# Characterization of Circulating MicroRNAs Related to HIV-1 and Opportunistic Infections

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**Abstract of**

**Characterization of Circulating MicroRNAs Related to HIV-1 and Opportunistic Infections**

Background: miRNAs are a group of ~22nt long small RNAs which can regulate gene expression post-transcriptionally. The miRNAs in circulating systems like human plasma or serum have been studied as potential biomarker for cancer diagnosis, since they are highly stable, easy to achieve, related to host physiological environment and reflect certain specific types of the cancer. Serological and nucleic acid assays are two commonly used methods for diagnosing of HIV-1 and opportunistic infections. However, a universal biomarker has not been discovered yet. We hypothesize that miRNA profile is specifically changed in response to HIV-1 and opportunistic infections and, therefore, some miRNAs may be used as specific biomarkers in the human circulating systems.

Result: Total RNA was extracted from HIV-1 mono-infected, HCV co-infected patients in chronic phase, and KSHV co-infected patients with Kaposi's sarcoma malignancy and healthy individuals as a control. Expression profile of each sample was detected by microarray hybridization and qRT-PCR. Data were statistically analyzed using hieratical clustering, correlation coefficient and *t*-test. The results indicated that miRNA level in KSHV co-infected samples changed dramatically, while HIV-1 mono-infected and HCV co-infected patients were comparably similar with healthy individuals. Data of several miRNAs (mir-92a, mir-29b, and mir-486-5p) were further confirmed by qRT-PCR.

Conclusion: The miRNA profiles in plasma of the patients, infected with HIV-1 and opportunistic pathogens have been changed. Selected miRNAs can be used as potential HIV-1 co-infection biomarkers in plasma.

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## Abbreviations

EBV: Epstein–Barr virus

HCV: hepatitis C virus

HIV: human immunodeficiency virus

HSV: herpes simplex virus

IFN-α: interferon alpha

KS: Kaposi’s sarcoma

KSHV/HHV-8: Kaposi's sarcoma-associated herpesvirus/ human herpesvirus 8

LANA: latency-associated nuclear antigen

LTR: long terminal repeats

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

TNF-α: tumor necrosis factor alpha

## Chapter 1: Background

*HIV and AIDS*

The human immunodeficiency virus (HIV), a member of lentivirus subfamily of the retrovirus family, is the causative agent of acquired immune deficiency syndrome (AIDS). It has been found that there are at least two types of HIV—HIV-1 and HIV-2. HIV-1 is more virulent and most AIDS patients are infected by this type of HIV [1] AIDS is a worldwide epidemic. Data estimated by World Health Organization indicates that in 2009, there had been 1.8 million people died from AIDS since the beginning of the epidemic, and among them, 260 thousand are children.

The most common routes of virus transmission are sexual intercourse, contaminated needles and infected blood products. Beside of body fluid, such as blood, breast milk, semen, and vaginal fluid, the virus can also be carried by infected CD4+ T cells, dendritic cells and macrophages, which have CD4 receptor and CCR5 or CXCR4 chemokine receptors on the membrane. Primary acute infection is defined as the first one to four weeks after virus transmission but before antibodies being detected. During this stage, CD4+ T cell count is dramatically decreasing and HIV virus increases in circulating system (around $10^6$ to $10^7$ RNA copies/ml in plasma). This acute infection activates immune response of cytotoxic CD8+ T cell to kill infected CD4+ T cells and control virus back to low level. With the subsequent adaptive immune response and antibody produced, CD4+ T cell count rise to around 800 cells ul-1. [1] Clinically, the symptom of acute infection stage is characterized like normal virus-like illness: headache, muscle aches, sore throat, fever, and retro-orbital pain [2]. After 3 to 5 months, usually these symptoms would pass, and virus

count in plasma keeps at a steady stage. Disease goes to a phase of apparent quiescence which is known as the asymptomatic period. This is the stage lasting from 6 months to 20 years until infected people finally develop AIDS [2].

HIV is enveloped virus, i.e. each virus particle or virion is covered by the lipid bilayer derived from the plasma membrane of virus-producing cell. The particle contains viral proteins and two molecules of genomic single-strand RNA. The RNA molecules are packed together with nucleocapsid protein p7 (NC) and enzymes, reverse transcriptase, protease and integrase, which are essential for HIV replication. All these components are enclosed in viral capsid which is made by the hexameric complexes of capsid protein – p24 (CA). The virion also contains two envelope glycoproteins gp120 and gp41.

The life cycle of HIV virus starts from virus-cell interaction. Cellular tropism of the virus is determined by the presence of CD4 molecules in combination with the molecules of CCR5 or CXCR4 co-receptors on the membrane of potential target cell. The HIV-1 targets are CD4+ T lymphocytes, dendritic cells and macrophages. Glycoprotein gp120 draws virus to CD4+ cell and attaches the surface. Before virus entering the cell, gp120 needs to recognize one of the chemokine receptors. CCR5 and CXCR4 are two common receptors expressed on cells. Once gp120 binds CD4 receptor and CCR5 or CXCR4 co-receptor, gp41 initiates the fusion of viral envelope to cell membrane. The envelope of virion is open after fusion, and components inside are released into the cytoplasm. After viral reverse transcriptase transcribes vrial RNA to cDNA in cytoplasm, cDNA is transported into nucleus with the

integrase and probably matrix and Vpr viral proteins and integrated to host genome by viral integrase. This integrated cDNA copy is called provirus [2].

Like other retroviruses, HIV genome is flanked by two regions of long terminal repeat sequence (LTR) at the ends, which is required for integration of becoming provirus and initiating viral transcription. [1] Protein products of three major genes *gag*, *pol* and *env* are included in all mature virions. Some other genes like *tat*, *rev*, *nef*, which have specific functions during virion development are formed by multiple splicing of virus transcripts.

The mature viral particle is produced from provirus once it is transcribed in activated T cell. Once the T cell is activated, transcription factor NF-κB enters nucleus and induces gene expression by binding with both cellular promoter and LTR of the provirus. Although in host genome, LTR itself can function as other transcription units to attract Polymerase II to the side of initiation, it is not able to elongate the whole viral genome [3]. NF-κB recruiting can up regulate silent provirus transcription by binding LTR region. Among the proteins of HIV, Gag and Pol are translated from non-spliced viral mRNA, while Env, Vpr, Tat, Rev and Nef are translated from single-spliced or multi-spliced mRNA. Tat and Rev can promote virus production in cell. [1] Associated together with cyclin T1, Tat binds on transcriptional activation region (TAR), which is a RNA region on LTR. The complex formed by Tat and positive-elongation factor B with TAR dramatically enhances viral gene expression and removes negative factors of elongation. [4] Incomplete spliced mRNA cannot be exported from nucleus in eukaryotic cells; however, Rev protein can bind on viral mRNA sequence together with CRM1, a cell pathcleocytoplasmic transported protein,

to export viral mRNA to cytoplasm. Once viral proteins are translated from spliced/unspliced mRNAs, new virus particles are packed at plasma membrane and budding out.

*HIV related opportunistic infections*

One of the major characteristics of HIV-1 pathogenesis is dramatic decrease of CD4+ T cell count. As a result, a patient becomes vulnerable to other pathogens causing opportunistic infections. There are a variety of opportunistic pathogens which infect patients with HIV. They can be categorized to parasites, bacteria, fungi and viruses. Some of these pathogens may increase HIV-1 infection through the activation of the virus replication. Earlier studies showed, that the in the HIV-EBV co-infected cell, the EBNA2 expressed by EBV can bind with the NF-κB associated with HIV-1 LTR and increase HIV production. [5]. Similarly, high expression level of HIV-1, induced by HSV co-infection was also detected due to the transactivation effect of HSV factors on HIV-1 LTR. [6]. On the level of organism, infection of some pathogens can increase the HIV-1 expression via regulating cytokine level, such as TNF-α, which has been considered as the regulator enhancing HIV replication. TNF-α induced by malarial antigen in peripheral blood mononuclear cells could increase HIV replication by stimulating LTR. This reaction could be inhibited by anti-TNF-α monoclonal antibody [7]. Some other mechanisms of HIV expression promoting by co-infectious pathogens include expression of the molecules in the cells, which are required for cell entry of HIV. For instance, *M.tuberculosis* can also enhance CXCR4 and CCR5 expression on plasma membrane of the primary CD4-positive

cells [8]. The member of herpesvirus family, HHV-6 is reported to be able to induce the expression of CD4 on the membrane of natural killer cells and CD8+ lymphocytes [9].

Herpesviruses together with HIV infection play an important role in generating of certain types of cancer, such as Kaposi's sarcoma caused by KSHV/HHV-8 and B cell lymphoma caused by EBV. Cancer occurs among almost 40% of HIV infected patients and it has been observed that cancer development in HIV carriers is more aggressive than in non-infected individuals. It is commonly accepted that generating of malignancy in patients is due to the immune system disturbance by HIV infection [10]. Host immune system is suppressed after being infected by HIV virus and immune surveillance of lymphocytes on tumor cells cannot work properly. Another theory of immune system disorder is that immune hypeactivation caused by HIV infection results in high level of cytokine secretion. The cytokines can induce both HIV replication and proliferation of malignant cells [11].

Kaposi's sarcoma (KS) is one of the most common types of cancer found in HIV advance infected patients. There is around 20% of HIV carriers in United States who suffer from KS [12]. When KS was first reported in Mediterranean region in nineteen century, it was used be considered as a tumor which rarely kills people and does not generate metastasis. However, in HIV infected group this cancer is much more aggressive. Immune suppression is known as the leading cause of KS, because the cases of both classic KS and HIV related KS indicate low host immune level. Furthermore, disregulation of the production of inflammatory cytokines, which include IL-6, TNFα, IFN-γ caused by HIV infection contributes to early stage of KS lesion. [13]. Other interesting observation showed

a cross talk between HIV and KSHV – the HIV-1 Tat could activate KSHV replication [14] and enhance KSHV entry into epithelial cells [15]. The critical transactivating protein ORF50, which induces KSHV reactivation, can also synergize with HIV-1 Tat and increase HIV-1 replication in CD4+ T cell [16].

Hepatitis C virus (HCV) is single-stranded RNA virus the member of *Flaviviridae* family. HCV is another common HIV co-pathogen which is found in almost 30% of HIV infected population. This is due to the same transmission route for HIV and HCV viruses such as contaminated needles and sexual intercourse. HCV infection causes severe liver damage. It has been found that HCV RNA levels are higher in HIV-positive multitransfused hemophiliacs than in HIV seronegative ones, and this finding suggested that co-infection processed liver damage more quickly than HCV mono-infection [17].

*HIV, HCV and KSHV Diagnostics*

The current methods of laboratory diagnostics of HIV infection can be categorized to two groups: serological assays and nucleic acid testing. In the phase of acute HIV infection (the first 1 to 4 weeks after virus transmission), virus particles are found in serum but antibodies have not been detected. Therefore, nucleic acid testing for viral RNA is commonly used during this stage, and this method is more sensitive than p24 antigen test. Anti-HIV antibodies can be detected in primary HIV infection phase (first 3 to 6 months after virus transmission). During this phase, diagnose is done by detecting HIV antibodies by enzyme-linked immunosorbent assay (ELISA) and subsequent immunofluorescence assay confirmation. Level of antibody variation in serum can be utilized to define different stages

of HIV infection. IgG3, a transient anti-p24 antibody, is detected by ELISA to define the first 1 to 4 months, due to its temporary activity during this stage [18]. Other stages can be detected by measuring virus changing in plasma via the measurement of level of anti-HIV antibodies variation by ELISA, western blot, and by viral RNA detection. Not only stages of infection, but HIV-1 subtypes also can be determined by immunoassay, which is sensitive to variety of IgGs specific for different HIV-1 subtypes [19].

Diagnostics of HCV and KSHV is similar. It also can be categorized to serological assays and nucleic acid assays. Specificity of anti-HCV antibody detection is more than 99%, while real time polymerase chain reaction (qRT-PCR) based assay is also high specific (from 98% to 99%). HCV can be classified to six groups according to its specific genotype which has around 30% diverse from other groups [20, 21]. Immunofluorescent assay for detecting of latency-associated nuclear antigen (LANA), which is playing an important role in KHSV viral replication, was used as the first generation to diagnose KSHV. Seropositivity of LANA is correlated with KS development; however, the sensitivity of this method is low (70~80% positive in KS sera) [22, 23]. In the second generation assay, the serological assays detecting different KSHV specific proteins were developed for KS diagnosis, such ORF65, 73, ORFs K8.1A and K8.1B [24]. However, the assays for latent-related antigens are less sensitive than for lytic-related antigens. Therefore, the nucleic acid detection assays were developed to monitor disease progression and the effect of antiviral therapy [24]. However, currently no universal biomarker is discovered to diagnose HIV and co-infections together.

*Dysregulation of miRNAs is related to virus infection and carcinogenesis*

MicroRNA (miRNA) is a class of small, non-coding RNAs which have post-transcriptional regulation activity in cell. MiRNAs are encoded by both exons and introns of chromosomal DNA. The molecules of primary microRNA or pri-miRNA are transcribed by RNA polymerase II (Pol II) or Pol III in the nucleus. These are 0.5-7 kb long single-strand RNA molecules folded into single or multiple hairpin structure. Then pri-miRNA is cleaved by enzyme called drosha, which results in formation of a 60-80 nt long hairpin structure precursor miRNA or pre-miRNA. Pre-miRNA binds with exportin 5 and then translocates through the nucleus pore to the cytoplasm, where the hairpin structure is processed by another RNase enzyme, dicer, into mature miRNA (18 to 25 nt long) [25]. The post-transcriptional regulation step of mature miRNA is mediated by RNA-induced silencing complex (RISC) containing an endonuclease called Argonaut (Ago2). Mature miRNA guides RISC targets on the 3'UTR region of mRNA. miRNA recognizes complementary sequence and then RISC complex accomplishes the mRNA cleavage. It results in inhibition of translation and the mRNA degradation in many cases [26] [27].

MicroRNAs can affect the interaction between cells and viruses. Cellular miRNA can inhibit virus activity by targeting viral RNA, while viral miRNA can increase virus production by regulating cellular environment, such as avoiding immune response [28]. Indeed, some viruses may produce their own miRNA. According to the recent studies, miRNA maturation takes few days, therefore, the virus enable to produce miRNA should present in the cell for this period, but do not destroy cellular organization and pathways. These characteristics are suitable for viruses that have period of long term latency in their

life cycle [29]. The viruses with the DNA genome, which transcribes in the nucleus of infected cell, are the candidates. The suitable virus family with the highest possibility to generate miRNA is *Herpesviridae*. The capacity to produce miRNA was revealed for different groups of viruses within this family, though, the virus-encoded miRNAs were detected for some other DNA viruses too [28].

The targets of viral miRNAs are cellular mRNA and viral mRNA itself. Simian virus 40 (SV40) generated miRNAs, SVmiRNAs, accumulate in cells at late stage of infection and perfectly target early viral mRNAs resulting in the degradation of these molecules. Since these early mRNAs encode viral antigen, the regulation by viral miRNAs could degrade antigens and avoid T cell response [30]. Some viral miRNAs can regulate cellar activity by targeting host mRNA. Herpes simplex virus type 1 (HSV-1) can establish a long lasting latent infection in neuron. During the quiescent stage, viruses only express one pri-miRNA latency-associtated transcripts (LATs) that able to resist neuron cells to apoptosis. Using this strategy, HSV-1 can keep itself on the stage of latent infection for a long time until reactivation [31]. So far as researchers have found, most viral miRNAs regulate cellular environment by inhibiting host activity, like innate immune response, interferon secretion, T cell response, and so on.

Cellular miRNA can also affect viral mRNA in the cells. It has been reported that the production of primate foamy virus 1 (PFV-1) can be inhibited by hsa-mir-32, while PFV-1 also expresses the protein Tas to inhibit this mir-32 regulated activities [32]. Besides of inhibiting, the effect of cellular miRNA can also include enhancing of virus activity. Mir-

122, a highly expressed in the liver miRNA, has been found having strong enhancing effect on HCV viral mRNA expression, which causes serious liver injury during infection [33]. In the HIV related research, it was been reported that in HIV-1 infected resting CD4+ T cell, several cellular miRNAs, including mir-28, mir-125b, mir-150, mir-223 and mir-382, targeted 3'LTR of HIV-1 mRNA. The level of this miRNA cluster in resting CD4+ T cell was much higher than in activated CD4+ T cell. It suggested that these miRNAs were related to HIV-1 latency [34].

Cancer is caused by uncontrolled cell proliferation and differentiation. There are several regulatory factors, such as tumor suppressor p53 and c-MYC, which are related to the control of cell cycle checkpoint and gene repair. Once these factors are switched on or off abnormally, cells will proliferate uncontrollably without repairing of damaged genes, and finally develop a tumor [35]. These critical regulatory factors are translated from mRNAs which are encoded by cellular DNA. It has been found that several miRNAs, which can interact with mRNA post-transcriptionally, have specific complementary sequence to the mRNAs encoding the proteins responsible for cell proliferation and apoptosis. The transcription factor c-MYC can stimulate expression of a cluster of six miRNAs located in the same chromosome. Two of these six miRNAs are capable to down regulate cell cycle stimulator E2F1, which, conversely, can be up-regulated by c-MYC [36]. Mir-29 family of miRNAs has found to activate p53 by suppressing p85α and CDC42. [37] Both proteins suppress p53 activity. Interestingly, p53 enhances the progression of maturation of a miRNA group which includes mir-16-1, mir143 and mir-145. All these miRNAs have a suppressing effect on cell proliferation. It was reported that p53 improved the pri-miRNA

processing by assisting the association between drosha and RNA helicase p68. Contrarily, once p53 is inactivated, it interrupts the assembly of drosha and p68 to inhibit miRNA maturation [38]. In summary, despite that many mechanisms of miRNA-related regulation of cell proliferation is still unclear, we can conclude that cancer malignancy is related to miRNA dysregulation, and these processes include complicated interaction between miRNAs and other cellular factors.

Multiple dysregulation of miRNAs in cancer cells implies the possibility that miRNA profile has the potential to be used as biomarker. Jun Lu et al. found that out of 217 miRNAs detected, 129 miRNAs were down-regulated in tumor tissue samples as compared with normal control. This result was consistent to the data of comparison of miRNA spectra between cancer cell lines and untransformed cells [39]. The comparison of the miRNA profiles between the cancer specimens from different organs (pancreases, kidney, bladder, prostate, ovary, brain, lung, uterus and so on) showed that the expression levels of almost all miRNAs were diverse between different cancer types. The hierarchical clustering of miRNAs showed that the tissues from haematopoietic origin clustered together, while some other types of cancer originated from epithelium, clustered together in heatmap. Furthermore, comparison between samples from acute lymphoblastic leukaemia indicated that miRNA profiles can differentiate between single developmental lineages [39]. In conclusion, the miRNA profiles have potential to be biomarker, which can reflect origin of cancer as well as the stage of cancer progression.

*MicroRNAs in circulating system*

While the miRNAs with cancer-related regulating activity have been discovered in cells, and miRNA profiles in cancer tissue have been found, different research groups reported that cell free miRNAs, including multiple cancer-related miRNAs, also presented in circulating system [40]. As compared with large molecule weight RNAs, the cell-free miRNAs are resistant to harsh conditions as RNAase digestion, multiple freeze-thaw cycles, and extremely high/low pH environment. It indicates that endogenous miRNAs in plasma and serum are very stable [40] [41].

So far, there are two theories which explain origin of circulating miRNAs. The first explanation is the passive release caused by tissue injury, such as myocardial injury or cancer cell lysis, which results in miRNAs leaking from injury cells [42]. According to the second theory, miRNAs in circulating system are positively secreted by cells in small particles, like exosomes. Exosomes are small extracellular particles with diameter from 50nm to 1μm, released by normal or damaged cells [43]. They are originated from variety of cells such as B cells, T cells, dendritic cells and some non-hematopoietic cells. Recently, it was reported that tumor released exosomes had the potential to suppress immune system by stimulating monocytes differentiating to myeloid-derived suppressor cells, inducing apoptosis of T cells, and suppressing response of natural killer cells [44]. Moreover, the miRNA profile in blood microvesicles was compared with the profile in peripheral blood monocytes and cancer tissues. The results showed significant difference between the profiles. Target prediction indicated that some of the miRNAs in microvesicles can regulate cellular differentiation and metabolism, and a few miRNAs had immune regulating activity [43]. Since recent study found evidence of cell-cell genetic communication by exosomes

[45], the hypothesis that miRNAs transferred in blood are associated with exosomes is becoming commonly accepted.

If the miRNAs in circulating system are mainly secreted active by cells, their potential to reflect physiological change and cancer processing can be predicted. The miRNA profiling of the same tissue species from healthy individuals showed that the level of miRNAs is stable and consistent with each other, but significantly different within patients [40]. Comparison among different types of cancer showed various circulating miRNAs profiles. Several cancer specific miRNAs were discovered to be potential biomarkers. The ratio of mir-92a/mir-638 was dramatically decreased in plasma from leukemia patients, and this down regulation could be a signal for leukemia diagnosis [46]. Mitchell et al. indicated that mir-141 significantly up regulated in serum from prostate cancer patients. Until now, many miRNAs have been reported having potential to be used for cancer diagnostics [41].

*Hypothesis*

MiRNA profile in circulating system is considered as a new biomarker for diagnostics of certain types of cancer because (1) it is non-invasive, (2) it is stable even in harsh conditions, (3) it can reflect different pathological and physiological conditions. Here we hypothesize that HIV-1 infection and/or co-infections with other viruses such as hepatitis C virus (HCV) and Kaposi sarcoma virus (KSHV) cause changes in the plasma miRNA profiles and these changes can serve as biomarkers for HIV-1 mono-infection and co-infection.

## Chapter 2. Methods and Materials

*Sample information*

Human plasma samples from AIDS and Cancer Specimen Resource (ACSR) were grouped into four groups: healthy control, HIV-1 mono-infected, HCV co-infected samples and KSHV co-infected samples. The last group of patients developed Kaposi sarcoma, whereas samples from HIV-1 mono-infected and HIV-1/HCV co-infected patients were from seropositive individuals with chronic, non-acute infection. In order to avoid the racial and gender effect on miRNA spectra, all samples were from black female individuals. Volume of each sample is 2ml, liquated into 0.5ml or 1ml. Samples should be stored in -80℃.

*Extrinsic control solution preparation*

Synthetic *C. elegans* miRNA cel-mir-238, which has no homolog with human miRNA, was added at the beginning of the extraction in order to avoid variance during experiment process. Cel-mir-238 was stored as the concentration of 5fmol/μl in -80℃. In order to avoid degradation due to multiple frozen-thaw cycles, each aliquot is 25μl in separate tube [47]. It was reported that RNAase in plasma can degrade synthetic miRNA immediately, so cel-mir-238 was added after lysis by TRI Reagent. (cel-mir-238 Fwd sequence: 5'-UUUGUACUCCGAUGCCAUUCAGA-3')

*RNA isolation*

Isolation of total RNA from human plasma samples were done by using TRI Reagent (Ambion p/n AM9738), together with mirVANA miRNA isolation kit (Ambion p/n AM1561). Plasma sample from same patient was first lysed by TRI Reagent of 3 times

volume. After 10min incubation, 5µl of cel-mir238 was added from 5 fmol/µl stock solution and mixed immediately. Then 1/5 volume of chloroform (Sigma) was added and the solution was centrifuged (Sorvall Biofuge Fresco) (4℃, 12,000rpm, 15min). Collected upper aqueous phase and filtered it with column from mirVANA to bind RNAs. After washing steps, purified RNAs were diluted with 100µl nuclease free water in 95C. RNA solution was concentrated by Speed-Vac (Savant) to 30µl, and concentration was detected by Nano drop ND-1000 spectrophotometer. Samples with 260/280> 1.5 were chosen to be detected by microarray hybridization. Extracted total RNA is kept in -80℃.

*Labeling*

From each sample, 150ng of total RNA was labeled by Agilent miRNA complete labeling and hybridization kit (Agilent p/n 5190-0456) according to the manufacture protocol. Briefly, RNAs were dephosphorylated by calf intestinal phosphatase, and then labeled with pCp-Cy3 by T4 RNA ligase catalyzing. Cytidine diphosphate together with Cy3 dye attached on 3' of RNA was for paring with the Guanine designed on probe, with the purpose of stabilizing miRNA on probe. After labeling, labeled RNA mix was purified by Micro Bio-spin 6 column (BIO-RAD p/n 732-6221), and then dried the sample by Speed-Vac.

*Hybridization and scanning*

Dried labeled RNA sample diluted in nuclease free water, together with 10X GE blocking reagent and 2X Hi-RPM hybridization buffer (Agilent p/n 5190-0456), was loaded on gasket slide (Agilent p/n G4470C). Then the array slide was placed on the gasket slide with

the side of probes facing down. There were 8 wells on one gasket slide, which were aligned with 8 arrays on array slide. This sandwich pair was hold firmly by chamber and incubated in incubator at 55℃ for 20 hours. During the process, labeled miRNAs were hybridized with probes on the array from Agilent human miRNA microarray kit (V3) (Agilent p/n G4470C) which can detect 866 human and 89 human viral miRNAs. After hybridization, array slide was washed by washing buffer 1 and 2 (Agilent p/n 5188-5327) and then processed to scanning by an Agilent G2565AA Dual Laser Microarray Scanner at 5 micron resolution. Tiff. images were produced.

*Feature extraction and data analysis by GeneSpring*

Feature extraction software (v.10.7.3.1) extracted signal from Tiff. images after scanning and generated QC metric report. Total gene signal intensity with background subtracted were log2 transferred by GeneSpring GX and normalized by quantile. In data analysis, coefficient correlation value was calculated to analyze the correlation between samples, and hieratical clustering and *t*-test was used to compare miRNA profiles between control and infected groups, and between HIV-1 mono-infected and co-infected groups.

*QuantiMir RT reaction and qPCR*

cDNAs of miRNAs were generated with QuantiMir Kit (SBI p/n RA420A-1) according to manufacture introduction. Briefly, in the first step, 5μl total RNAs were incubated with 2μl 5XPolyA buffer, 1μl 25mM MnCl2, 1.5 μl of 5mM ATP and 0.5μl polyA polymerase, for 30min at 37℃. This step was to attach polyA tail on the 3' end of miRNA. Then dT adaptor was anchored complementarily on polyA tail, with the reaction of heating for 5min

at 60℃ then cooling to room temperature for 2min. In the third step, cDNAs were synthesized by reaction with 4μl 5X RT buffer, 2μl dNTP mix, 1.5μl 1M DTT and 1μl reverse transcriptase. Incubation lasted for 60min at 42 ℃ and 10min at 95 ℃.

The QuantiMir mix was ready for subsequent real-time qPCR with SYBR Green super mix (BIO-RAD p/n 170-8882). In one 20μl reaction system, 10μl 2X SYBR Green supermix, 0.5μ universal reverse primer and 1μl designed primer (10μM) and 1μ QuantiMir mix were mixed. The mix was incubated for 2min at 50℃ for 2min, 95℃ for 5min, 95℃ for 15 seconds and 60℃ for 1min. Disassociation stage was needed to generate melting curve. Comparative Ct (ΔΔCt) method was used to analyze data.

## Chapter 3. Results

We analyzed miRNA profiles from 18 human plasma samples obtained from AIDS and Cancer Specimen Resource (ACSR). The specimens were from four groups of patients: healthy donors (control)-4 samples, HIV-1 mono-infected-6 samples, HCV co-infected with HIV-1-4 samples and KSHV co-infected with HIV-1-4 samples. Human microRNA microarray V3.0 from Agilent detects 866 human and 89 human viral miRNAs. For each miRNA probe, the complementary oligonucleotide replicates 16 times on every array. Total probe signal represents the mean value for each miRNA calculated after subtraction of the background probe signal.

*Normalization of microarray data*

The normalization of data has been done before analysis to resolve the systematic bias and errors from RNA extraction and microarray platform. The GeneSpring software allows using several methods of normalization, such as normalization by positive control probes, median/mean value, percentile, scaling and quintile (GeneSpring GX manual). For the miRNA microarray, the method of positive control probes is not consistent. The method of median or mean value is not stable because the dramatic variation of miRNA level. The $90^{th}$ percentile is assuming that about 75% of the genes do not have significant change, and the signal from different arrays will be normalized by the median of remaining 25% of genes. Quantile is a non-parametric normalizing method, which revalues every miRNA according to its rank in the array. Therefore, standard deviation and distribution will be the same between arrays. Both percentile and quantile are very robust, however, according to the data presented by Lopez-Romero et al., two methods did not show difference when normalizing high-expressed miRNAs, but percentile normalized low signal had larger variability among replicates [48]. Since the signal of many miRNAs in plasma was very low, we chose quintile to normalize signal between arrays.

*Correlation coefficient value*

The correlation coefficient is usually engaged to calculate the correlation between two samples. Technically, higher value represents more similar experimental conditions, and conversely, lower value represents higher variation (GeneSpring GX manual). After analyzing the correlation coefficient value among all microarray data, the samples with low value calculated by comparison with samples from the same group were excluded from further data analysis. Finally, four control, four samples from mono-infected individuals,

two samples from HIV-1/ HCV-coinfected patients, and two samples from HIV-1/KSHV coinfected patients were chosen. We found comparably high correlation between the control, HIV-1 mono-infected and HIV-1/HCV co-infected groups (CE value is around 0.7), while KSHV co-infected group had very low CE value (~0.4) when paired with samples from other groups. (Supplementary table 1)

*Clustering and t-test on GeneSpring*

The normalized data from different samples were subjected to hierarchical clustering to compare the miRNA expression profile among 4 groups (Fig. 1). The miRNA profile changed between different groups, especially from HIV-1/KSHV co-infected individuals. Consistent with the result of correlation coefficient, clustering analysis indicated that miRNA profile of control, HIV mono-infected and HCV co-infected groups did not change very significantly, while the profile of KSHV co-infected group was dramatically different. Different profile between individuals from the same group may due to the variant progression of disease, however, further information should be given to confirm.

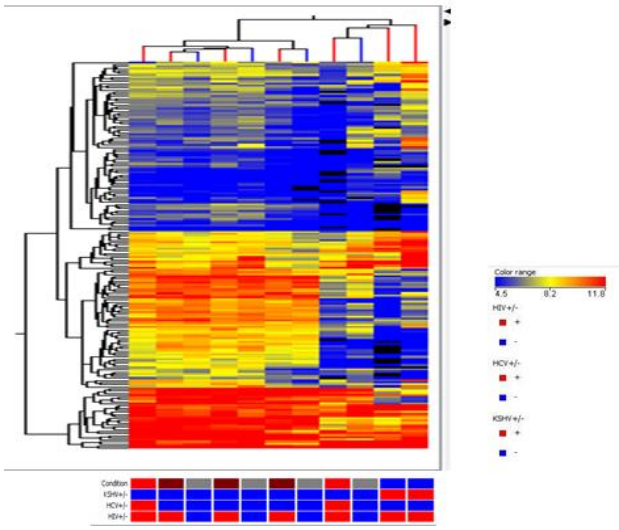

**Fig. 1. Hieratical clustering of miRNAs** Hieratical clustering was done to compare miRNA profile among different groups. All entities were firstly filtered by expression (raw data>10), then 183 filtered miRNAs were used in clustering. We chose "Euclidean" as distance metric, "Average" as linkage rule, and data was clustered on "conditions and entities" [49].

In order to find out specific miRNAs that changed significantly, *t*-test was done between groups. Comparison of HIV-1 mono-infected with control group revealed 8 up-regulated and 2 down-regulated miRNAs in HIV-1 mono-infected group ($p<0.05$). Within these 10 miRNAs, mir-520b and mir-520e were detected only in HIV-1 mono-infected samples but not in any other groups. Alternatively, the signal for several miRNAs (mir-92a and mir-486-5p) was strong in all samples, and at the same time, up-regulated in HIV-1 mono-infected samples. (Data is shown in supplementary table 2)

As we presented before, the miRNA profile of the samples from HIV-1/KSHV-co-infected patients was dramatically different. Subsequent *t*-test analysis between HIV-1/KHSV co-infected and HIV-1 mono-infected groups showed that 19 miRNAs were up-regulated and 29 miRNAs were down-regulated ($p<0.05$), while the analysis between KSHV co-infected and control groups showed that 24 miRNAs were up-regulated and 26 miRNAs were down-regulated ($p<0.05$). These two groups had 28 miRNAs in common. (Data is shown in Supplementary table 3). Clustering result of these 28 miRNAs indicated that some miRNAs were highly ranked in all groups, such as mir-638; some miRNAs were highly expressed in KSHV-positive samples (mir-483-5p, mir-1268, mir-1281-3p, mir-765); and several

miRNAs were significantly down regulated in HIV-1/KSHV co-infected groups (mir-20a, 20b, mir-17a). Also, mir-765 level was readily increased as compared with control, HIV-1 mono-infected and to KSHV co-infected individuals. Moreover, we found that mir-92a was down-regulated in KSHV co-infected group comparing with HIV-1 mono-infected, but did not show significant change between KSHV co-infected and control groups. Since the level of mir-92a was also increased in HIV-1 mono-infected group compared with control (Fig. 2), we can suggest that mir-92a up-regulation was specific for HIV-1 mono-infection.

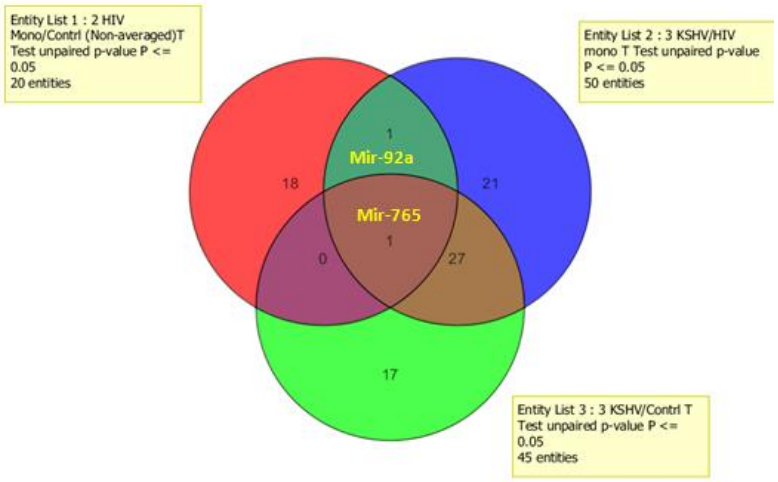


**Fig. 2. KSHV co-infected/Control and KSHV co-infected/HIV-1 mono-infected shared 28 miRNAs in common.** Mir-765 (in the overlap of 3 circles) was also up-regulated in HIV-1 mono-infected samples. Mir-92a, which was up-regulated in HIV-1 mono-infected samples, was down-regulated in KSHV co-infected samples. Detail data is shown in supplementary table 3.

The miRNA profile of HCV co-infected group was comparably similar to HIV-1 mono-infected group. Therefore, even using less robust *t*-test ($p<0.1$), we found only 2 up-

regulated miRNAs and 5 down-regulated miRNAs in HIV-1/HCV co-infected group compared with control; one miRNA up-regulated and 7 miRNAs down-regulated as compared with HIV-1 mono-infection. (Supplementary table 4).

*qRT-PCR Confirmation on result from microarray*

Microarray analysis gave a lot of information, at the same time it generated false positive results for some miRNAs. To gain convincing results, we tried to filter data with low *p*-value and high fold change, and confirmed the result by subsequent qRT-PCR. We selected four miRNAs, mir-92a, mir-29b, mir-486-5p and mir-765, to do qRT-PCR detection in 2 RNA samples from HIV-1 mono-infected, 2 – from HIV-1/KSHV co-infected, and 3 – from control individuals.

In the first step, we needed to choose a stable expressed gene as a control to normalize data in qRT-PCR quantitative assay. So far, commonly accepted endogenous control gene has not been found for miRNA analysis. Here, we tried to use different method to normalize qPCR data: synthetical *C. elegans* miRNA cel-mir-238, which was added at the beginning of total RNA extraction, small nuclear RNA U6 snRNA, and endogenous miRNA.

The endogenous miRNA control should be (1) highly expressed, (2) consistently expressed across all samples, and (3) detectable by qRT-PCR. We ranked all the miRNAs according to their modified *z*-score (Table 1.) [50], and chose let-7b and mir-1207-5p as intrinsic controls. Mir-16 is commonly used as control in cancer related research, because it is highly and stably expressed in tissues, cell lines, and serum samples. However, because of

the low *z*-score value, mir-16 in HIV-1 infected plasma samples was stable across samples. This phenomenon was coincident with the result from analysis of HIV-1 infected cells. (Done by S. Iordanskiy, data is not shown here)

| systematic name | Rank | Modified *z*-score | Mean |
|---|---|---|---|
| hsa-miR-923_v12.0 | 1 | 5.241 | 13.391 |
| hsa-miR-320d | 2 | 4.278 | 10.876 |
| hsa-let-7b | 3 | 4.201 | 11.911 |
| hsa-miR-320c | 4 | 4.05 | 11.111 |
| hsa-miR-720 | 5 | 3.762 | 12.789 |
| hsa-miR-1207-5p | 6 | 3.42 | 11.097 |
| hsa-miR-638 | 7 | 3.111 | 12.272 |
| hsa-miR-1202 | 8 | 2.502 | 10.532 |
| hsa-miR-320b | 9 | 2.49 | 10.314 |
| hsa-miR-1225-5p | 10 | 2.4299 | 10.925 |
| hsa-mir-16 | 33 | 0.666 | 9.789 |

**Table 1. Rank of miRNAs according to their modified *z*-score.** Quantile normalized data from microarray was calculated to gain *z*-score. The miRNAs with high *z*-score value were expected to be stable and highly expressed.

Multiple peaks in melting curves of mir-1207-5p and U6 indicated that primer recognition was not specific, so these two RNAs caould not be used as intrinsic control (Supplementary Fig.1). The qRT-PCR data showed that the level of cel-mir-238 was comparably stable across all samples, which confirmed stability of experiment process. Since it was known

that miRNAs in KSHV co-infected was low, let-7b level, which was low only in samples from HIV-1/KSHV co-infected patients, reflected this condition, but cel-mir-238 did not (Table 2). Therefore, let-7b was the only option met all requirements. Thus, we selected this miRNA as an intrinsic control for normalization.

| | Contrl_1 | Contrl_1 | Contrl_1 | HIV mono_1 | HIV mono_2 | KSHV_1 | KHSV_2 |
|---|---|---|---|---|---|---|---|
| let-7b | 25.13126 | 31.19549 | 20.1812 | 20.97497 | 23.42814 | NQ | 23.42814 |
| cel-mir-238 | 17.0345 | 32.7615 | 15.96815 | 15.3502 | 16.34675 | 26.5911 | 21.85995 |
| U6 | 32.0485 | NQ | 27.09 | 29.0425 | 31.5855 | NQ | NQ |

**Table 2. Raw Ct value of let-7b, cel-mir-238 and U6 from qRT-PCR; NQ means not qualified.**

Data of qRT-PCR was converted to ΔCt value normalized by let-7b. The result of mir-92a, mir-29b and mir-486-5p showed the same regulation trend with hybridization, while mir-765 was opposite (Table 3) [51].

| | Control | | HIV-1 mono | | Relative quantification HIV-1 mono/Control 2^-ΔΔCt | Regulation | Regulation in hybridization |
|---|---|---|---|---|---|---|---|
| | Raw Ct | ΔCt | Raw Ct | ΔCt | | | |
| **mir-29b** | 32.33575 | 9.67952 | 33.039 | 10.83745 | -2.231363 | **down** | **down** |
| **mir-92a** | 26.12552 | 4.99815 | 21.60906 | 4.592963 | 1.324261 | **up** | **up** |
| **mir-486-5p** | 28.32617 | 2.823517 | 22.47975 | 0.278195 | 5.8373827 | **up** | **up** |
| mir-765 | 30.369 | 3.59827 | 31.0305 | 8.828945 | -37.54828 | down | up |

**Table 3. Expression data of miRNAs was presented by Ct value.** ΔCt is Ct after normalized by let7b. (ΔCt=Ct(miRNA)-Ct(let-7b)) And 2^-ΔΔCt, which equates to 2^-

(ΔCt(HIV-1 mono)-ΔCt(control)), reflected the fold change of the miRNAs in HIV-1 mono-infected samples compared with control.

## Chapter 4. Discussions and Conclusions

*Technical approach*

The amount of miRNA is very low in circulating system. During the extraction process, so far, the common used method of miRNA extraction is based on phenol/chloroform, and there are several commercial kits being used. Group of researchers from Agilent compared different extraction methods: Trizol, mirVANA (ABI), and miRNeasy (Qiagen). Trizol prepared samples gave the highest amount of total RNA and highest total gene signal in hybridization, but the phenol contamination was also the worst in 3 methods (Robert A Ach, 2008[59]). According to QC metric reports of samples we have done, total RNA which was directly extracted by TRI reagent gave high signal in microarray detection, but at the same time, abnormal %CV value (Supplementary Fig. 2). MirVANA prepared samples showed low reproductively and low total gene signal compared with other two methods, but the peak at 230nm was smaller than in Trizol prepared samples. MirNeasy seems to be the ideal preparation method. But despite of the method was chosen, the level of each miRNA was still consistent across different methods.

Another problem caused by low amount of circulating miRNA is the difficulty in quantification. Once concentration is lower than 10ng/μl, it cannot be detected accurately by Nanodrop. A stem-loop reverse transcription which is highly sensitive and reproductive has been used for circulating miRNA detection. Another reverse transcription method for miRNA by QuantiMir miRNA kit includes attachment of a poly A tail at the 3' end of miRNA, and subsequent reverse transcription of the miRNA to a cDNA with an oligodT anchor at the 5' end. Compare to stem-loop reverse transcription, QunatiMir is much

cheaper, but it cannot differentiate mature miRNA, pre-miRNA, and mRNA with poly A tail. Although the large majority of miRNA in plasma is mature, it is still important to check the melting curves in qPCR to see the specificity of the reaction.

Finding a control to normalize miRNA in qRT-PCR is also a big challenge. Total miRNA is a small part of the whole RNA mass. Moreover, it is changed dramatically during different stages of cell regulation. It is very important to find out a control which can represent the global miRNA variation. Here we tried different types of control, including small nuclear U6, external synthetic cel-mir-238 and intrinsic hsa-let-7b. Multiple peaks of the melting curve for snU6 and irregular data of qRT-PCR were due to the non-specific recognition of primer. Cel-mir-238 was ideal to correct the variation between samples during experiment process, however, could not reflect internal global miRNA changing. MiRNAs were destroyed in KSHV co-infected samples due to improper transportation, and tis generated additional difficulties for analysis. In this condition, it was impossible to normalize data by cel-mir-238. Among these 3 different normalizing methods, let-7b was the best choice. It is highly ranked with *z*-score among all miRNAs in plasma, and normalized data of chosen miRNAs (mir-92a, mir-29b, mir-486-5p) showed the same regulation trend with microarray result.

*Identification of miRNA biomarker*

The key role of cellular miRNA is regulation of proliferation, differentiation and apoptosis. In healthy individuals, though miRNAs expression in individual cells may be different, the level of each miRNA in circulating fluid is very stable [40]. In the acute phase of HIV-1

infection, the virus copy number boosts in circulating system and CD4+ T cell count drops down. This effect is related to massive death of CD4 positive T cells in the organism of HIV-1 infected patient. This cell death may be the result of necrosis or/and apoptosis induced by different HIV-mediated stimuli. Necrosis of cell causes miRNA release in circulating system. Apoptosis certainly results in dramatic change of cellular miRNAs level [52], associated with passive and active exchange of miRNAs between body fluid and cells.

Our data showed that the HIV-1 positive and HIV-1/HCV positive plasma specimens were obtained from patients who had already passed the acute phase of HIV infection and had chronic infection. Therefore, the expression of majority of miRNAs returned to the normal level, and profile of miRNAs in plasma was similar to healthy groups. The microarray data was consistent with physiological condition of these patients.

The HIV-1/KSHV co-infected patients had Kaposi's sarcoma associated with HIV-1 infection. Since Kaposi's sarcoma (KS) is very aggressive in advanced HIV infected patients, it dysregulates cellular functions and environment. The progression of KS is related to immune suppression, and several studies indicated that KS development caused more dramatic decrease of CD4+ cell and increase of HIV-1 RNA. As a member of herpesvirus family, KSHV infection has two stages, latent and lytic [53]. During the latent stage, latency-associated nuclear antigen (LANA) was found to dysregulate G1/S cell cycle checkpoint [53] and inhibit p53 dependent cell apoptosis activity [54][55]. At the same time, KSHV can enhance cell proliferation and survive by stimulating NF-κB. Once infection enters lytic stage, thousands of virus particles are formed and secreted from cell,

which directly result in massive cell death. This is similar to other tumor progression. KSHV encoded viral miRNAs were reported as reprogramming cell differentiation [56]. Since it is known that tumor progression is related to intensive miRNA dysregulation and cellular environment change, the level of miRNA changes dramatically in KSHV co-infected patients. The plasma miRNA profile of KS patients reflects these changes. Our data showed that the level of many miRNAs in plasma was significantly different between HIV-1/KSHV positive individuals and other groups.

Mir-29b, as a member of mir-29 family, can inhibit viral production in cells by binding on HIV-1 3'-UTR region to interrupt the interaction between HIV-1 and RISC proteins [57]. According to the data of hybridization we got, mir-29b was decreased in plasma of HIV-1 mono-infected patients. The authors of recently published study divided HIV-1 seropositive patients to 4 groups according to their viral load in plasma, CD4+ cell count, and scanned miRNA profile in PBMCs. Compared with normal group, expression level of mir-29b in PBMCs was down-regulated only in the two groups of patients with high level of virus copy in blood, but did not show significant change in the patients with low viral load [52]. Our data show that the level of mir-29b in plasma reflects its decreased synthesis on in the PBMCs of HIV-1 infeted patients. Thus, this miRNA may be considered as potential marker for HIV-1 infection.

Mir-92a belongs to mir-17~92 family, which includes mir-17, mir-18a, mir-19a, mir-19b, mir-20a and mir-92. These miRNAs can activate cell proliferation by stimulating expression of NF-κB, which is the crucial transcriptional factor to initiate HIV-1 replication.

It is possible that mir-92a up-regulated in the plasma of HIV-1 mono-infected patients was due to secretion of mir-92a from the cells [46] [58]. According to the hybridization data, in plasma samples from HIV-1/KSHV co-infected patients, mir-92a dropped down dramatically, as well as mir-17, mir-19, mir-20a and mir-20b. This is a common phenomenon for all types of tumor. Taken together, both the whole miRNA profile changing in plasma as well as variations of specific miRNAs reflected physiological conditions of the patients with chronic HIV-1 mono-infection or acute HIV-1 with KS.

The ideal biomarker miRNA should be either highly expressed or significantly decreased in the specific disease or stage as compared with control specimens. In our samples, the mir-483-5p was highly expressing and mir-17~92 clusters were down regulated only in KSHV co-infected group. However, these miRNAs were comparably low expressed and gave very low signal in microarray analysis (raw data<150). For low expressed miRNAs, microarray data is less repeatable by qRT-PCR compared with high expressed miRNAs. Therefore, some result from microarray could not be confirmed by qRT-PCR. Within the samples from HIV-1 mono-infected and HIV-1/HCV co-infected patients, there was no miRNA boosted in either condition. So we selected a group of miRNAs for qRT-PCR confirmation, which were highly expressed in samples and changed dependent on the type of diseaese. Variation of these miRNAs can also be potential biomarker.

Since many miRNAs in the samples from KSHV co-infected patients were destroyed before extraction, it was difficult to prove whether the down regulation of miRNAs was due to the disease procession or external effect, therefore, now we concerned only up-

regulated miRNAs. Down-regulated miRNAs should be further confirmed by properly prepared samples from KS patients.

## Conclusions

The miRNA profiles in plasma of the patients, infected with HIV-1 and opportunistic pathogens are changed as compared with healthy donors. The differences were found non-significant in the profiles of miRNA from the plasma of HIV-1 mono-infected and HIV-1/HCV co-infected patients with non-acute HIV and HCV infection. Dramatic variations in plasma miRNA profiles were found in the HIV-1/KSHV co-infected patients, who had been diagnosed with KS malignancy. Analysis by correlation efficiency, hieratical clustering, and *t*-test using GeneSpring software indicated that the level of 47 miRNAs was significantly changed in the plasma. The qRT-PCR quantitation for selected miRNAs (mir-92a, mir-29b, mir-486-5p) confirmed the microarray hybridization data. In summary, the miRNA profile in plasma can reflect physiological and pathological condition of HIV-1 mono-infected and HIV-1/KSHV co-infected patients. Our data support the hypothesis that miRNA profile can be potential universal biomarker for HIV-1 mono-infection and opportunistic viral pathogens.

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## Appendics

| Sample | Contrl_1 | Contrl_2 | Contrl_3 | Contrl_4 | HIV -1 Mono_1 | HIV-1 Mono_2 | HIV-1 Mono_3 | HIV -1 Mono_4 | HCV_1 | HCV_2 | KSHV_1 | KHSV_2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Contrl_1 | 1 | 0.943 | 0.707 | 0.960 | 0.959 | 0.927 | 0.695 | 0.931 | 0.904 | 0.737 | 0.447 | 0.458 |
| Contrl_2 | 0.943 | 1 | 0.756 | 0.893 | 0.954 | 0.938 | 0.737 | 0.914 | 0.916 | 0.787 | 0.559 | 0.463 |
| Contrl_3 | 0.707 | 0.756 | 1 | 0.649 | 0.736 | 0.760 | 0.807 | 0.692 | 0.799 | 0.888 | 0.738 | 0.611 |
| Contrl_4 | 0.960 | 0.893 | 0.649 | 1 | 0.906 | 0.871 | 0.620 | 0.948 | 0.841 | 0.667 | 0.340 | 0.330 |
| HIV-1 Mono_1 | 0.959 | 0.954 | 0.736 | 0.906 | 1 | 0.938 | 0.758 | 0.958 | 0.921 | 0.771 | 0.506 | 0.460 |
| HIV-1 Mono_2 | 0.927 | 0.938 | 0.760 | 0.871 | 0.938 | 1 | 0.769 | 0.912 | 0.943 | 0.847 | 0.574 | 0.449 |
| HIV-1 Mono_3 | 0.695 | 0.737 | 0.807 | 0.620 | 0.758 | 0.769 | 1 | 0.717 | 0.808 | 0.806 | 0.811 | 0.733 |
| HIV-1 Mono_4 | 0.931 | 0.914 | 0.692 | 0.948 | 0.958 | 0.912 | 0.717 | 1 | 0.885 | 0.736 | 0.458 | 0.388 |
| HCV_1 | 0.904 | 0.916 | 0.799 | 0.841 | 0.921 | 0.943 | 0.808 | 0.885 | 1 | 0.838 | 0.603 | 0.547 |
| HCV_2 | 0.737 | 0.787 | 0.888 | 0.667 | 0.771 | 0.847 | 0.806 | 0.736 | 0.838 | 1 | 0.643 | 0.594 |
| KSHV_1 | 0.447 | 0.559 | 0.738 | 0.340 | 0.506 | 0.574 | 0.811 | 0.458 | 0.603 | 0.643 | 1 | 0.679 |
| KSHV_2 | 0.458 | 0.463 | 0.611 | 0.330 | 0.460 | 0.449 | 0.733 | 0.388 | 0.547 | 0.594 | 0.679 | 1 |

**Supplementary table 1**. Coefficience correlation value was calculated between samples. The ones with too low CE value when compared with other samples from same group are excluded. Finally, 4 control, 4 HIV-1 mono-infected, 2 HCV co-infected and 2 KSHV co-infected samples were chosen for analysis in GeneSpring.

| systematic name | p-value | regulation | Fold change |
|---|---|---|---|
| hsa-miR-1183 | 0.0264811 | up | 2.324533 |
| hsa-miR-122 | 0.0292106 | up | 2.8622286 |
| hsa-miR-196a | 0.0222458 | up | 3.1161466 |
| hsa-miR-486-5p | 0.039238 | up | 3.5002546 |
| hsa-miR-520b | 0.0404155 | up | 2.50662 |
| hsa-miR-520e | 0.0226017 | up | 2.5667841 |
| hsa-miR-765 | 0.0077625 | up | 2.6603944 |
| hsa-miR-92a | 0.0376823 | up | 1.8419418 |
| hsa-miR-301a | 0.0144109 | down | -1.9167688 |
| hsa-miR-301b | 0.0107795 | down | -3.706739 |
| hsa-miR-338-3p | 0.0104715 | down | -2.320595 |
| hsa-miR-33a | 0.0034767 | down | -3.9031315 |
| hsa-miR-381 | 0.0315041 | down | -2.3611577 |
| hsa-miR-497 | 0.0314097 | down | -2.7919326 |
| hsa-miR-654-3p | 0.0405872 | down | -1.6563256 |

**Supplementary Table 2**. miRNAs changed between HIV-1 mono infected and control groups. Data has been filtered by expression ($p$<0.05, raw data>1.2 in all conditions)

| | KSHV coinf/HIV-1 mono | | | KSHV coinf/Control | | | HIV-1 mono/Control | | |
|---|---|---|---|---|---|---|---|---|---|
| Systematic name | p-value | regulat | Fold change | p-value | regulat | Fold change | p-value | regulat | Fold change |
| hcmv-miR-UL70 3p | 0.0343 | up | 5.0079 | 0.0258 | up | 7.3624 | | | |
| hsa-miR-1202 | 0.0013 | up | 2.9266 | 0.0175 | up | 2.4511 | | | |
| hsa-miR-1225-3p | 0.0022 | up | 5.1466 | 0.0231 | up | 5.6680 | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| hsa-miR-1225-5p | 0.0136 | up | 2.5480 | 0.0234 | up | 3.8678 | | | |
| hsa-miR-1246 | 0.0256 | up | 7.4127 | 0.0209 | up | 18.3510 | | | |
| hsa-miR-1268 | 0.0037 | up | 5.6567 | 0.0047 | up | 8.3581 | | | |
| hsa-miR-1290 | 0.0399 | up | 7.6669 | 0.0142 | up | 17.3030 | | | |
| hsa-miR-1308 | 0.0208 | up | 4.1034 | 0.0181 | up | 6.9471 | | | |
| hsa-miR-483-5p | 0.0152 | up | 3.3576 | 0.0108 | up | 5.9009 | | | |
| hsa-miR-671-5p | 0.0351 | up | 4.1861 | 0.0013 | up | 7.5250 | | | |
| **hsa-miR-765** | **0.0012** | **up** | **6.2350** | **0.0014** | **up** | **16.8295** | **0.0078** | **up** | **2.6604** |
| **hsa-miR-92a** | **0.0412** | **down** | **-3.2101** | | | | **0.0377** | **up** | **1.8419** |
| hsa-miR-557 | 0.0198 | up | 1.9280 | 0.0046 | up | 2.5001 | | | |
| hsa-miR-601 | 0.0335 | up | 2.7970 | 0.0400 | up | 2.2957 | | | |
| hsa-miR-638 | 0.0364 | up | 6.4928 | 0.0369 | up | 6.2816 | | | |
| hsa-miR-940 | 0.0325 | up | 3.1483 | 0.0110 | up | 3.2168 | | | |
| kshv-miR-K12-3 | 0.0420 | up | 6.1664 | 0.0455 | up | 6.5497 | | | |
| hsa-miR-1915 | 0.0261 | up | 8.0329 | 0.0481 | up | 7.3076 | | | |
| hsa-let-7a | 0.0407 | down | -34.0147 | 0.0042 | down | -30.2929 | | | |
| hsa-let-7b | 0.0135 | down | -5.5271 | 0.0076 | down | -4.0888 | | | |
| hsa-let-7c | 0.0213 | down | -16.3803 | 0.0218 | down | -9.2122 | | | |
| hsa-let-7d | 0.0374 | down | -33.0723 | 0.0022 | down | -30.5872 | | | |
| hsa-let-7e | 0.0394 | down | -68.2140 | 0.0000 | down | -97.9463 | | | |
| hsa-let-7f | 0.0420 | down | -32.1953 | 0.0074 | down | -33.0217 | | | |
| hsa-let-7g | 0.0401 | down | -42.1111 | 0.0004 | down | -60.6664 | | | |
| hsa-let-7i | 0.0252 | down | -12.1107 | 0.0028 | down | -19.1127 | | | |
| hsa-miR-103 | 0.0438 | down | -40.8473 | 0.0068 | down | -78.9148 | | | |

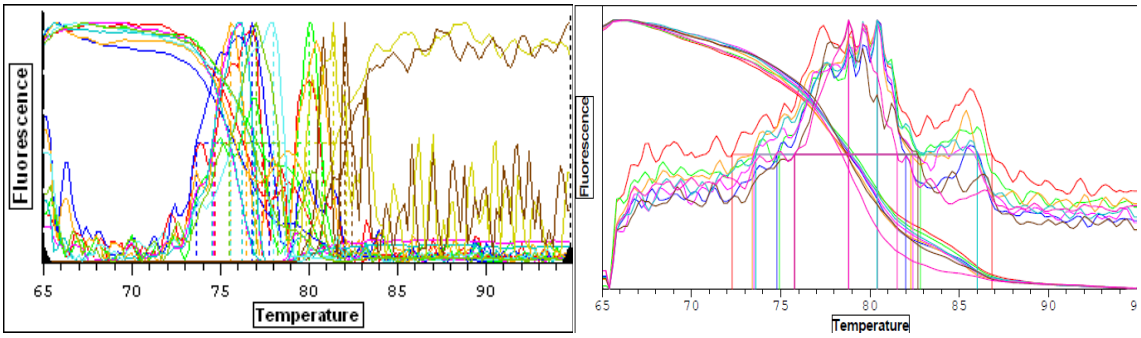
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| hsa-miR-107 | 0.0327 | down | -39.2588 | 0.0218 | down | -43.7631 | |
| hsa-miR-17 | 0.0116 | down | -44.6635 | 0.0168 | down | -32.7522 | |
| hsa-miR-199a-3p | 0.0395 | down | -20.0797 | 0.0027 | down | -38.2711 | |
| hsa-miR-19a | 0.0402 | down | -9.3075 | 0.0173 | down | -16.3032 | |
| hsa-miR-20a | 0.0139 | down | -35.3685 | 0.0224 | down | -34.4424 | |
| hsa-miR-20b | 0.0081 | down | -71.4792 | 0.0379 | down | -44.1015 | |
| hsa-miR-361-5p | 0.0226 | down | -37.4828 | 0.0057 | down | -41.9625 | |
| hsa-miR-93 | 0.0064 | down | -33.6219 | 0.0011 | down | -28.5511 | |
| hsa-miR-126 | 0.0345 | down | -13.3575 | 0.0348 | down | -23.2066 | |
| hsa-miR-130b | 0.0321 | down | -20.9756 | 0.0192 | down | -28.8494 | |
| hsa-miR-185 | 0.0230 | down | -21.4071 | 0.0309 | down | -20.2214 | |
| hsa-miR-221 | 0.0486 | down | -7.1652 | 0.0094 | down | -8.7111 | |
| hsa-miR-24 | 0.0201 | down | -6.5005 | 0.0188 | down | -8.5512 | |
| hsa-miR-27a | 0.0302 | down | -9.5005 | 0.0128 | down | -17.3531 | |
| hsa-miR-27b | 0.0221 | down | -23.0780 | 0.0082 | down | -36.6862 | |
| hsa-miR-320a | 0.0040 | down | -5.3680 | 0.0230 | down | -4.4341 | |

**Supplementary Table 3**. miRNAs changed between KSHV co-infected and control, KSHV co-infected and HIV-1 mono-infected. ($p$<0.05)

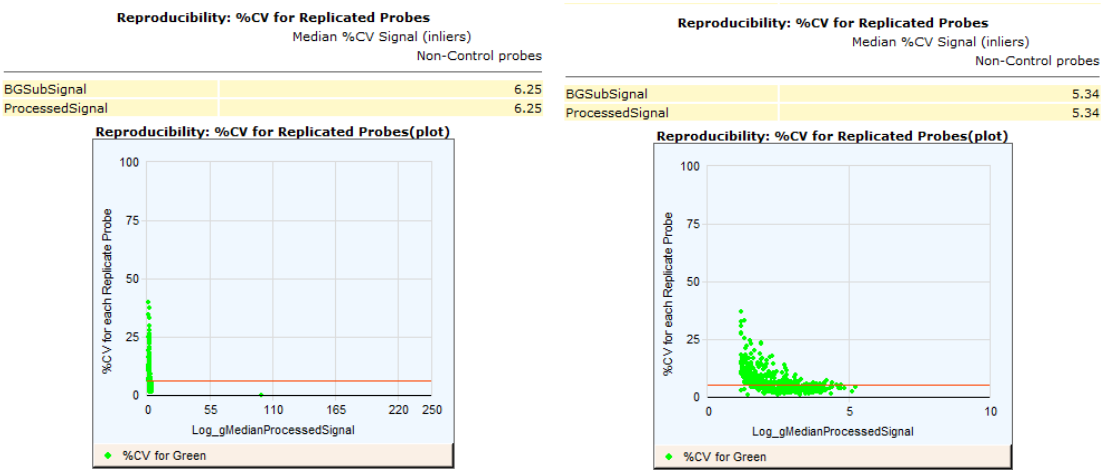
| | HCV Coinf/Control | | | HCV Coinf/HIV-1 mono | | | |
|---|---|---|---|---|---|---|---|
| systematic_name | p-value | regulation | Fold change | systematic_name | p-value | regulation | Fold change |
| hsa-let-7e | 0.0689 | down | -1.9336 | ebv-miR-BART12 | 0.0590 | down | -9.7305 |
| hsa-miR-196a | 0.0259 | up | 4.4202 | hcmv-miR-UL70-3p | 0.0764 | down | -3.2879 |
| hsa-miR-199a-3p | 0.0931 | down | -4.1865 | hsa-miR-1471 | 0.0859 | down | -2.8387 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| hsa-miR-328 | 0.0362 | down | -4.2153 | hsa-miR-328 | 0.0865 | down | -3.0450 |
| hsa-miR-331-3p | 0.0868 | down | -3.4865 | hsa-miR-451 | 0.0516 | down | -15.4198 |
| hsa-miR-766 | 0.0391 | down | -3.1112 | hsa-miR-505* | 0.0250 | down | -5.6481 |
| | | | | hsa-miR-575 | 0.0567 | up | 11.9765 |
| | | | | hsa-miR-933 | 0.0113 | down | -1.5290 |
| | | | | hsv1-miR-H1_v14.0 | 0.0564 | down | -3.5087 |

**Supplementary Table 4**. miRNAs changed between HCV co-infected and control, HCV co-infected and HIV-1 mono-infected. ($p$<0.1)




**Supplementary Fig. 1**. Melting curves of snU6 (left one) and mir-1207-5p(right one) in qRT-PCR.



**Supplementary Fig. 2.** %CV from QC metric report. Lower the %CV value is, higher the reproducibility of replicated probes is. The left sample was prepared with TRIreagent only, and right one was prepared with mirVANA.