Unique Circulating microRNA Profiles in Epidemic Kaposi's Sarcoma

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Abstract

Background

The 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. 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 the unique serum miRNAs in patients with epidemic KS to generate baseline data to aid in developing a miRNA-based non-invasive 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 respectively. Nested PCR for circulating HHV-8 DNA was performed on the isolated DNA, whereas miRNA library preparation and sequencing for circulating miRNA was performed on the RNA samples. The miRge2 pipeline and EdgeR were used to analyze 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 down regulated. 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.

Background

Kaposi sarcoma (KS) is the most common HIV-related malignancy worldwide and the 3rd most frequently diagnosed cancer amongst 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 (Phipps et al., 2010). 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 anti-cancer genes in these cells. In Majority of infected individuals, the virus may remain latent for years or decades, only to be activated by compromised immunity, such 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 (Mosam et al., 2012), and despite standard treatment with highly active antiretroviral therapy (HAART) and chemotherapy, about one-third of these patients succumb to the disease after 2 years (Freeman et al., 2016; Okuku et al., 2017).

This relatively high disease morbidity and mortality partly results from delayed diagnosis arising from a lack of a reliable diagnostic assay for detection of latent or early stage disease. Nevertheless, the identification of reliable biomarkers for KS remains a challenge to-date. In resource-limited settings, 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 (Yaqub et al., 2019). Moreover, even though histopathological analysis is considered the gold standard diagnostic test for KS (Corey Casper, Martin S Hirsch, & Bloom, 2019), 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 inter-observer variability among pathologists (Amerson et al., 2016). 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 sub-clinical 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 (Ablashi, Chatlynne, Whitman, & Cesarman, 2002). PCR-based techniques that detect HHV-8 DNA in tissues and serum, albeit highly sensitive, are also limited by low specificity (Auten, Kim, Bradley, & Rosado, 2017), particularly in the viral latent phase.

A novel class of molecules called microRNAs (miRNAs) have recently gained traction in healthcare management for their potential as biomarkers for human diseases (Dave et al., 2019). miRNAs are small (19-24 nucleotides), evolutionary conserved, endogenous non-coding 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 (O'Brien, Hayder, Zayed, & Peng, 2018). During the past decade, our knowledge about the role of miRNAs in human diseases has grown exponentially (Gilad et al., 2012; O'Brien et al., 2018). Abnormal expression of a single miRNA can have implications on the activity of multiple genes (Dave et al., 2019; O'Brien et al., 2018). It's 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, cardiovascular, autoimmune, neurodegenerative, hepatic, and inflammatory diseases (Dave et al., 2019). The utility of miRNA's in diagnostics is derived from their specificity for a

particular type of tissue or cell, their abundance, as well as their remarkable stability in body tissues and fluids such as serum, urine, saliva, milk and cerebrospinal fluid (Dave et al., 2019; Gustafson, Tyryshkin, & Renwick, 2016). 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 (Ahmed et al., 2009; Calin et al., 2005; Eitan et al., 2009; Gustafson et al., 2016; Ji et al., 2009). HHV8 infection or HHV8-related malignancies can induce a unique signature of human and viral intracellular and extracellular miRNAs (Qin, Peruzzi, Reiss, & Dai, 2014), and this has been confirmed in vitro studies (Hoshina et al., 2016). These data though highly suggestive, cannot be extrapolated to epidemic KS, due to the additive effects of HIV-infection on cellular miRNA signaling.

This study aimed at profiling the unique miRNAs found in 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 Kaposi's Sarcoma, which would greatly facilitate the detection and management of a disease that has a high rate of recurrence and mortality.

Methods

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 manifestation of KS at 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. The pregnant, severely sick, and 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 the Fleiss's equation (1980) with continuity correction, commonly used for comparative studies. All patients were taking anti-retroviral 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 a non-nucleoside reverse transcriptase inhibitors (NNRTI), 6 were taking cART consisting a two NRTIs and a protease inhibitors (PI), whereas 7 received an Integrase Strand Transfer Inhibitor (INSTI) therapy based ART. The proportion of participants taking cART was similar among Epidemic KS and none-KS participants. A questionnaire was used to obtain participants demographic information, including age, gender, as well as base line 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 were collected from study participants in specialized vacutainer tubes, of which 2mls were stored at 4^0C for DNA isolation, whereas 3mls were left to stand on ice for 30 minutes, centrifuged for 20 minutes at 1300g to collect serum that was then aliquoted in 2ml Eppendorf tubes containing RNA later (Thermo Fisher Scientific), and stored at -80^0C for further analysis.

DNA extraction

DNA was extracted from whole blood using the QIAamp DNA midi blood kit (QIAGEN) in accordance with the manufacturer's instructions. Briefly, 100µl of the Protease enzyme was pipetted into a 15ml Centrifuge tube and 200µl of the sample, 800µl PBS, and 200µl of Buffer AL added. The resulting mixture was vortexed for 15 seconds, and then incubated at 70°C for 10 minutes to lyse the cells. 1 ml of absolute ethanol was then added to the mixture and vortexed to allow DNA precipitation. The resulting mixture was then added to the QIAamp Midi spin column, centrifuged at 8000 rpm for 1 minute and the supernatant discarded. 2ml of Wash buffer AW1 was added to the column and the centrifuged at 5000rpm for 1 minute and the supernatant discarded. Then, 2ml of Wash buffer AW2 was added to the column, centrifuged at 5000rpm for 15 minute and the supernatant was discarded. DNA was eluted in 200µl Buffer AE. The DNA quality was assessed using the NanoDrop^{TM} 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USAs)

Nested PCR for HHV-8 DNA

Two amplifications were done 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 (Machado, Farias, Pereira, Freitas, & Fonseca, 2015). Briefly, for the first run, a total reaction mixture of 25µl was prepared from a mixture of the following; 12.5µl 1X Master Mix (BioLabs, New England), 1.25µl of 10pMol of each sense (5'AGCCGAAAGGATTCCACCAT-3') and antisense (5'-TCCGTGTTGTCTACGTCCAG-3') primers (Euro films Genomics, Vienna), 7µl nuclease free water and 3µl sample DNA. During this run, 35 cycles of PCR amplification of HHV-8 DNA was done on a SimpliAmp^{TM} Thermal cycler (Thermo Fisher Scientific, Applied Biosystems, Singapore). The PCR conditions were as follows; initial denaturation at 95 °C for 5 minutes, followed by further denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, extension at 68°C for 45 seconds and a final extension step at 68 °C for 10 minutes. The second PCR run targeted a 211bp region in the first amplicon. The nested PCR amplification mixture contained 5µl of the first RCR mixture, 25µl of the 1X master mix, 15µl of nuclease free water and 2.5µl of 10pMol of each of the internal forward primer (5'TTCCACCATTGTGCTCGAAT-3') and reverse primer (5'-TACGTCCAGACGATATGTGC-3'). During the run, 35 cycles of amplification were done under the following conditions; initial denaturation at 95°C for 5 minutes, further denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 68 °C for 30 seconds and a final extension of 10 minutes at 68°C. Five microlitres (5µl) of the final PCR product were loaded into a 1.2% agarose gel, and

electrophoresed at 120V for 90 minutes. Positive reactions yielded an amplicon of 211 bp, which was easily viewed on a UV trans-illuminator after ethidium bromide staining.

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

RNA extraction and quality control

Serum total RNA was extracted from 200µl of serum samples using the Qiagen miRNeasy Serum/Plasma kit (QIAGEN, Valencia, CA, USA) according to 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

Library preparation and sequencing

First, small RNA fragments between 18 to 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 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 110bp to 130bp 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.

Sequencing data analysis

After Sequencing, data was analyzed using the miRge2 pipeline (Lu, Baras, & Halushka, 2018) for alignment and quantification of miRNAs, whereas all statistical analyses of differentially expressed miRNAs were conducted in EdgeR (Robinson, McCarthy, & Smyth, 2010). In brief, Fastq files underwent quality control, removal of adapters sequences, and formation of unique reads. Specifically, unique reads were annotated to mature miRNAs, mRNA hairpins, and other RNA types by alignment toward miRbase v.22 (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006). Only quantified mature miRNAs were used in subsequent data analysis. Low read counts were filtered out 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 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 downregulated. Differentially expressed miRNAs were visualized on volcano plots. Unsupervised clustering was performed by using log2 transformed values and using Euclidian distances between samples. All statistical analysis was conducted in R environment (version 3.6.3).

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 also registered with the Uganda National Council for Science and Technology (UNCST); (study number: HS 2405). Participants' confidentiality was maintained at all times.

Results

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 the 27 KS diseased 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 KS patients in this study are shown in table 1.

Table 1. Sociodemographic and Clinical characteristics of 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)
	Non-formal	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)	<3months	7 (29)
	3-6months	2 (8)
	6-12months	6 (25)
	>12months	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)

Differential expression of miRNA

To determine whether patients with Epidemic Kaposi's sarcoma differentially express miRNAs relative to HIV patients without Kaposi's sarcoma, we performed differential expression testing using EdgeR, using normalized read counts. 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 *figure 2,* and heatmap in *figure 3*).

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

Relationship between miRNA expression and 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 (Stenfeldt et al., 2017). 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 presence of circulating HHV-8 DNA on the serum expression profile of circulating

miRNAs. A 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 that tested positive for circulating HHV-8 DNA compared to those that tested negative revealed a differential expression of 4 miRNAs that were downregulated and only one miRNA that was up regulated (*table 3* and *figure 4*). Further stratification with regards to 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 that tested negative for HHV-8 DNA (*table 4*).

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

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

Differential miRNA expression in latent HHV-8 infection.

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

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				

Differential miRNA expression and duration from first HIV Diagnosis

Participants were stratified in to four (4) groups from when they were diagnosed with HIV (i.e., 0-2 years, 2-5 years, 5-10 years, and above 10 years). Consequently, 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 down regulated, whereas 20 circulating miRNAs where up regulated (volcano plot in *figure 5*). There were no significant differences in the circulating miRNA expression when the remaining categories were compared.

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

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 prostate cancer, renal cell carcinoma, and lung cancer. In the current study, we profiled the unique miRNAs found in 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 analyzed 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) differentially upregulated circulating levels of miR-193a compared to their counterparts (HIV-positive, HHV-8 DNA negative). Further analysis of 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 down regulated 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 non-invasive biomarkers in molecular diagnostics of disease (Blondal et al., 2013). The utility of miRNA's in diagnostics is derived from their specificity for a particular type of tissue or cell, their abundance, as well as their remarkable stability in body tissues and fluids such as serum, urine, saliva, milk and cerebrospinal fluid (Dave et al., 2019; Gustafson et al., 2016). Even though the precise roles of circulating miRNA 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, and extended storage, as well as degradation from RNAse enzymes. (Blondal et al., 2013; Wang, Peng, Wang, Qin, & Xue, 2018). In contrast to miRNAs, common RNA species like messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) are degraded within several seconds after being placed in a nuclease rich extracellular environment (Chen et al., 2008; Turchinovich, Samatov, Tonevitsky, & Burwinkel, 2013). 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 (Wang et al., 2018; Yu, Guan, Cuk, Zhang, & Brenner, 2019). In the current study, we determined the differential expression profile of circulating miRNA by comparing the serum miRNA

expression profiles of patients with Epidemic KS to that 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 down regulated. 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 the infection, only a limited number of HHV-8 genes are expressed in a bid to promote persistent infection, evade host immune responses, and induce HHV-8-related malignancies, such as KS (Ye, Lei, & Gao, 2011). 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 reprograming naïve B-cells towards supporting 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 none-invasive biomarker of latent epidemic KS infection. Indeed miR-193a-5p has been reported to be upregulated in primary lymphatic endothelial cells with in 72hrs of HHV-8 infection (Lagos et al., 2010), 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 (Yang et al., 2017) and hepatocellular carcinoma (Ji et al., 2009), as well as during hypoxic conditions (Viollet et al., 2017).

miRNAs are actively secreted via "exosomes". Exosomes are small vesicles released from cells, into which miRNAs are specifically sorted and accumulated. Exosomes help to protect circulating miRNA from degradation by RNases in the extracellular environment (Sohel, 2016). Host-encoded miRNAs have previously been found in exosomes released from KSHV-infected lymphoma cell lines (Hoshina et al., 2016). Indeed, some of the dysregulated miRNA 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 that 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 (Punj et al., 2010; Wu et al., 2015), implying that they originated in the KS tumor lesions. Regarding the roles of these miRNA in KS pathophysiology, KSHV infection activates the transcription factor nuclear factor kappa B (NFκB), which is believed to up-regulate the expression of miR-146. miR-146a then down-regulates the chemokine ligand CXCR4, which promotes the release of KSHV-infected endothelial cells into the circulation (Hussein & Akula, 2017). miR-126a is believed to promote growth of KS by inducing the expression of Vascular Endothelial Growth Factor -A (VEGF-A), whereas miR-199a promotes KS development by inducing the proliferation and survival of endothelial cells (Shatseva, Lee, Deng, & Yang, 2011).

During human immunodeficiency virus type 1 (HIV-1) infection, host miRNA profiles are altered either as a host response against the virus or as a mechanism for the virus to facilitate viral replication and infection, or to maintain latency (Su et al., 2018). Moreover, it has been shown that HIV-infected subjects have unique circulating miRNA profiles compared to HIV-uninfected persons. However, what is currently unknown is whether this pattern of differential expression stays constant during the lifecycle 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 down regulation of four miRNAs in patients diagnosed with 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 miRNA 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. From amongst 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 health HIV-negative controls (Qi et al., 2017). 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 (Munshi, Panda, Holla, Rewari, & Jameel, 2014). Additionally, differential co-expression of miR-150 in plasma has been used to identify cognitive impairment in HIV-infected patients (Kadri et al., 2016). These results add to the existing findings that HIV infection alters the differential expression of circulating miRNAs.

Use of highly active antiretroviral therapy (HAART) reduces the incidence of epidemic KS in HIV positive, KS negative individuals. In individuals with epidemic KS, use of HAART alone can lead to resolution of KS (Stebbing, Portsmouth, & Gazzard, 2003). 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 response to chemotherapy in pancreatic ductal adenocarcinoma, colorectal cancer, and osteosarcoma (Review by (Wang et al., 2018)), we did not find any differential circulating miRNA expression when we compared epidemic KS patients who had received only three or less cycles of chemotherapy, compared 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 diagnosis of epidemic Kaposi's sarcoma. As it is 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.

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.

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 Non-nucleoside 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

Declarations

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 the **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.

Competing interests

The authors declare that they have no competing interests.

Funding

Author's contributions

HM, HK, DN, DPK, and FB contributed to study conceptualization and design. HM, HK and FN conducted the laboratory analysis. CA conducted bioinformatics analysis on sequencing data for the study, and contributed to manuscript writing and revision. HM wrote the first draft of the manuscript. JK, AL, IK, JN, SLF, AMD, DN, DPK, FB, and FS contributed critical revisions and important intellectual content to the manuscript. All authors read and approved the final manuscript.

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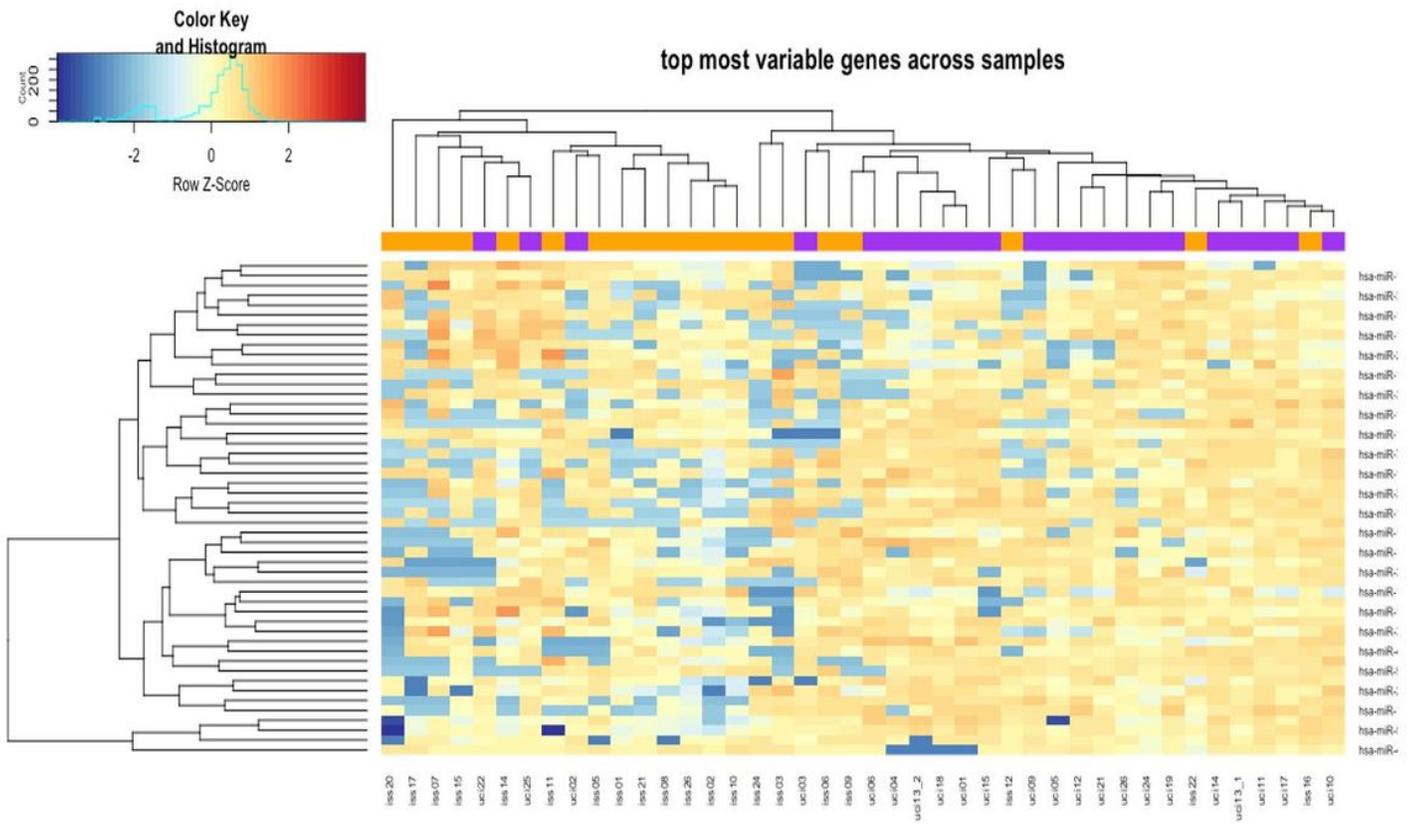
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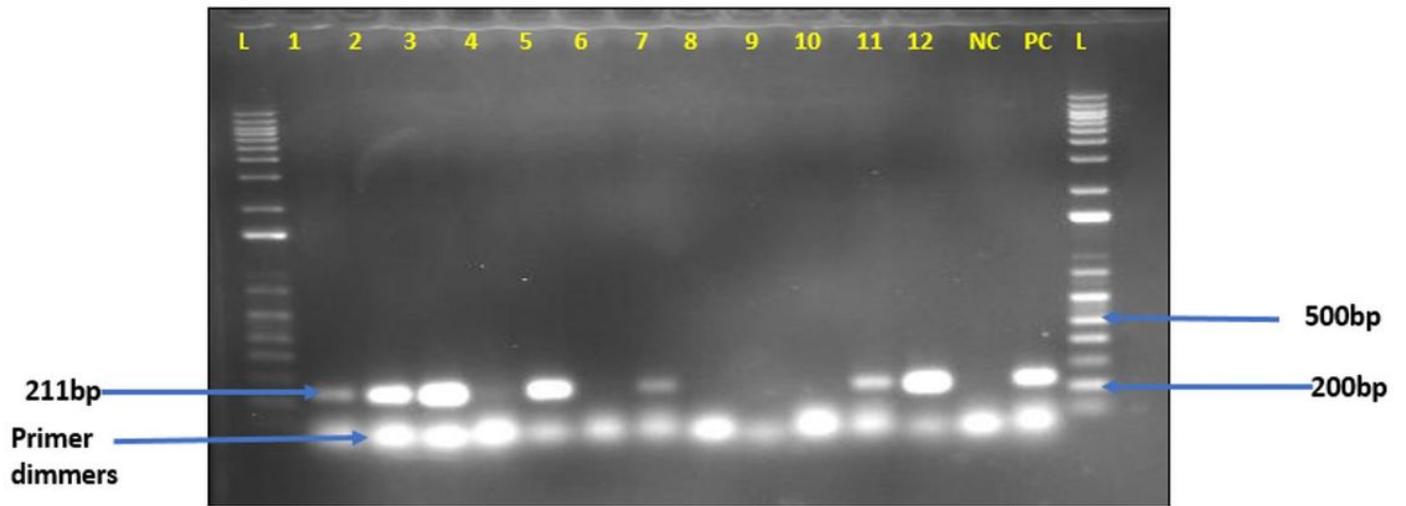
Figure 1

Gel electrophoresis image after amplification for the HHV 8 DNA. Legend: Lanes labeled L - 1000bp DNA ladder. Lane NC - negative control, while 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.
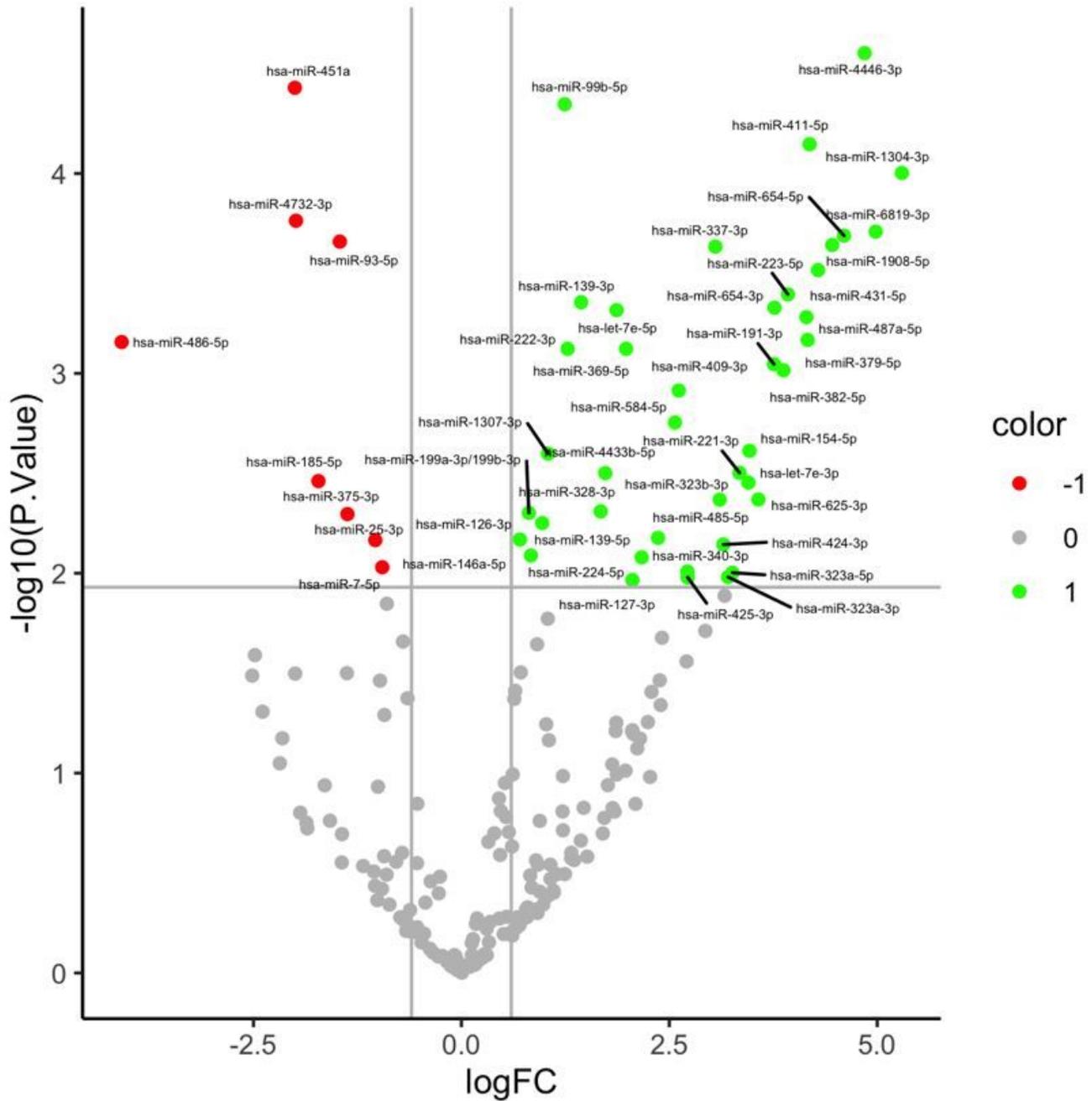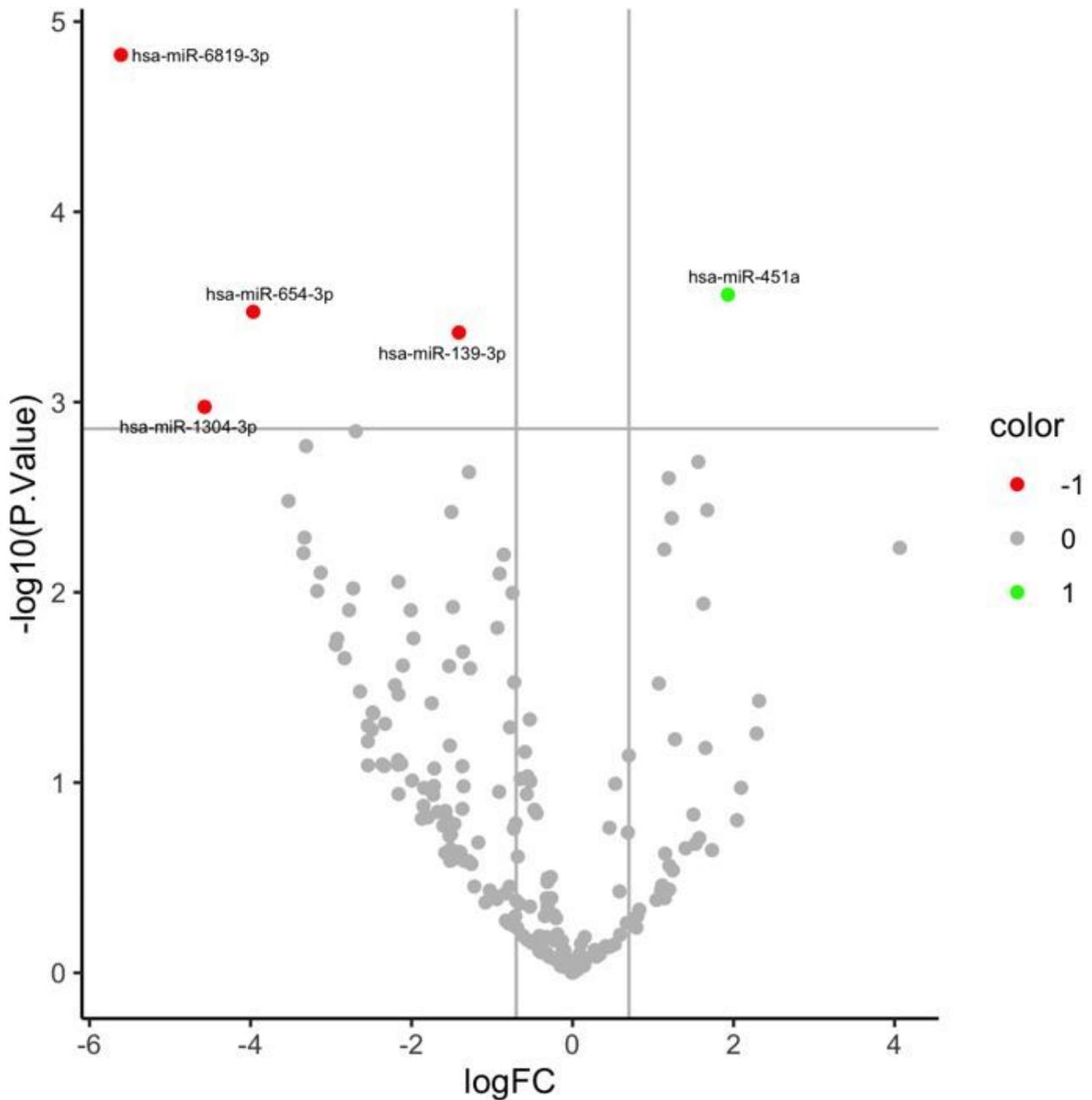
Figure 2

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.
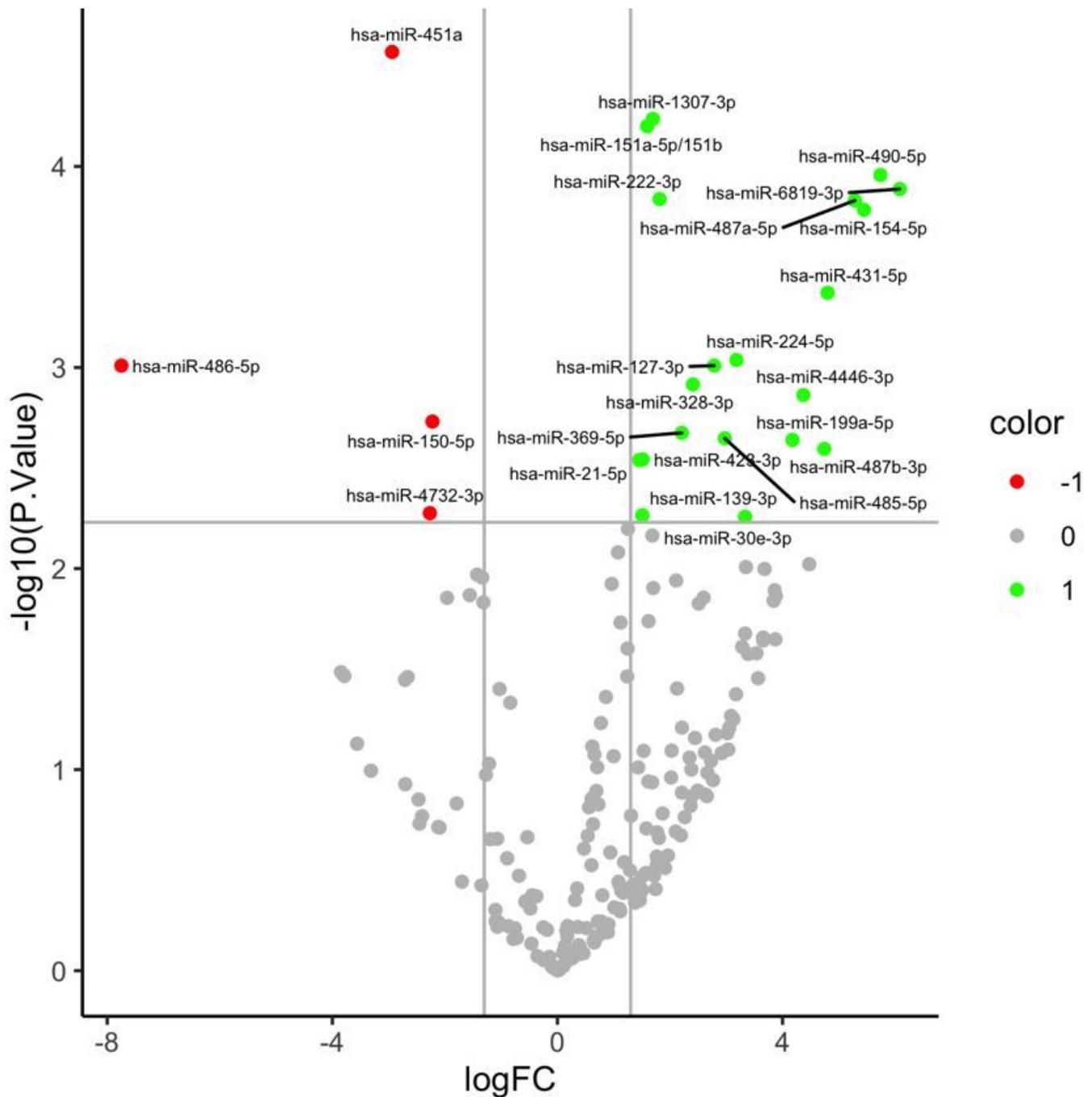
Figure 3

The most variable circulating miRNAs expressed in patients with and without Epidemic KS. Legend: Z-score expression heat map 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

Figure 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.

Figure 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. miRNAs differentially expressed with an adjusted p-value < 0.005 are depicted in red for down regulation and green for up regulation.

Supplementary Files

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