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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 (Ambros, 2003; Arroyo et al., 2011; Bhutani et al., 2015). KSHV is a causative factor for Kaposi sarcoma, primary effusion lymphoma, and some subtypes of multicentric Castleman disease (Boss et al., 2011). 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%) (Bustin et al., 2009; Cai et al., 2005). 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) (Calin 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 (Calin et al., 2005). 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 (Bhutani et al., 2015; Cai et al., 2005).

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 (Chen et al., 2008; Fabbri et al., 2011; Fabris and Calin, 2016; Ferrajoli et al., 2013). Previous studies led to the discovery of virally encoded miRNAs that play important roles in regulating the latent-lytic switch of gamma-Herpesviruses infections (Bhutani et al., 2015; Ferrajoli et al., 2015). 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 (Frances et al., 2009). 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) (Ganem, 2010; Giza et al., 2016; Goedert et al., 1997; Herman et al., 2015; Kamm and Smith, 1972; Levy, 1997; Luppi and Torelli, 1996). These miRNAs remain in circulation in a stable form, being highly resistant to acute changes in pH, endogenous RNase activity, and variations in temperature (Mesri et al., 2010). 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 (Muller et al., 2014; Nishimura et al., 2013). Moreover, we previously reported that higher plasma levels of KSHV miRNAs are associated with a worse clinical outcome in patients with sepsis (Kamm and Smith, 1972). 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 (Giza et al., 2016; Kamm and Smith, 1972; Osmond et al., 2002) (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 ref Kamm and Smith (1972). We used a validation cohort containing 43 patients with sepsis from The University of Texas MD Anderson Cancer Center (UT-MDACC), Houston, Texas, US (Kamm and Smith, 1972). 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 (Kamm and Smith, 1972); and 95 patients with chronic lymphocytic leukemia (CLL) from UT-MDACC as reported previously in refs (Giza et al., 2016; Osmond et al., 2002). 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) (Giza et al., 2016; Kamm and Smith, 1972; Osmond et al., 2002; Rohner et al., 2014; Samols et al., 2005; Schatz et al., 2001). Briefly, total plasma

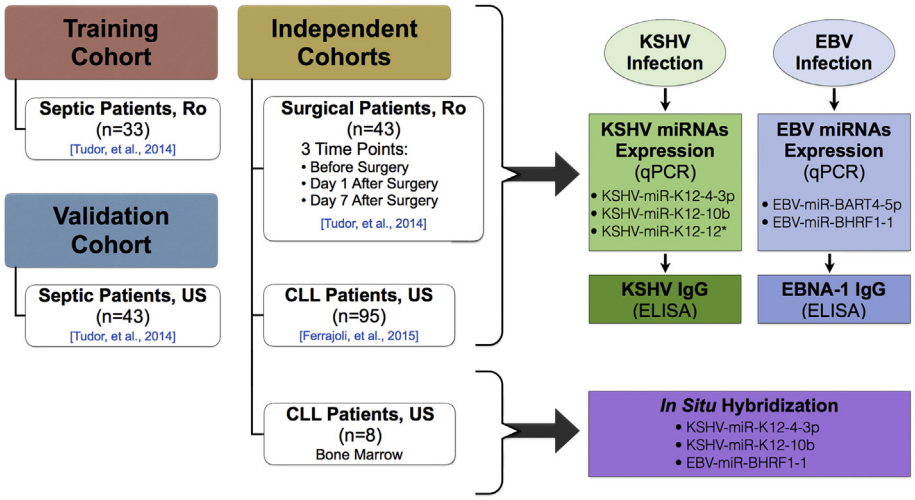

Fig. 1. Schematic representation of the set of patient plasma samples used for the present study. Workflow of the plasma sample collection from four independent patient cohorts and a set of bone marrow samples, and the processing steps followed in the study. Ro: Fundeni Clinical Hospital (FCH), Romania;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

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