Non-coding RNA Research 7 (2022) 114–122

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

Unique circulating microRNA profiles in epidemic Kaposi's sarcoma

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ARTICLE INFO

Keywords:
microRNA
Circulating microRNA
Kaposi's sarcoma
Biomarker
Blood
Serum

ABSTRACT

Background: Human herpesvirus 8 (HHV-8) causes Kaposi's sarcoma (KS). Kaposi sarcoma in HIV/AIDS patients is referred to as epidemic KS and is the most common HIV-related malignancy worldwide. The lack of a diagnostic assay to detect latent and early-stage disease has increased disease morbidity and mortality. Serum miRNAs have previously been used as potential biomarkers of normal physiology and disease. In the current study, we profiled unique serum miRNAs in patients with epidemic KS to generate baseline data to aid in developing a miRNA-based noninvasive biomarker assay for epidemic KS.

Methods: This was a comparative cross-sectional study involving 27 patients with epidemic KS and 27 HIV-positive adults with no prior diagnosis or clinical manifestation of KS. DNA and RNA were isolated from blood and serum collected from study participants. Nested PCR for circulating HHV-8 DNA was performed on the isolated DNA, whereas miRNA library preparation and sequencing for circulating miRNA were performed on the RNA samples. The miRge2 pipeline and EdgeR were used to analyse the sequencing data.

Results: Fifteen out of the 27 epidemic KS-positive subjects (55.6%) tested positive for HHV-8 DNA, whereas only 3 (11.1%) out of the 27 HIV-positive, KS-negative subjects tested positive for HHV-8 DNA. Additionally, we found a unique miRNA expression signature in 49 circulating miRNAs in epidemic KS subjects compared to subjects with no epidemic KS, with 41 miRNAs upregulated and 8 miRNAs downregulated. Subjects with latent KS infection had a differential upregulation of circulating miR-193a compared to HIV-positive, KS-negative subjects for whom circulating HHV-8 DNA was not detected. Further analysis of serum from epidemic KS patients revealed a miRNA signature according to KS tumor status and time since first HIV diagnosis.

Conclusions: This study reveals unique circulating miRNA profiles in the serum of patients with epidemic KS versus HIV-infected subjects with no KS, as well as in subjects with latent KS. Many of the dysregulated miRNAs in epidemic KS patients were previously reported to have crucial roles in KS infection and latency, highlighting their promising roles as potential biomarkers of latent or active KS infection.

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https://doi.org/10.1016/j.ncrna.2022.02.002
Received 19 October 2021; Received in revised form 24 January 2022; Accepted 12 February 2022
Available online 10 April 2022
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1. Background

Kaposi sarcoma (KS) is the most common HIV-related malignancy worldwide and the 3rd most frequently diagnosed cancer among men in sub-Saharan Africa. Uganda has one of the highest rates of KS in the world, partly due to the HIV pandemic, with the incidence of KS in women surpassing that of cervical cancer as the most common female malignancy [1]. Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV8), is the etiological agent of Kaposi's sarcoma, and in vivo, it mainly infects B cells and endothelial cells, impairing the expression of pro- and anticancer genes in these cells. In the majority of infected individuals, the virus may remain latent for years or decades and may only be activated by compromised immunity, as seen in human immune virus (HIV) infection and organ transplantation. Consequently, epidemic Kaposi's sarcoma is only found in patients who have HIV acquired immune deficiency syndrome (HIV/AIDS). In Africa, KS is typically aggressive, and patients with epidemic KS often present late with widely disseminated and rapidly progressive disease [2]. Despite standard treatment with highly active antiretroviral therapy (HAART) and chemotherapy, approximately one-third of these patients succumb to the disease after 2 years [3,4].

This relatively high disease morbidity and mortality partly results from delayed diagnosis arising from a lack of a reliable diagnostic assay for the detection of latent or early-stage disease. Nevertheless, the identification of reliable biomarkers for KS remains a challenge to date. In resource-limited settings, the diagnosis of KS is usually clinical based on the presence of cutaneous or mucosal KS lesions, as well as associated symptoms. The challenge with the clinical approach for KS diagnosis is the fact that KS skin lesions are usually mimicked by other non-KS lesions [5]. Moreover, even though histopathological analysis is considered the gold standard diagnostic test for KS [6], this test is wrought with challenges, such as the prior requirement of a tissue sample acquired from an invasive tissue-biopsy procedure, as well as the subjective interpretation of test results leading to interobserver variability among pathologists [7]. Additionally, histopathological diagnosis can only be performed for patients who have manifested KS-like lesions on the skin or mucosa membranes and thus may not apply to patients with subclinical or latent disease. Similarly, ELISA-based assays used to augment other tests during KS detection have limitations of low specificity and cross-reactivity with other antigens, such as Epstein Bar Virus (EBV) proteins [8]. PCR-based techniques that detect HHV-8 DNA in tissues and serum, albeit highly sensitive, are also limited by low specificity [9], particularly in the viral latent phase.

A novel class of molecules called microRNAs (miRNAs) has recently gained traction in healthcare management for their potential as biomarkers for human diseases [10]. miRNAs are small (19–24 nucleotides), evolutionarily conserved, endogenous noncoding RNA molecules that bind to the 3′UTR of target mRNA transcripts with partial or perfect sequence complementarity, resulting in translational repression and/or mRNA destabilization [11]. During the past decade, our knowledge about the role of miRNAs in human diseases has grown exponentially [11,12]. Abnormal expression of a single miRNA can have implications for the activity of multiple genes [10,11]. It is not surprising, therefore, that changes in miRNA expression have been found to contribute to a wide variety of human disease states and disorders, such as cancer and cardiovascular, autoimmune, neurodegenerative, hepatic, and inflammatory diseases [10]. The utility of miRNAs in diagnostics is derived from their specificity for a particular type of tissue or cell, their abundance, and their remarkable stability in body tissues and fluids such as serum, urine, saliva, milk and cerebrospinal fluid [10,13]. These characteristics demonstrate a remarkable potential for microRNAs to be used as biomarkers for disease. miRNAs have been successfully used to classify cancer, identify cancer tissue origin, determine prognosis and disease progression, predict resistance to chemotherapy, monitor therapy, and screen for disease [13–17]. HHV8 infection or HHV8-related malignancies can induce a unique signature of human and viral intracellular and extracellular miRNAs [18], and this has been confirmed in vitro [19]. These data, although highly suggestive, cannot be extrapolated to epidemic KS due to the additive effects of HIV infection on cellular miRNA signaling.

This study aimed to profile the unique miRNAs found in the serum of patients with epidemic KS by comparing circulating miRNAs in patients with epidemic KS to those of a comparative group of patients with no KS. These data will provide baseline data that will aid in developing a miRNA-based minimally invasive PCR assay for epidemic KS, which would greatly facilitate the detection and management of a disease that has a high rate of recurrence and mortality.

2. Methods

2.1. Study setting and population

This was a comparative cross-sectional study conducted from August 2018–July 2019. Whole blood samples were obtained from 27 HIV-positive adult patients with epidemic Kaposi's sarcoma, confirmed by histopathology at the Uganda Cancer Institute (UCI), and 27 HIV adult patients without clinical manifestations of KS at the Makerere Joint AIDs Program (MJAP) clinic for comparison. The Skin Cancer Clinic at UCI provides care for ambulatory patients with skin cancer. The MJAP clinic is an outpatient facility for ambulatory patients seeking continued HIV care services and provides comprehensive HIV prevention, treatment, care, and support. The ISS clinic provides general care to over 15,000 HIV-infected patients and opens 5 days a week with daily attendance of >300 patients. Pregnant, severely sick, HIV-positive, KS-negative individuals with mucosal and skin lesions that could mimic KS were excluded. The total number of study participants was fifty-four (54), and study participants were purposively sampled. The sample size was determined using Fleiss's equation (1980) with continuity correction, which is commonly used for comparative studies. All patients were taking antiretroviral therapy (ART). Of the 50 participants who had available information regarding their ART medication, 34 were taking combined antiretroviral therapy medicines (cART) consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) in combination with nonnucleoside reverse transcriptase inhibitors (NNRTIs), 6 were taking cART consisting of two NRTIs and protease inhibitors (PIs), and 7 received integrase strand transfer inhibitor (INSTI) therapy-based ART. The proportion of participants taking cART was similar among epidemic KS and non-KS participants. A questionnaire was used to obtain participants' demographic information, including age, sex, and baseline clinical characteristics, such as KS lesion morphotype, presence of edema, disease comorbidities and treatments received at presentation or after KS diagnosis.

Five (5) milliliters of venous blood was collected from study participants in specialized vacutainer tubes, of which 2 mL was stored at 4 °C for DNA isolation, whereas 3 mL was left to stand on ice for 30 min and centrifuged for 20 min at 1300 g to collect serum that was then aliquoted into 2 ml Eppendorf tubes containing RNA later (Thermo Fisher Scientific) and stored at −80 °C for further analysis.

2.2. DNA extraction

DNA was extracted from whole blood using the QIAamp DNA Midi Blood Kit (QIAGEN) in accordance with the manufacturer's instructions. Briefly, 100 μl of the protease enzyme was pipetted into a 15 ml centrifuge tube, and 200 μl of the sample, 800 μl PBS, and 200 μl of Buffer AL were added. The resulting mixture was vortexed for 15 s and then incubated at 70 °C for 10 min to lyse the cells. Then, 1 ml of absolute ethanol was added to the mixture and vortexed to allow DNA precipitation. The resulting mixture was then added to a QIAamp Midi spin column and centrifuged at 8000 rpm for 1 min, and the supernatant was discarded. Two milliliters of wash buffer AW1 was added to the column and centrifuged at 5000 rpm for 1 min, and the supernatant was

discarded. Then, 2 ml of wash buffer AW2 was added to the column and centrifuged at 5000 rpm for 15 min, and the supernatant was discarded. DNA was eluted in 200 µl Buffer AE. The DNA quality was assessed using a NanoDrop™ 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USAs).

2.3. Nested PCR for HHV-8 DNA

Two amplifications were performed by the nested PCR technique. The first amplification targeted a region in the open reading frame (ORF)-26 of HHV-8 as was done previously [20]. Briefly, for the first run, a total reaction mixture of 25 µl was prepared from a mixture of the following: 12.5 µl 1X Master Mix (BioLabs, New England), 1.25 µl of 10 pMol of each sense (5′AGCCGAAAGGATTCCACCAT-3′) and antisense (5′-TCCGTGTTGTCTACGTCCAG-3′) primers (Euro films Genomics, Vienna), 7 µl nuclease-free water and 3 µl sample DNA. During this run, 35 cycles of PCR amplification of HHV-8 DNA were performed on a SimpliAmp™ Thermal cycler (Thermo Fisher Scientific, Applied Biosystems, Singapore). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by further denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 68 °C for 45 s and a final extension step at 68 °C for 10 min. The second PCR run targeted a 211 bp region in the first amplicon. The nested PCR amplification mixture contained 5 µl of the first RCR mixture, 25 µl of 1X master mix, 15 µl of nuclease-free water and 2.5 µl of 10 pMol of each of the internal forward primers (5′TTCCACCATTGTGCTCGAAT-3′) and reverse primers (5′-TACGTCCAGACGATATGTGC-3′). During the run, 35 cycles of amplification were performed under the following conditions: initial denaturation at 95 °C for 5 min, further denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 68 °C for 30 s and a final extension of 10 min at 68 °C. Five microliters (5 µl) of the final PCR product was loaded onto a 1.2% agarose gel and electrophoresed at 120 V for 90 min. Positive reactions yielded an amplicon of 211 bp, which was easily viewed on a UV transilluminator after ethidium bromide staining.

As a positive control, confirmed HHV-8 DNA was used, and a set of negative controls of nuclease-free and negative clinical samples for HHV-8 DNA were used.

2.4. RNA extraction and quality control

Serum total RNA was extracted from 200 µl of serum samples using the Qiagen miRNeasy Serum/Plasma kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions (QIAGEN; Haldane, Germany). RNA abundance and integrity were determined after isolation using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with an RNA integrity number (RIN) > 9 were subjected to subsequent library preparation.

2.5. Library preparation and sequencing

First, small RNA fragments between 18 and 30 nucleotides were extracted from total RNA on a PAGE gel. Then, a 5-adenylated, 3-blocked single-stranded DNA adapter was ligated to the 3′ end of the RNA fragments. Thereafter, reverse transcription (RT) primers with unique molecular identifiers (UMIs) were added to the resulting mixture to enable hybridization to the ligated 3′ adaptors in RNA and dissociative 3′ adapters. Next, 5′ adapters were linked to the 5' end of the resulting ligation product, followed by one-strand cDNA synthesis with RT primers. The resulting cDNA was then amplified by PCR. Fragments between 110 bp and 130 bp of the cDNA library were selected by PAGE electrophoresis, quantified, and then pooled with libraries from other samples. The cDNA libraries were validated using the Agilent Technologies 2100 bioanalyzer and thereafter subjected to high-throughput DNA nanoball sequencing using Phi29 DNA polymerase.

2.6. Sequencing data analysis

After sequencing, data were analysed using the miRge2 pipeline [21] for alignment and quantification of miRNAs, whereas all statistical analyses of differentially expressed miRNAs were conducted in EdgeR [22]. In brief, Fastq files underwent quality control, removal of adapter sequences, and formation of unique reads. Specifically, unique reads were annotated to mature miRNAs, mRNA hairpins, and other RNA types by alignment with miRbase v.22 [23]. Only quantified mature miRNAs were used in subsequent data analysis. Low read counts were filtered out by setting at least five reads as the minimum cut-off. Filtered counts were then normalized and transformed to log2 counts per million (log CPM) using the R package EdgeR. miRNAs with an FC > 1 and FDR <0.05 were considered upregulated, and those with an FC < 1 and FDR <0.05 were considered downregulated. Differentially expressed miRNAs were visualized on volcano plots. Unsupervised clustering was performed by using log2 transformed values and Euclidian distances between samples. All statistical analyses were conducted in the R environment (version 3.6.3).

2.7. Ethics

The study was approved by the Research and Ethics Committee of the School of Biomedical Sciences (SBS-REC) with study number SBS SBS-519 and registered with the Uganda National Council for Science and Technology (UNCST) (study number: HS 2405). Participants' confidentiality was maintained at all times.

3. Results

3.1. Patient demographics

A total of 54 participants participated in this study. Half of the participants (27) had epidemic KS, whereas the other half were HIV-positive with no confirmed diagnosis of KS. All 27 kS disease patients had cutaneous lesions, with most of them localized to the lower extremities. A few of the participants had further manifestations of ulcerated tumors, swollen and painful lymph nodes and palate lesions. Details of the sociodemographic and clinical characteristics of the KS patients in this study are shown in Table 1.

3.2. Differential expression of miRNA

To determine whether patients with epidemic KS differentially expressed miRNAs relative to HIV patients without KS, we performed differential expression testing using EdgeR with normalized read counts (see Table 2). We found that 49 miRNAs were differentially expressed, with 8 miRNAs downregulated and 41 miRNAs upregulated (adjusted p value < 0.05) (Table 2, volcano plot in Fig. 2, and heatmap in Fig. 3).

3.3. Relationship between miRNA expression and the presence or absence of KSHV DNA

Changes in the levels of circulating miRNAs in the serum of humans and animals have been detected as a result of infection with a variety of viruses [24]. Because epidemic KS is caused by HHV-8, in addition to the fact that HHV-8 DNA has been detected in the blood of epidemic KS patients, we examined the effect of the presence of circulating HHV-8 DNA on the serum expression profile of circulating miRNAs. Nested PCR targeting a region in the open reading frame (ORF)-26 of HHV-8 DNA was conducted on DNA extracted from the blood of both epidemic KS patients and the comparative group. Analysis of samples from patients who tested positive for circulating HHV-8 DNA compared to those who tested negative revealed a differential expression of 4 miRNAs that were downregulated and only one miRNA that was upregulated (Table 3 and Fig. 4). Further stratification with regard to

Table 1
Sociodemographic and clinical characteristics of the study participants.

Characteristic		Frequency n (%)
Gender	Male	46 (85)
	Female	8 (15)
Age in years (n = 54)	21–30	5 (19)
	31–40	14 (52)
	41–50	6 (22)
	51–80	2 (7)
Nature of residence (n = 54)	Urban	43 (80)
	Rural	11 (20)
Occupation (n = 54)	Employed	34 (62)
	Unemployed	20 (38)
Educational level (n = 54)	Primary	24 (44)
	Secondary	20 (37)
	Tertiary	7 (13)
	Nonformal	3 (6)
Duration on ART (n = 54)	<6 months	3 (5)
	6–24 months	10 (19)
	24–60 months	22 (41)
	60–120 months	12 (22)
	>120 months	7 (13)
Duration from index KS diagnosis (n = 24)	<3 months	7 (29)
	3–6months	2 (8)
	6–12 months	6 (25)
	>12 months	9 (38)
Form of KS (n = 27)	Skin	27 (100)
	Other	0 (0)
No. of Chemotherapy cycles completed (n = 20)	One cycle	2 (10)
	Two cycles	6 (30)
	Three cycles	3 (15)
	Four cycles	4 (20)
	Five cycles	2 (10)
	Six cycles	3 (15)
Positive HHV-8 DNA (n = 54)	KS (n = 27)	15 (55.6)
	Non-KS (n = 27)	3 (11.1)
Tumor appearance (n = 27)	Ulcerated	5 (19)
Lymph node involvement (n = 27)	Yes	8 (24)
Palate involvement (n = 27)	Yes	6 (22)

epidemic KS status did not reveal any significant differences in the differential expression of circulating miRNA between epidemic KS patients who tested positive for circulating HHV-8 DNA compared to epidemic KS patients who tested negative for HHV-8 DNA (Table 4).

3.4. Differential miRNA expression in latent HHV-8 infection

Three participants from the comparative group (HIV-positive, KS negative) tested positive for HHV-8 DNA on nested PCR, indicating that they could be latently infected with KS. Consequently, serum miRNAs from these patients were compared to those of HIV-positive, KS-negative patients who tested negative for HHV-8 DNA. Analysis of differential miRNA expression revealed a significant upregulation of miR-193a-5p in latently infected HHV-8 patients (Table 5).

3.5. Differential miRNA expression and duration from first HIV diagnosis

Participants were stratified into four (4) groups when they were diagnosed with HIV (i.e., 0–2 years, 2–5 years, 5–10 years, and above 10 years). Consequently, the differential expression of circulating miRNAs was compared between these different groups. Analysis revealed significant differences in miRNA expression between patients diagnosed HIV positive 0–2 years and 2–5 years ago (Table 6). Between these two groups, 4 circulating miRNAs were downregulated, whereas 20 circulating miRNAs were upregulated (volcano plot in Fig. 5). There were no significant differences in circulating miRNA expression when the remaining categories were compared.

Table 2
Differentially expressed miRNAs in patients with epidemic KS compared to HIV patients with no KS.

miRNA	Fold change (log10)	Up/Down regulation	p value	Adjusted p value
hsa-miR-4446-3p	4.84849	Up	2.50E-05	0.00317
hsa-miR-451a	−2.003	Down	3.72E-05	0.00317
hsa-miR-99b-5p	1.24184	Up	4.50E-05	0.00317
hsa-miR-411-5p	4.18601	Up	7.14E-05	0.00376
hsa-miR-1304-3p	5.29579	Up	9.94E-05	0.00419
hsa-miR-4732-3p	−1.9894	down	0.00017	0.00445
hsa-miR-6819-3p	4.98167	UP	0.0002	0.00445
hsa-miR-654-5p	4.60077	Up	0.0002	0.00445
hsa-miR-93-5p	−1.4618	Down	0.00022	0.00445
hsa-miR-1908-5p	4.45843	Up	0.00023	0.00445
hsa-miR-337-3p	3.05436	Up	0.00023	0.00445
hsa-miR-431-5p	4.28894	Up	0.0003	0.00535
hsa-miR-223-5p	3.92606	Up	0.0004	0.00637
hsa-miR-139-3p	1.44008	Up	0.00044	0.00637
hsa-miR-654-3p	3.76475	Up	0.00047	0.00637
hsa-let-7e-5p	1.86566	Up	0.00048	0.00637
hsa-miR-487a-5p	4.14846	Up	0.00052	0.00649
hsa-miR-379-5p	4.16306	Up	0.00068	0.00758
hsa-miR-486-5p	−4.0858	Down	0.0007	0.00758
hsa-miR-369-5p	1.97987	Up	0.00075	0.00758
hsa-miR-222-3p	1.27732	Up	0.00075	0.00758
hsa-miR-191-3p	3.75839	Up	0.0009	0.00863
hsa-miR-382-5p	3.87227	Up	0.00097	0.00885
hsa-miR-409-3p	2.61324	Up	0.00122	0.01071
hsa-miR-584-5p	2.56752	Up	0.00176	0.01487
hsa-miR-154-5p	3.4633	Up	0.00244	0.01972
hsa-miR-1307-3p	1.04218	Up	0.00252	0.01972
hsa-miR-221-3p	3.34441	Up	0.00314	0.02296
hsa-miR-4433b-5p	1.73024	Up	0.00316	0.02296
hsa-miR-185-5p	−1.7193	Down	0.00346	0.02396
hsa-let-7e-3p	3.45092	Up	0.00352	0.02396
hsa-miR-625-3p	3.57103	Up	0.00428	0.0274
hsa-miR-323b-3p	3.10619	Up	0.00429	0.0274
hsa-miR-328-3p	1.67445	Up	0.00491	0.02964
hsa-miR-199a-3p/199b-3p	0.81156	Up	0.00499	0.02964
hsa-miR-375-3p	−1.3701	Down	0.00506	0.02964
hsa-miR-139-5p	0.97288	Up	0.00561	0.03199
hsa-miR-485-5p	2.36411	Up	0.00666	0.03602
hsa-miR-126-3p	0.70315	Up	0.00678	0.03602
hsa-miR-25-3p	−1.0357	Down	0.00683	0.03602
hsa-miR-424-3p	3.15035	Up	0.00719	0.03698
hsa-miR-146a-5p	0.83632	Up	0.00815	0.04088
hsa-miR-224-5p	2.16699	Up	0.00833	0.04088
hsa-miR-7-5p	−0.9513	Down	0.00933	0.04475
hsa-miR-340-3p	2.71832	Up	0.00981	0.04559
hsa-miR-323a-5p	3.26012	Up	0.00994	0.04559
hsa-miR-323a-3p	3.20691	Up	0.01045	0.04619
hsa-miR-425-3p	2.72079	Up	0.01051	0.04619
hsa-miR-127-3p	2.05625	Up	0.0108	0.04651

4. Discussion

Serum miRNAs have been proposed as potential biomarkers of normal physiology and disease, and serum microRNA signatures are currently used as diagnostic and prognostic markers of disease in

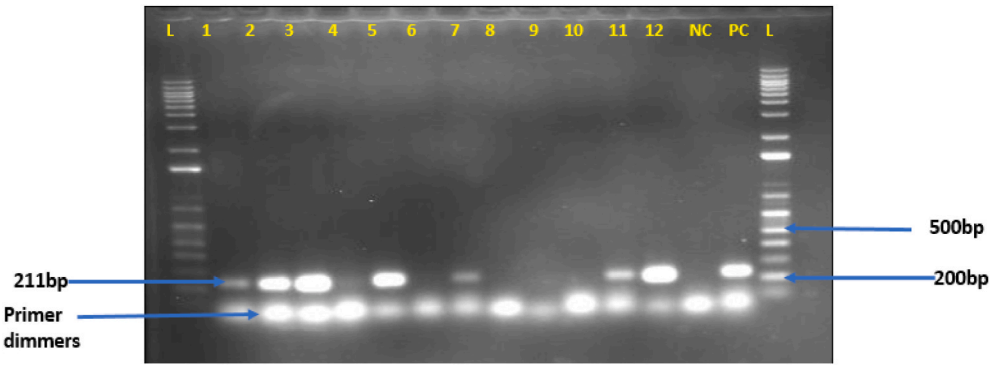

Fig. 1. Gel electrophoresis image after amplification of HHV 8 DNA. **Legend:** Lanes labeled L - 1000 bp DNA ladder. Lane NC - negative control, lane PC positive control. Lanes 1, 2, 3, 5, 7, 11, and 12 – are from samples positive for HHV 8 DNA. Lanes 4, 6, 8, 9, and 10 – are from samples negative for HHV 8 DNA.

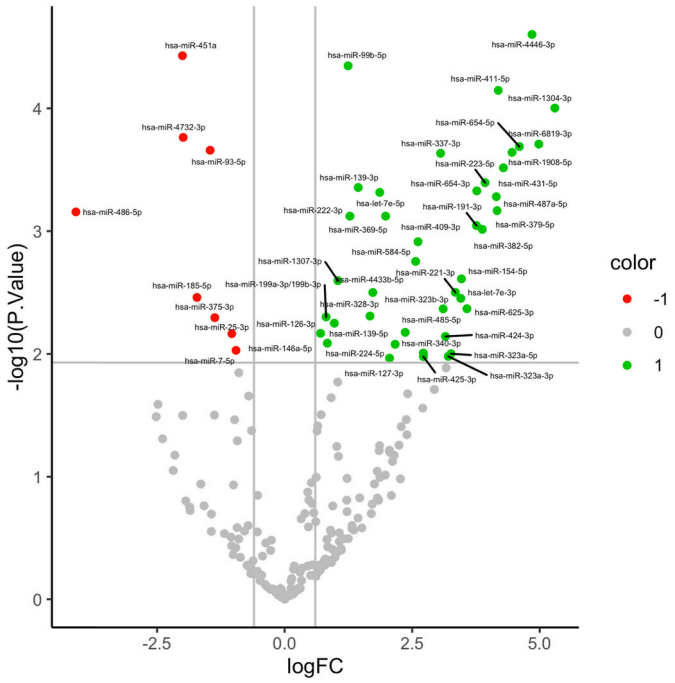

Fig. 2. Volcano plot of miRNA differentially expressed in patients with KS verse those no KS. Legend: The plot is depicted with vertical and horizontal lines indicating the threshold for a relative expression fold change of 2 0r −2 compared with no KS (controls), at a 0.05 P value. Down regulated miRNAs are depicted in red, whereas upregulated miRNAs are depicted in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

prostate cancer, renal cell carcinoma, and lung cancer. In the current study, we profiled the unique miRNAs found in the serum of patients with epidemic KS by comparing circulating miRNAs in patients with epidemic KS to those of a comparative group of patients with no KS. Additionally, we analysed the associations between the level of expression of dysregulated miRNAs and participant clinical data. We found a unique miRNA expression signature in 49 miRNAs when comparing serum collected from patients with epidemic KS to those with no epidemic KS. Moreover, three participants from the comparative group who tested positive for HHV-8 DNA (latent KS infection) had differentially upregulated circulating levels of miR-193a compared to their counterparts (HIV-positive, HHV-8 DNA negative). Further analysis of the serum of epidemic KS patients revealed a miRNA signature according to KS tumor status (1 downregulated miRNA) and time since first HIV diagnosis (1 upregulated and 4 downregulated miRNAs). However, no differential expression was found when comparing chemotherapeutic cycles, nature of KS lesions and duration since the first KS diagnosis in the epidemic KS patient group.

miRNAs are prime candidates for use as noninvasive biomarkers in molecular diagnostics of disease [25]. The utility of miRNAs in diagnostics is derived from their specificity for a particular type of tissue or cell, their abundance, and their remarkable stability in body tissues and fluids such as serum, urine, saliva, milk and cerebrospinal fluid [10,13]. Although the precise roles of circulating miRNAs are still largely unknown, they have been found to be stable and survive conditions such as extreme variations in pH, boiling, multiple freeze thaw cycles, extended storage, and degradation from RNAse enzymes [25,26]. In contrast to miRNAs, common RNA species, such as messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA), are degraded within several seconds after being placed in a nuclease-rich extracellular environment [27,28]. In certain cancers, such as non-small-cell lung cancer, differential expression of circulating miRNAs has been observed at different stages of disease, thereby contributing to diagnosis, treatment and prognosis [26,29]. In the current study, we determined the differential expression profile of circulating miRNAs by comparing the serum miRNA expression profiles of patients with epidemic KS to those of a comparative group of HIV-positive patients with no KS. We found a unique miRNA expression signature in 49 miRNAs in patients with epidemic KS compared to those with no epidemic KS, with 41 miRNAs upregulated and 8 miRNAs downregulated. The top 10 significantly upregulated miRNAs were miR-4446-3p, miR-411-5p, miR-1304-3p, miR-6819-3p, miR-654-5p, miR-1908-5p, miR-431-5p, miR-223-5p, miR487a-5p, and miR-379-5p. The significantly downregulated miRNAs were miR-451a, miR-4732-3p, miR-93-5p, miR-486-5p, miR-185-5p, miR-375-3p, miR-25-3p, and miR-7-5p. These findings imply that the above patterns of differential expression of circulating miRNAs could be used to diagnose epidemic KS in HIV-infected individuals.

The life cycle of HHV-8 consists of latent and lytic replication phases. During the latent phase of infection, only a limited number of HHV-8 genes are expressed to promote persistent infection, evade host immune responses, and induce HHV-8-related malignancies, such as KS [30]. In vitro studies conducted in cell lines derived from HHV-8-associated tumors have implicated the HHV-8 viral miRNA miR-k12-11 as a key player in reprogramming naive B cells to support long-term latency. In the current study, three out of the 29 kS-negative, HIV-positive subjects in the comparison group tested positive for HHV-8 DNA, pointing to possible latent HHV-8 infection. Differential profiling of circulating miRNA in the latently infected individuals in our study revealed a significant upregulation in the expression of miR-193a-5p compared to HIV-positive, HHV-8 PCR-negative participants. This could imply that circulating levels of miR-193a-5p could be used as a noninvasive biomarker of latent epidemic KS infection. Indeed, miR-193a-5p has been reported to be upregulated in primary lymphatic

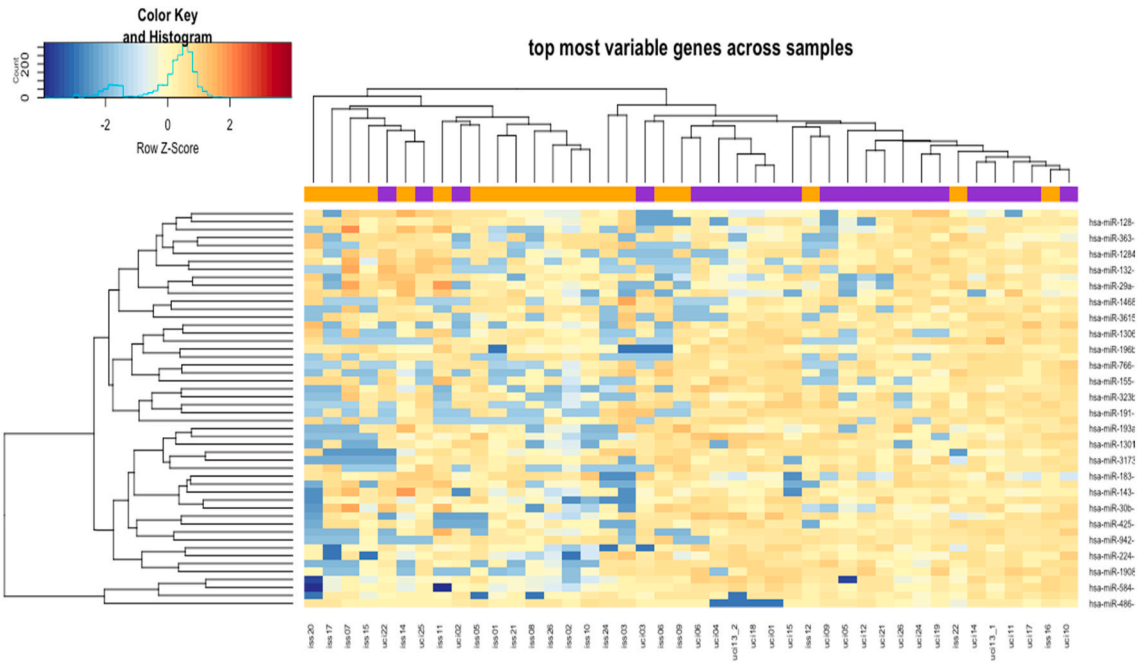

Fig. 3. The most variable circulating miRNAs expressed in patients with and without Epidemic KS
Legend: Z score expression heatmap of the most variable circulating miRNAs expressed in patients with and without epidemic KS. Patients with epidemic KS are denoted as *uci,* whereas the comparative group of HIV-positive patients with no KS are denoted as *iss*.

Table 3
Differential circulating miRNA expression analysis in participants testing positive or negative for HHV-8 DNA.

miRNA	Fold change (log10)	Up/Down regulation	p value	Adjusted p value
hsa-miR-451a	1.930507	Up	2.72E-04	0.02273
hsa-miR-6819-3p	−5.610377	Down	1.50E-05	0.00316
hsa-miR-654-3p	−3.967188	Down	3.35E-04	0.02273
hsa-miR-139-3p	−1.411567	Down	4.31E-04	0.02273
hsa-miR-1304-3p	−4.57006	Down	1.06E-03	0.04470

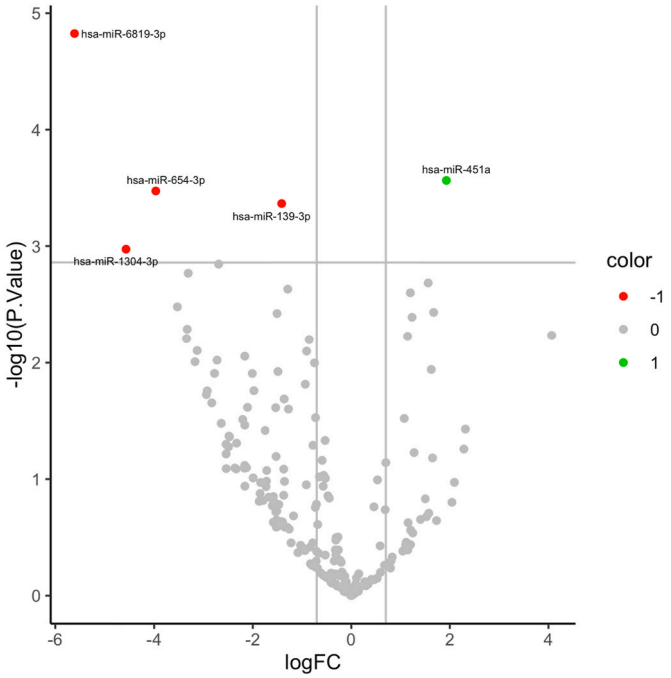

Fig. 4. Differential circulating miRNAs expressed in participants testing positive or negative for HHV-8 DNA
Legend: Volcano plot showing differentially expressed miRNAs in patients testing positive or negative for HHV-8 DNA. miRNAs differentially expressed with an adjusted p value < 0.005 are depicted in red for down regulation and green for up regulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

endothelial cells after 72 h of HHV-8 infection [31], further strengthening the fact that they could be used as early disease biomarkers in KS. miR-193a-5p has also been reported to be upregulated in other cancers, such as prostate cancer [32] and hepatocellular carcinoma [16], as well as during hypoxic conditions [33].

miRNAs are actively secreted via "exosomes". Exosomes are small vesicles released from cells, into which miRNAs are specifically sorted and accumulate. Exosomes help to protect circulating miRNAs from degradation by RNases in the extracellular environment [34]. Host-encoded miRNAs have previously been found in exosomes released from KSHV-infected lymphoma cell lines [19]. Indeed, some of the dysregulated miRNAs in patients with epidemic KS in the current study have been reported to be expressed in KS endothelial tumor cells. Specifically, miR-146a-5p, miR-199a-3p, and miR-126-3p, which we found to be upregulated in serum collected from patients with epidemic KS in our study, were also upregulated in tumor specimens collected from patients with both classical and epidemic KS in earlier studies [35,36], implying that they originated in KS tumor lesions. Regarding the roles of these miRNAs in KS pathophysiology, KSHV infection activates the transcription factor nuclear factor kappa B (NFκB), which is believed to upregulate the expression of miR-146. miR-146a then downregulates the chemokine ligand CXCR4, which promotes the release of KSHV-infected endothelial cells into the circulation [37]. miR-126a promotes the growth of KS by inducing the expression of vascular endothelial growth factor-A (VEGF-A), whereas miR-199a promotes KS

Table 4
Differentially expressed circulating miRNAs in epidemic KS patients testing positive or negative for HHV-8 DNA.

miRNA	Fold change (log10)	Up/Down regulation	p value	Adjusted p value
hsa-miR-150-5p	−1.9696311	n.s	0.00314924	0.6644893
hsa-miR-16-5p	4.4026519	n.s	0.00901365	0.9509403
hsa-miR-1301-3p	4.1115834	n.s	0.01907221	0.9774167
hsa-miR-363-3p	3.8036035	n.s	0.01995521	0.9774167
hsa-miR-486-5p	−3.6520209	n.s	0.02860547	0.9774167
hsa-miR-15b-5p	3.5675304	n.s	0.03616976	0.9774167
hsa-miR-25-3p	3.3827188	n.s	0.04592067	0.9774167
hsa-miR-532-5p	0.9929975	n.s	0.05097679	0.9774167
hsa-miR-451a	2.5280154	n.s	0.05651414	0.9774167
hsa-miR-29a-3p	−1.2541687	n.s	0.05970539	0.9774167

Table 5
Differentially expressed circulating miRNAs in KS-negative patients testing positive or negative for HHV-8 DNA.

miRNA	Fold change (log10)	Up/Down regulation	p value	Adjusted p value
hsa-miR-193a-5p*	**6.565646**	**Up**	**2.13E-06**	**0.00026667**
hsa-miR-10a-5p	2.786356	n.s	1.25E-03	0.05386547
hsa-miR-99a-5p	3.72719	n.s	1.42E-03	0.05386547
hsa-miR-143-3p	4.498237	n.s	1.72E-03	0.05386547
hsa-miR-375-3p	2.964285	n.s	2.64E-03	0.06611451
hsa-miR-483-3p	4.872215	n.s	6.32E-03	0.1217676
hsa-miR-942-5p	−8.418699	n.s	6.82E-03	0.1217676
hsa-miR-122-5p	2.75821	n.s	2.59E-02	0.37944146
hsa-miR-10b-5p	3.901264	n.s	2.73E-02	0.37944146
hsa-miR-100-5p	3.984967	n.s	3.10E-02	0.38552523

*Indicates significantly dysregulated miRNA, n.s- Not significant.

development by inducing the proliferation and survival of endothelial cells [38].

During human immunodeficiency virus type 1 (HIV-1) infection, host miRNA profiles are altered either as a host response against the virus, as a mechanism by which the virus facilitates viral replication and infection, or to maintain latency [39]. Moreover, it has been shown that HIV-infected subjects have unique circulating miRNA profiles compared to HIV-uninfected persons. However, it is currently unknown whether this pattern of differential expression remains constant during the life-cycle of HIV infection. In the current study, we compared the differential miRNA expression patterns at different time points from index HIV diagnosis up to a period of more than 10 years after index HIV diagnosis. We found an upregulation of 20 differentially expressed miRNAs, as well as downregulation of four miRNAs in patients diagnosed with HIV in 0–2 years of the study compared to 2–5 years from index HIV diagnosis at the time of the study. The 10 most significantly upregulated miRNAs were miR-1307-3p, miR-151a-5p, miR-151b-5p, miR490-5p, miR-222-3p, miR-6819-3p, miR-487a-5p, miR-154-5p, miR-431-5p, and miR-224-5p, whereas miR-451a, miR-486-5p, miR-150-5p, and miR-4732-3p were significantly downregulated. Among the upregulated miRNAs, miR-151 has previously been reported as an early biomarker of HIV-1 infection in a panel of circulating miRNAs that distinguished HIV-1-infected individuals from healthy HIV-negative controls [40]. Conversely, downregulation of circulating miR-150, as described in the current study, was previously reported to predict HIV/AIDS disease progression and therapy. miR-150 is a key regulator of immune cell differentiation and activation and is expressed in monocytes, as well as in mature and resting B and T lymphocytes [41]. Additionally, differential coexpression of miR-150 in plasma has been used to identify cognitive impairment in HIV-infected patients [42]. These results add to the existing findings that HIV infection alters the differential expression of circulating miRNAs.

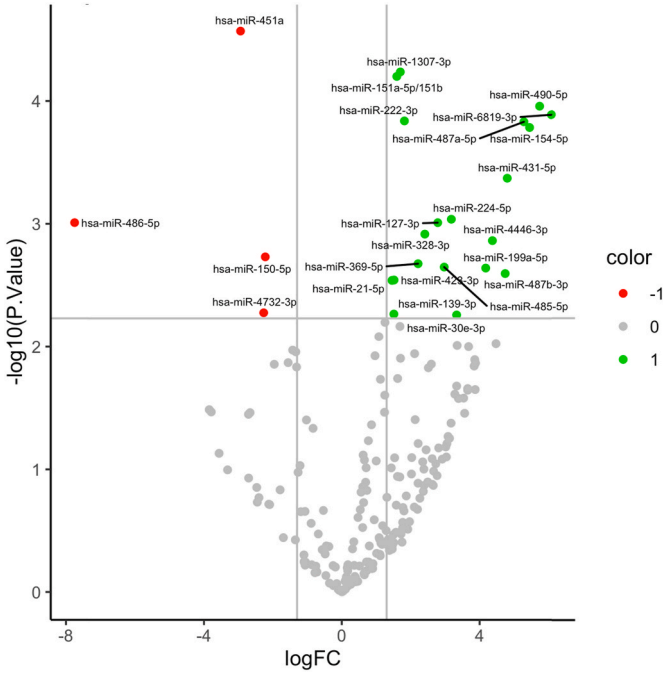

Fig. 5. Differential miRNA expression in patients 0–2 years versus 2–5 years after index HIV diagnosis. **Legend:** Volcano plot showing differential miRNA expression in patients 0–2 years versus 2–5 years after index HIV diagnosis. Differentially expressed miRNAs with an adjusted p value < 0.005 are depicted in red for downregulation and green for upregulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Number of differentially upregulated or downregulated miRNAs at different time points from index HIV diagnosis.

Time since HIV Diagnosis (Years)							
Expression	0–2 Vs 2-5	Expression	0–2 Vs 2-5	Expression	0–2 Vs 2-5	Expression	0–2 Vs 2-5
Down	4	Down	4	Down	4	Down	4
Not significant	187	Not significant	187	Not significant	187	Not significant	187
up	20	up	20	up	20	up	20

The use of highly active antiretroviral therapy (HAART) reduces the incidence of epidemic KS in HIV-positive, KS-negative individuals. In individuals with epidemic KS, the use of HAART alone can lead to the resolution of KS [43]. In addition to HAART, patients with epidemic KS at the Uganda Cancer Institute are treated with chemotherapy consisting of a combination of bleomycin and vincristine for varying treatment cycles, depending on the patients' response. Although miRNAs have previously been used to predict the response to chemotherapy in pancreatic ductal adenocarcinoma, colorectal cancer, and osteosarcoma (reviewed by Ref. [26]), we did not find any differential circulating miRNA expression when we compared epidemic KS patients who had received only three or fewer cycles of chemotherapy to epidemic KS patients who had received more than 3 cycles of chemotherapy. Taken together, these results imply that circulating miRNAs are poor predictors of chemo-response or chemo-resistance in epidemic KS.

Our results are encouraging, as they advance the potential of circulating miRNAs as biomarkers for the diagnosis of epidemic KS. As with all observational studies, causality cannot be inferred from this study. Thus, further work is needed, using a larger, controlled prospective study to fully validate the differential circulating miRNA expression patterns observed in the current study. Moreover, the differential fold expression results for circulating miRNAs in the current study have to be validated by real-time polymerase chain reaction (RT–PCR). Additionally, the methodology adopted for the current study does not provide insights into the causes of the differential expression of the circulating miRNAs observed, which could have resulted from factors other than epidemic KS.

5. Conclusions

In conclusion, this study reveals unique circulating miRNA profiles in the serum of patients with epidemic KS versus HIV-infected subjects with no KS, as well as in subjects with latent KS. Many of the dysregulated miRNAs in epidemic KS patients were previously reported to have potential roles in KS infection and latency, highlighting their promising roles as potential biomarkers of latent or active KS infection. Additionally, duration from index HIV diagnosis also significantly contributed to alterations in the differential expression of circulating miRNAs in all study subjects.

Ethics approval and consent to participate

This research was carried out in compliance with research principles adopted from the Helsinki Declaration, and approval to conduct the study was obtained from the Institutional Review Board of **Makerere University, College of Health Sciences, School of Biomedical Sciences Research and Ethics Committee (SBS-REC) (Protocol number: SBS-519)**. Written informed consent was obtained from all study participants.

Consent for publication

Not applicable.

Availability of data and material

The datasets used are available from the corresponding author on reasonable request.

Funding

This work is part of a research fellowship supported by Grant Number D43TW010132 supported by the Office of the Director, National Institutes of Health (OD), National Institute of Dental and Craniofacial Research (NIDCR), National Institute of Neurological Disorders and Stroke (NINDS), National Heart, Lung, and Blood Institute (NHLBI), Fogarty International Center (FIC), National Institute on Minority Health and Health Disparities (NIMHD), and the Microbiology and Immunology Training in HIV and related infection in Uganda (MITHU) fellowship under the Uganda Case Western Research collaboration. The funding agencies were not involved in the design, data collection, analysis, and interpretation of data or in writing the manuscript. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the supporting offices.

CRediT authorship contribution statement

Haruna Muwonge: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Hassan Kasujja:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Project administration. **Nixon Niyonzima:** Conceptualization, Writing – review & editing. **Carolyne Atugonza:** Methodology, Software, Formal analysis, Writing – review & editing. **Josephine Kasolo:** Writing – review & editing. **Allan Lugaajju:** Writing – review & editing. **Joshua Nfambi:** Writing – review & editing. **Sembajwe Larry Fred:** Writing – review & editing. **Ali Moses Damani:** Writing – review & editing. **Ivan Kimuli:** Writing – review & editing. **Robert Zavuga:** Writing – review & editing. **Faith Nakazzi:** Methodology, Investigation, Writing – review & editing. **Edgar Kigozi:** Methodology, Validation, Investigation, Writing – review & editing. **Damalie Nakanjako:** Conceptualization, Writing – review & editing, Supervision. **David Patrick Kateete:** Conceptualization, Writing – review & editing, Supervision. **Freddie Bwanga:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

The authors deeply appreciate the study subjects for agreeing and volunteering time to participate in this study. The authors also appreciate the contributions Mr. Ashaba Fred made to arrange for experimental procedures to be conducted for this study. We would also like to thank Seeding Labs for providing access to American Journal Experts (AJE) English language editing services.

LIST OF ABBREVIATIONS

3′UTR	three prime untranslated region
AIDS	Acquired immune deficiency syndrome
ART	Anti-retroviral therapy
cART	Combined Antiretroviral Therapy
cDNA	Complementary DNA
DNA	deoxyribonucleic acid
EBV	Epstein Bar Virus
ELISA	Enzyme-linked Immunosorbent Assay
FC	Fold change
FDR	False discovery rate
HAART	Highly Active Antiretroviral Therapy
HHV-8	Human Herpes Virus 8
HIV	Human Immune Virus
INSTI	Integrase Strand Transfer Inhibitor
ISS clinic	Immune suppression syndrome clinic
KS	Kaposi's sarcoma
KSHV	Kaposi's Sarcoma-associated Herpesvirus
log CPM	log2 counts per million
miRNA	microRNA
mRNA	messenger RNA
NFκB	nuclear factor kappa B

NNRTI	Nonnucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
ORF	Open Reading Frame
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PI	Protease Inhibitors
RIN	RNA Integrity Number
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT primers	Reverse Transcription primers
SBS-REC	School of Biomedical Sciences Research Ethics Committee
tRNA	transfer RNA
UCI	Uganda Cancer Institute
UMIs	Unique Molecular Identifiers
UNCST	Uganda National Council for Science and Technology
VEGF-A	Vascular Endothelial Growth Factor-A

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