in CLL patients EBV miRNA BHRF1-1 expression levels were significantly higher in the plasma of patients compared with healthy individuals and observed a correlation between higher BHRF1-1 expression levels and shorter patients survival (Ferrajoli et al., 2015). Patients under stress due to surgery also showed increases in KSHV viral miRNAs and KSHV mRNAs expression levels (Ferrajoli et al., 2015; Tudor et al., 2014). Here our results showed that surgery can uncover those patients presenting false negative results by immune testing profiling, but positive to KSHV/HHV8 infection as shown by the viral miRNAs detection (Fig. 2, Fig. S2, Table S6 and Table S7). All these data support a potential important role of these miRNAs in the pathogeny and poor clinical outcome of sepsis and CLL.

EBV (HHV-4) infection is quite ubiquitous in all human populations (Young et al., 2016). The viral miRNA selection for detection in our study was based on expression array profile combined with the identification of targeting tumor suppressor TP53 (Ferrajoli et al., 2015). As

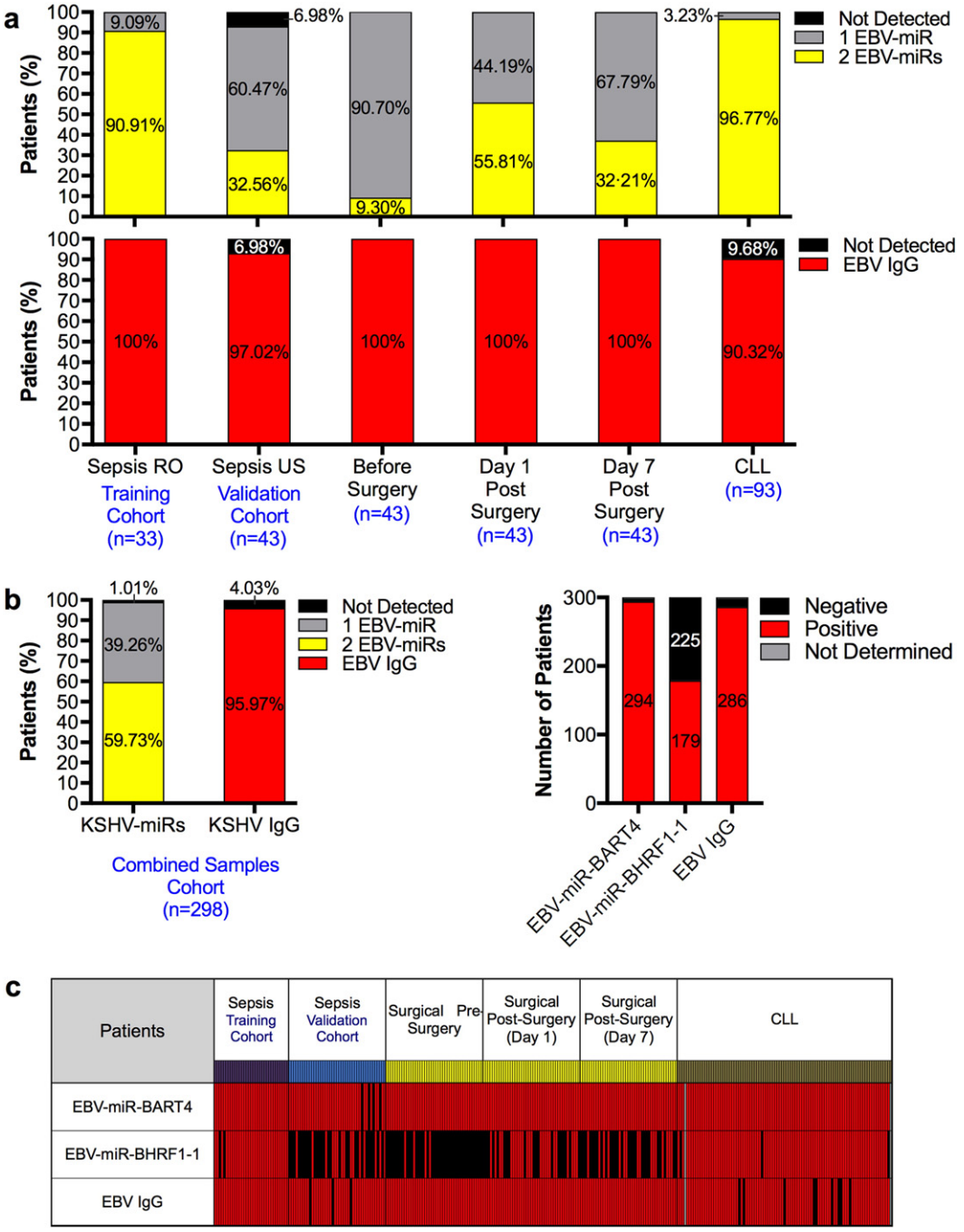

Fig. 4. EBV-miRNAs vs. EBNA-1 IgG immunoassay as plasma biomarkers for EBV infection. a. Percentage of patients positive (Ct value ≤ 35) for the plasma expression of one or two EBV-miRNAs (EBV-miR-BART4 and EBV-miR-BHRF1-1) (upper panel). Percentage of patients positive for the plasma EBNA-1 IgG (bottom panel). $P < 0.001$ as analyzed with Chi-square test. See individual analysis of the patient cohorts in Tables S9 and S10. b. Number of patients (out of 214 patients) positive (Ct value ≤ 35) for each of the plasma EBV-miRNAs (EBV-miR-BART4 and EBV-miR-BHRF1-1) and EBNA-1 IgG. See statistical analysis details in Table S11. c. Detection of the plasma EBV-miRNAs (EBV-miR-BART4 and EBV-miR-BHRF1-1) and EBNA-1 IgG in 214 patients (including 43 surgical patients day 1 post-surgery). Red color = positive for expression (Ct value ≤ 35), black color = negative for expression, and grey color = not determined. See statistical analysis details in Table S11.

seropositivity is >90% in adults, the miRNA RT-qPCR will be useful in determining the presence of EBV infection in immunocompromised states. Of note, if the miRNA detection in multiple human populations by multiple groups in the next future will confirm that quite 100% of human population is EBV-positive this will have a great significance, and consequently the testing of EBV positivity will be performed only in instances where the viral quantification is essential, such as cancer diagnosis and prognosis. In contrast with EBV, KSHV seropositivity is much lower; we showed that latent infection is highly prevalent and KSHV miRNAs can be detected almost ubiquitously after surgery or in the immunocompromised state of sepsis, both of which are conditions that can reactivate latent KSHV infection. Finally, here we identified KSHV infection prevalence via detection of KSHV-miRNAs by RT-qPCR in patients with leukopenia and patients with lymphocytopenia. Detection of KSHV-miRNAs was statistically significant higher compared to the results via ELISA detection of KSHV IgG in these patients. Therefore, our data suggest that the prevalence of KSHV infection is grossly underestimated by immunological methods. Measurement of KSHV-miRNAs by RT-qPCR for KSHV/HHV8 viremia detection may be an important tool for screening the donated blood supply or transplanted organs to prevent the spread of KSHV infection. Our group and other groups have shown that infection with members of the gamma-herpes virus family represent a risk factor

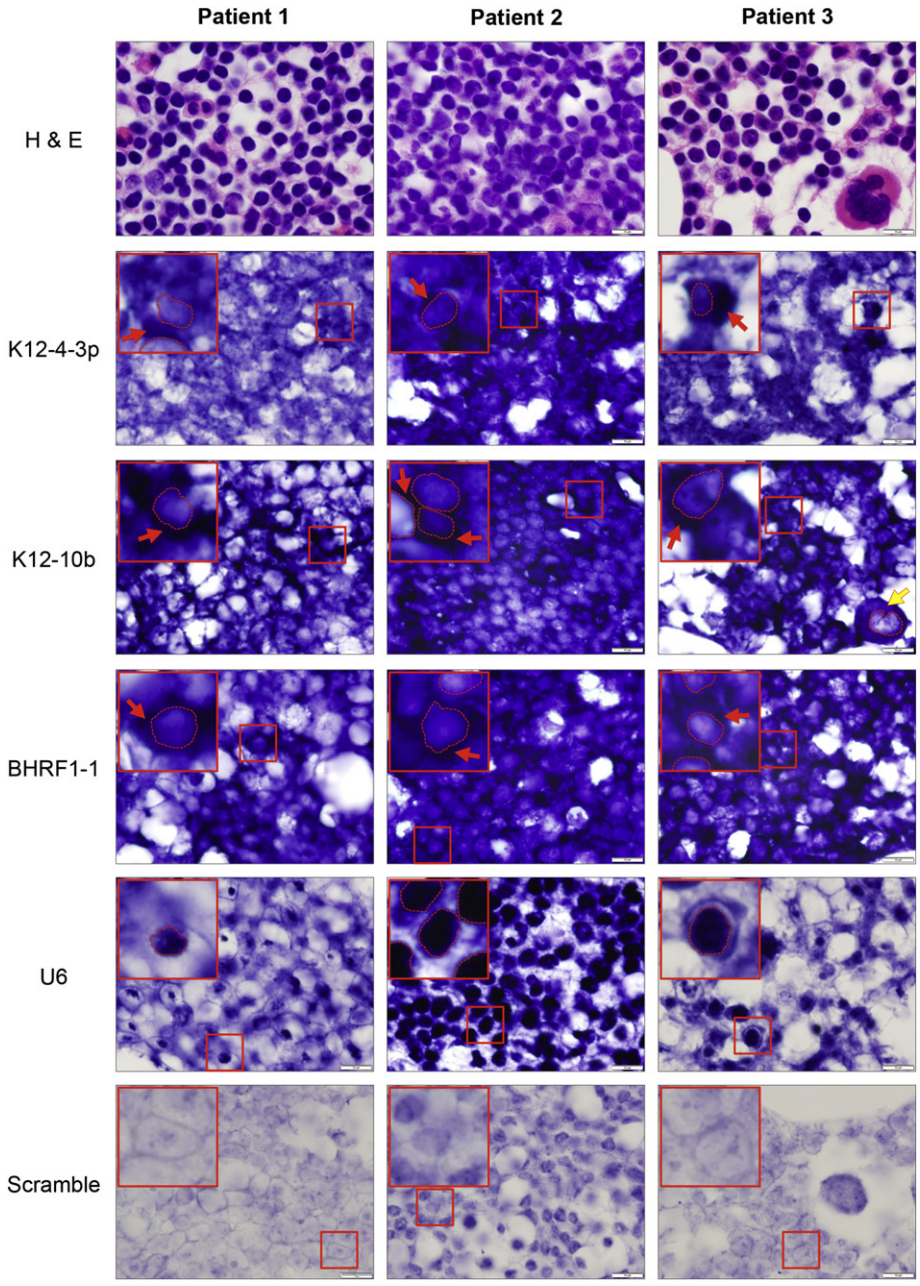

Fig. 5. Detection of viral miRNAs by In Situ *Hybridization* in Bone Marrow Biopsies. In Situ Hybridization (ISH) for the KSHV-miR-K12-4-3p and KSHV-miR-K12-10b, and the EBV-miR-BHRF1-1 were performed in bone marrow tissue samples. Red dashed lines show the nucleus boundaries, red arrows point to cytoplasmic localization of the miRNA in lymphocytes-derived cells, and yellow arrows point to cytoplasmic localization of the miRNA in megakaryocytes. Images were taken at 1000× magnification and the scale bar = 10 μm.

for diseases (e.g., CLL, severe sepsis) and development of infection-related complications. The screening of day 1 post-surgery patients for plasma KSHV-miRNAs could be an application of our study – the identification of high levels of KSHV miRNAs will classify the patient for potential prophylactic large spectrum antibiotic therapy to prevent severe sepsis in case of infections with hospital bacteria.

We tested Caucasian patients in order to increase the statistical power of the study by excluding race as a variable. Moreover, the Caucasian population has a low reported incidence of HHV-8 infection potentially due to underestimation of seropositivity prevalence with current serological detection, which has low sensitivity that can lead to false negative results. Larger prospective studies with thousands of patients from multiple geographic regions and races are needed to fully assess the advantage of using miRNA RT-qPCR with or without ELISA for detection of KSHV infection. This method is suitable to the detection in human body fluids (and not only plasma) of any virus codifying miRNAs (29 viruses according to miRBase at http://www.mirbase.org/). Identification of viral exposure is currently important in the management of patients receiving stem cell transplantation or other highly T-cell immunosuppressive therapies. Clearly, current KSHV/HHV8 serology methods are failing to detect the majority of KSHV latency. Measurement of the viral miRNAs described here allows a direct assessment of the levels of viral derived regulators of the patient's immune system and may present advantages over the current ELISA method. Circulating cell-free nucleic acids in plasma has been previously shown many decades ago (Kamm and Smith, 1972). Circulating miRNAs exhibit remarkable stability in various types of body fluids (e.g., whole blood, serum, plasma, urine, saliva) (Weber et al., 2010), being resistant to plasma RNase activity, as well as to severe physicochemical conditions, such as freeze-thawing and extreme pH (Chen et al., 2008). This can be

explained by their encapsulation into vesicular bodies (e.g., exosomes, microvesicles), or by their binding to RNA-binding proteins (e.g., nucleophosmin 1, HDL, AGO-2) (Arroyo et al., 2011; Vickers et al., 2011; Wang et al., 2010). The reduction of the KSHV miRNAs levels between Day-1 and Day-7 post-surgery can be potentially explained by their translocation from plasma in cells that represent the "sanctuaries" for persistence, such as bone marrow B cells (as we showed by ISH, see Fig. 5 and Supplementary Figs. S4 and S7) or endothelial cells. Moreover, the time/labor cost of this new method can represent about one third of the cost of the current ELISA method for KSHV. Our findings set the ground for the development of KSHV viral infection detection approach by multiplex RT-qPCR detection method of multiple viral miRNAs. This strategy to detect viral infection based on the measurement of viral miRNAs has the potential to become a "gold" standard method in the clinical practice to detect viremia or latency of viruses in the general population and in individuals with immunocompromised states.

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Conflict of Interest Disclosures

The authors declare no conflict of interest.

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Appendix A. Supplementary Data

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