JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Use of Flaviviral genetic fragments as a potential prevention strategy for HIV-1 Silencing

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Abstract

Introduction: Coinfection with certain members of the *Flaviviridae*, such as Dengue Virus (DV), West Nile Virus (WNV) Yellow Fever Virus (YFV) and most importantly, GBV-C have been documented to reduce HIV-1 viral load *in vivo*. Numerous studies strongly support the notion that persistent coinfection with non-pathogenic virus prolongs survival in HIV-1 infected individuals. Coinfected individuals show higher CD4+ cell counts, lower HIV-1 RNA viral loads and live three times longer than clinically matched HIV-1 monoinfected patients. We have previously shown that one of the major anti-HIV defenses conferred by GBV-C coinfection is the upregulation of intracellular miRNAs in CD4+ cells that share significant mutual homologies with GBV-C and HIV-1 (>80%) genomes.

Methodology: Genome-wide bioinformatics analyses were carried out to search for miRNA binding sites in mutual homologies between HIV and several members of the *Flaviviridae*

Results: Several miRNAs shared significant mutual homology with HIV-1 genetic sequences and GBV-A, B, C, DV, WNV and YFV. These may be responsible for beneficial effects in HIV-1 infected individuals. Three highly mutual homologous miRNAs (i.e. miR-627-5, miR-369-5 and miR-548f), expressed in CD4+ cell lines, reduce HIV-1 replication by up to 90% whereas miRNAs with low mutual homologies (i.e. miR-34-1 and miR-508) impart only slight inhibition of HIV-1.

Conclusion: We hypothesize that a recombinant GBV-C-based vector can be constructed which expresses several beneficial genetic motifs of the *Flaviviridae* without causing any side effects while stimulating a wide array of beneficial miRNAs that can more efficiently prevent HIV-1 infection.

Key words: Flaviviruses; HIV-1; miRNAs; vaccine.

J Infect Dev Ctries 2016; 10(8):870-879. doi:10.3855/jidc.7386

(Received 06 July 2015 – Accepted 16 January 2016)

Introduction

HIV-1 infection is often accompanied by co-infection with other pathogens that generally result in accelerating the progression of the disease and early development of AIDS *i.e.* tuberculosis, HBV, CMV, EBV, etc. [1,2]. However, certain co-infections may sometimes have beneficial effects [3-5]. Several reports have documented the favorable properties of flaviviral coinfection [3-5]. Currently, the *Flaviviridae* is divided into four genera: *Flavivirus*, that includes WNV, DV and YFV; *Hepacivirus* that includes hepatitis C viruses, which are divided into six types, A, B, C, D, E and G; *Pestivirus* that includes swine fever virus (SFV) and bovine diarrhea virus (BDV); and the newly-designated *Pegivirus* that includes GBV-A, B and C in humans and GBV-C_{cpz} in the chimpanzee [6]. Members of the *Flaviviridae* contain a single-stranded, positive sense RNA genome encoding a polyprotein that is post-translationally cleaved into structural and nonstructural proteins. The Flaviviridae genome contains about 9,400 nucleotides and has a single open reading frame (ORF) [7]. This particular ORF is capable of encoding a polyprotein made up of about 2,844 amino acids, which is then cleaved by both viral and cellular proteases, resulting in its functional and non-structural proteins. E1 and E2 (envelop surface glycoproteins) represent structural proteins, whereas non-structural proteins embody the protease NS2, the serine protease/RNA helicase NS3, the RNA dependent polymerase NS5B, as well as NS4, and NS5A. The members of the Flaviviridae replicate through their respective intermediary negative strands. All flaviviruses are generally similar in their genome structure and replication strategies with a few important differences [7]. For example, the nonstructural protein 5 (NS5) of pestiviruses, hepaciviruses, and pegiviruses are processed into two products termed NS5A and NS5B. NS5A is a multifunctional phosphoprotein required for infectivity and RNA replication, and NS5B contains polymerase activity [7]. In contrast, the NS5 proteins of

the *Flavivirus* genus are not processed and retain polymerase activity [7,8]. Members of *Flavivirus* are pathogenic to humans, whereas members of *Hepacivirus* (i.e. HCV) and *Pegivirus* (i.e. GBV-C) cause persistent infections [7]. For example, HCV may cause hepatic failure and is the major cause of hepatocellular carcinoma in infected individuals. While most studies have reported the beneficial effects of GBV-C in HIV-1 co-infected patients, data are accumulating to show varying degrees of benefit for other flaviviruses [8-12]. GBV-C is a close relative of GB virus A, GB virus B, and HCV. Pegiviruses are known to be non-pathogenic [13, 14]. We propose the development of a recombinant GBV-C-based viral vaccine that can incorporate the major "beneficial anti-HIV-1 genetic fragments" that can serve as a preventive vaccine against HIV-1.

During the course of viral infections, viruses from similar species compete with each other to gain access to the host's replication machinery. Successful viruses eventually block the entry of competitors into the host cell by receptor modulation or by intracellular interference in its replication by various mechanisms [1-3]. Viruses from heterologous species can nonetheless co-infect cells cooperatively where one virus provides another with a useful factor that it co-opts for its own use [1-3]. Rarely, one type of virus becomes dependent on another virus. One such example is hepatitis Delta, which requires the presence of hepatitis B virus in order to replicate. Viruses also interact with each other directly at molecular levels [15,16]. The aim of the present investigation is to gain an insight into viral RNA-host miRNAs interactions *in situ* between HIV-1 and co-infecting members of the *Flaviviridae* that have demonstrated beneficial effects at the intracellular level and to dissect the mechanisms of HIV-1 inhibition mediated by miRNAs modulated by the Flaviviridae [3, 16]. Eventually, it is our goal to develop a recombinant Pegiviral-based vaccine that incorporates the numerous beneficial genetic motifs of members of the *Flaviviridae* without any adverse effects.

Methodology

Mature miRNA database and gene alignment

At the time of our studies (Sept 2013) 2,578 mature *hsa-miRs* were listed in the Sanger database. By utilizing the human miRBase sequences database [http://microrna.sanger.ac.uk/sequences /version 20.0), the *hsa-miR* sequences were first downloaded from the database and then aligned with each of the flaviviral genomes, individually, as described previously [17]. For each virus the full length sequences were downloaded in FASTA format from the Nucleotide and gene bank database (NCBI). The specific gene sequences were utilized for each of the six viruses: GBV-A NC_001837, GBV-B NC_001655, GBV-C AF121950.1, GBV-C_{cpz} AF070476, DV EF629366, WNV HM147822 and YFV NC_002031 were used. Before analysis, each U of *hsa-miRs* was converted to a T. In addition, since alignment tools are generally programmed in the FASTA format, all the genomic sequences were annotated in FASTA format before the alignment process. The reference genome sequences of both viruses were obtained from http://www.ncbi.nlm.nih.gov/. Following this, we utilized multiple alignment tools to search for miRNAs that shared identities with both viruses as previously described [17-19].

Determination of miRNA alignment to viral sequences

To determine the suitability of each of the *hsa-miRNAs* as a potential therapeutic agent, we developed and refined the algorithm that incorporates the three critical elements that increases the suitability of a miRNA as a successful silencing agent [17]. These include: 1) the length of the complementary pairing between *hsa-miRNAs* and their target sites in viral genomes: generally, in plants miRNA targets exhibit complete homology at the ORFs, whereas the binding between animal miRNAs and their targets show incomplete homology in base-pairing, binding sites at 3'UTRs, 5'UTRs and the coding regions of target genes. Therefore, it is important that the length of the targeting miRNAs is 19 bp or above for silencing to take place. In this case we 1) downloaded the available miRNAs from miRbase and aligned both of the members of the Flaviviridae and HIV-1 for the mutual homologies in each of the genomes; 2) seed sequence complementarity: a near-perfect alignment at miRNA seed sequences located at the 3'-untranslated region (UTR) base pair 2 to 8 that signals a successful silencing match [20] and 3) high degree of complementarity: an 80%-90% degree level of homology of the sequences of miRNAs with each of the selected members of the Flaviviridae genomes being considered as highly significant ($p<0.001$) and reported to significantly reduce the "off-target" silencing of other genes [17-20].

Cell lines

The Jurkat cell line was obtained from NIH AIDS Research & Reference Reagent Program (Catalogue numbers 3233, 100. 1109 and 175, respectively) and

were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 UI/mL penicillin, 100 µg/mL streptomycin and 2 mM/L-Glutamine [17].

Intracellular Expression of hairpin miRNAs

pEGP vector, purchased from Biolab [21], was utilized for expression of each of the five miRs' gene sequences (miR-627-5, miR-369-5, miR-548f**,** miR-34-1 and miR-508). Positive clones were confirmed by sequencing on an ABI PRISM 3130 sequencer as well as by BgIII-HindIII restriction enzyme digestion [21]. A total of six different miRNAs gene constructs were generated, five expressing the respective duplex miRNA, and one was used as a control that contained an empty vector without any miRNA insert [6]. The six transfected cell lines were used to develop stably transfected cell lines as described previously [6]. Three (*i.e.* miR-627-5, miR-369-5 and miR-548f) contained miRNAs with a high degree of mutual homologies to HIV-1 and flaviviruses. Two contained miRNAs with a low degree of homology to HIV-1 and flaviviruses (miR-34-1 and miR-508). The Jurkat cell line was transfected by electroporation utilizing 10 ng of the cloned plasmids (Neon™ Transfection System, Cat. No. MPK5000, Korea). The G418-(neomycin)-selected clonal cell lines were first maintained in G418-free medium for at least 2 weeks prior to HIV-1 infection as described before [17].

HIV-1 challenge studies

Molecular clones of HIV-1 (pNL4-3: Cat #114) were obtained from the NIH AIDS Research & Reference Reagent Program. All the studies were conducted using the HIV-1 strain pNL4-3. Viral stock was prepared as described previously [22]. The Jurkat cell lines expressing various miRNA were incubated with infectious NL4-3 virus for 12 h. Cells were then washed four times with pre-warmed, serum-free medium. Cells were maintained in complete RPMI 1640. Every six days, cells were split 1:4 to maintain a cell density of approximately 10^6/ml, and the culture supernatants were collected for HIV-1 p24 antigen analyses. The HIV-1 p24 antigen levels in supernatants were determined by enzyme-linked immunosorbent assay (ELISA; Zeptomatrix) [17, 22]. Cell viability was monitored using the Eosin-Y viability assay. Each experiment was repeated at least 3 times.

Results

We analyzed the homologous sequences of *hsa-miRs* submitted to the miRNA database (www.miRbase.org/) and full length coding sequences of six members of the Flaviviridae *viz.*, *Pestivirus* (GBV-A, GBV-B, GBV-C) and *Flavivirus* (DV, WND, YFV). We discovered 69 *hsa-miRs* that showed near 80% mutual homology to different genomic motifs of the six different members of the *Flaviviridae* and HIV-1. Seven miRNAs exhibited >80% mutual homology to more than one *Flavivirus* and to HIV-1 (Table 1). As shown in Figure 1, these seven miRNAs shared homologies to *E2, NS2B, NS3, NS5A* and *NS5*B as well as targeted various motifs of HIV-1 including *5" LTR, gag, pol, tat* and *env*. As presented in Table 1, seven miRNAs exhibited significant mutual homology to two or more members of the Flaviviridae. Consequently, *hsa-miR-218-5p* showed homologies to GBV-A and YFV, *hsa-miR-3611* showed homologies to GBV-C and DV*, hsa-miR-3612* showed homologies to GBV-A, GBV-B and DV*, hsa-miR-2276-3p* showed homologies to GBV-C and YFV*, hsa-miR-3918* showed homologies to GBV-C and DV*, hsa-miR-200b-5p* showed homologies to GBV-C, DV and YFV and *hsa-miR-4754* showed homologies to GBV-A, DV and YFV. None of the seven miRNAs displayed homology to WNV. By utilizing information from Table 1, we developed a composite alignment map of the seven

Table 1. The miRNAs showing identity with HIV 1 and three or more than three other Flaviviruses.

No	miRNA	Flaviviruses					
		A	**B**	**C**	**D**	**W**	**Y**
1	hsa-miR-218-5p	✓					✓
2	hsa-miR-3611			✓	✓		
3	hsa-miR-3612	✓	✓		✓		
4	hsa-miR-2276-3p			✓			✓
5	hsa-miR-3918			✓	✓		
6	hsa-miR-200b-5p			✓	✓		✓
7	hsa-miR-4754	✓			✓		✓

A = Hepatitis GB virus A; B = Hepatitis GB virus B; C = GB Virus C variant troglodytes; D = Dengue Virus Type 3 strain BR DEN# 95-04; I = Hepatitis G virus strain Iowa; W = West Nile virus from South Africa; Y = Yellow Fever Virus.

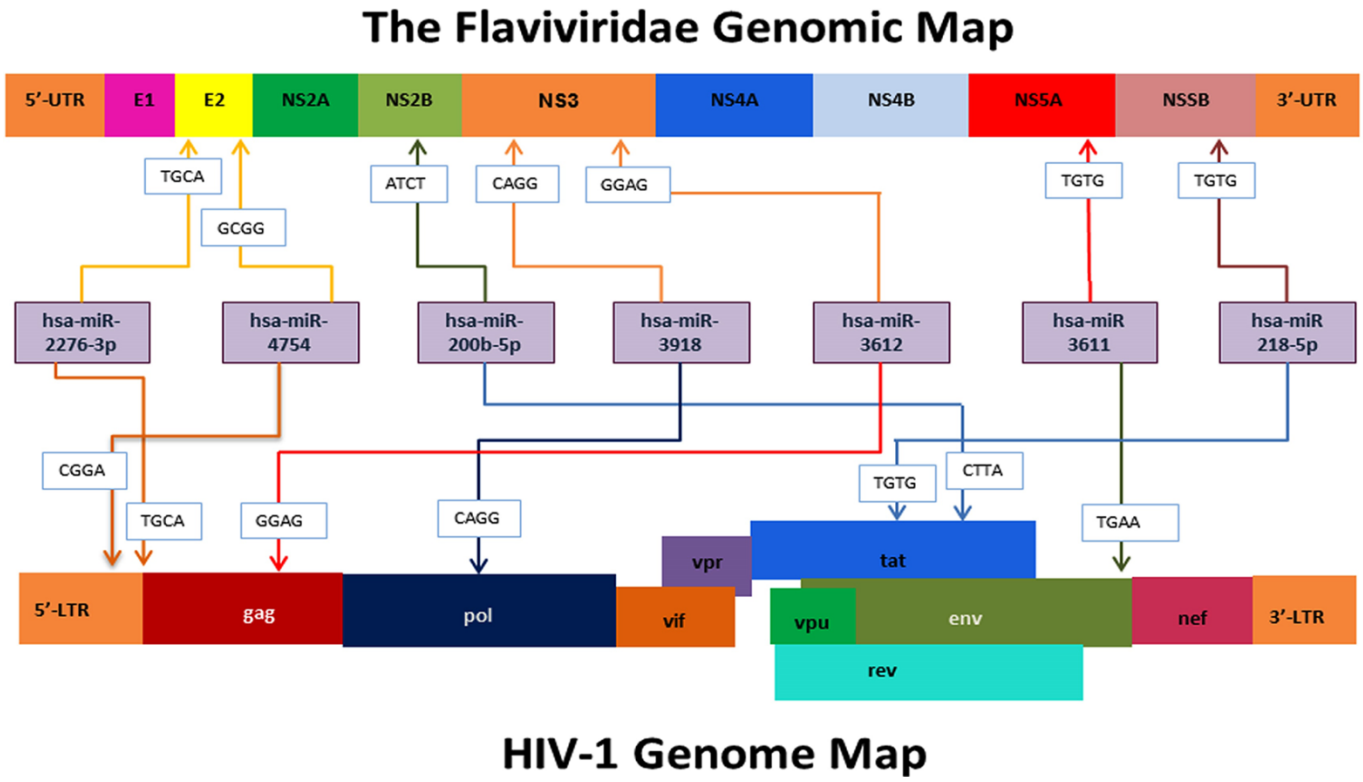
miRNAs that aligned with the HIV-1 genome. Figure 1 shows the composite genomic map of the Flaviviridae. As revealed, two human miRNAs: *hsa-miR-2276-3p* and *hsa-miR-4754* exhibited significant homology to the envelop 2 (*E2*) gene of the Flaviviridae. Similarly, *hsa-miR-200b-5p* expressed homology to the non-structural protein gene NS2B, whereas *hsa-miR-3918* and *hsa-miR-3612* illustrated homology to NS3. *hsa-miR-3611* and *hsa-miR-218-5p* expressed homology with NS5A and NS5B, respectively.

Nine miRNAs shared >80% homology to HIV-1 and GBV-A (data not shown), 16 miRNAs had mutual homology to HIV-1 and GBV-B (data not shown), 17 miRNAs were mutually homologous to GBV-C (data not shown), 14 miRNAs shared homology to GBV-C_{cpz} (data not shown), 14 miRNAs shared homology to DV (data not shown), 18 miRNAs to WNV (data not shown) and 22 miRNAs to YFV (data not shown) and HIV-1. As summarized in Table 1, we discovered seven *hsa-miR*s that showed alignments to two or more members of the Flaviviridae and a significant mutual homology to HIV-1. All the miRNAs listed in Table 1 fulfilled the three fundamental criteria that we established previously [17, 19] as critical elements that increase the suitability of a miRNA as a successful silencing agent (*vide supra*).

Eight nucleotide Seed Sequence base-pairing value in seed region of the binding site

MiRNA targets commonly have at least one region that has Watson-Crick pairing to the 5' part of miRNA. Normally a miRNA seed is defined as the consecutive 8-nt sequence starting from the second base at the 5' end of a miRNA. The seed region has the most important features for target recognition [20,23]. In order to get better results for predicting HIV-1 RNA targets from human microRNAs, we conducted base-pairing comparative studies between whole genomes of HIV-1 and six members of the Flaviviridae (i.e. GBV-A, B and C, DV, YFV and WNV) from the NCBI and human miRNAs from miRBase. The hypothesis is that upon infection with any of the six members of the Flaviviridae the intracellular immune defenses will be activated and upregulate numerous miRNAs, some amongst them showing homology to HIV-1 RNA and hence being able to block its replication at various stages of the life cycle [3,24].

Figure 1. Locations of homologous miRNAs in Flaviviridae/HIV-1 genomic structures.



Genomic Structures of both Flaviviridae genome (top) and HIV-1 genome (bottom) showing the location of seven mutually homologous miRNAs, carried out by multiple alignment tools.

We discovered that *hsa-miRs*: *hsa-miR-218-5p*, *-6833-5p. -6787-3p, -3912-3p, -410-3p, -365b-3p, -4788, -633* and *-4754* exhibited significant mutual homologies, varying from 100% to 62% identity at the seed sequence to both HIV-1 and GBV-A genomes. Overall, whole genome searches for mutual homologies showed significant homologies (i.e. ~80%).

In addition, 16 human miRNAs that have mutual homologies to GBV-B and HIV-1. Three among them: *hsa-miR-3132, hsa-miR-4637* and *hsa-miR-505-3p*, expressed 100% homology to HIV-1 genomic motifs at the seed sequences.

Homology analyses showed that 17 human miRNAs that have mutual homologies to *Homo sapien*s' GBV-C and HIV-1. All of miRNAs showed high degrees of identity to HIV-1, varying from 80-84%. Three among them, namely *hsa-miR-6788-5p, hsa-miR-4719* and *hsa-miR-298*, expressed 100% homology to HIV-1 genomic motifs at the seed sequences.

Similarly, 14 human miRNAs that have mutual homologies to chimpanzee GBV-C_{cpz} and HIV-1. One amongst the 14, *hsa-miR-34b-5p*, revealed 100% homology to HIV-1 at the seed sequence, whereas seven of them *viz.* human miRNAs *hsa-miR-361, hsa-miR-4528, hsa-miR-4726-5p, hsa-miR-1284, hsa-miR-3912-3p, hsa-miR-141-3p* and *hsa-miR-486-3p* illustrated 87.5% homology at the seed sequences with HIV-1.

Further homology alignment showed 14 human miRNAs that have mutual homologies to DV and HIV-1. Five of the human miRNAs, namely *hsa-miR-3611, hsa-miR-3612, hsa-miR-1178-3p, hsa-miR-3915, hsa-miR-150-3p* and *hsa-miR-6778-5p* displayed 87.5% homology at the seed sequences with HIV-1.

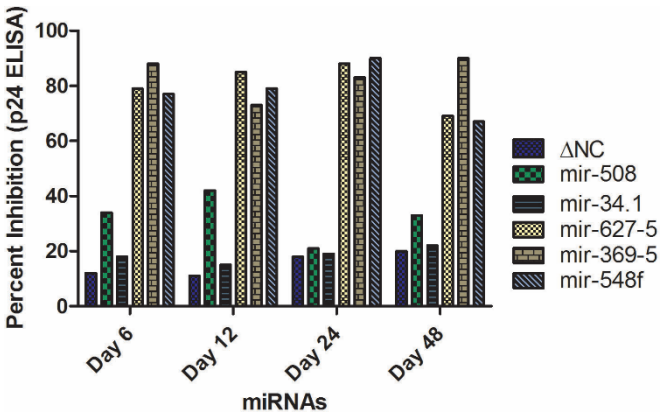
Analyses of homology showed 22 human miRNAs that have mutual homologies to YFV and HIV-1. Two among them, *hsa-miR-4719* and *hsa-miR-34b-5p*, exhibited 100% homology to the HIV-1 genomic motifs at the seed sequences. Nine human miRNAs: *hsa-miR-218-5p, hsa-miR-4436b-3p, hsa-miR-4411, hsa-miR-141-3p, hsa-miR-3672, hsa-miR-6785-5p, hsa-miR-3669, hsa-miR-4644-5p* and *hsa-miR-6506-5p* exhibited 87.5% homology at the seed sequences with HIV-1.

Finally, analyses of homology showed 18 human miRNAs that have mutual homologies to WNV and HIV-1. Five among them, *hsa-miR-3132, hsa-miR-4422, hsa-miR-6788-5p, hsa-miR-298,* and *hsa-miR-4782-5p* presented 100% homology to HIV-1 genomic motifs at the seed sequences. Six human miRNAs: *hsa-miR-6750-5p, hsa-miR-3914, hsa-miR-6892-3p, hsa-miR-2278, hsa-miR-6870-3p,* and *hsa-miR-1295a* expressed 87.5% homology at the seed sequences with HIV-1.

Flaviviral-regulated miRNA-induced gene silencing of HIV-1

As a proof of concept we randomly selected three miRNAs with high homologies to three different members of the Flaviviridae and HIV-1 from Table 1. *miR-627-5* showed also high degrees of mutual homology to YFV (76%) and HIV-1 (80%) at the genomic levels and 100% and 85% at the seed sequences (Table 1). *miR-369-5* showed mutual homologies to GBV-C, 76% and HIV-1, 80% at genomic levels and 85% and 71% at the seed sequences respectively. Similarly, *miR-548f* exhibited mutual homologies for GBV-B and HIV-1 at 82% and 80% at genomic levels and 71% and 85% at the seed sequences, respectively. The HIV-1 inhibitory activity of each of these three mutually homologous miRNAs was examined by cloning them into a miRNA expression vector (pEGP, a miRNA retroviral based expression vector) [21]. Comparison with un-transfected Jurkat cells showed that the three cell lines expressing a high degree of homologous miRNAs significantly inhibited HIV-1 during the 48 day period of observation. The three other cell lines, two expressing non-homologous miRNAs (*miR-34.1* and *miR-508*) and a third one, an empty vector (ΔNC), were used as the negative controls [8,9]. The empty vector was used to determine whether expression of EGP adversely interfered with HIV-1 replication since a concern was that over-expression of EGP may occupy the protein synthesis machinery that might indirectly slow down HIV-1 replication by

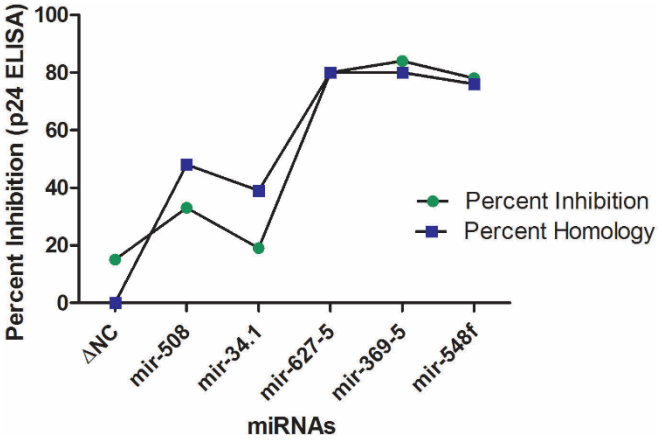
Figure 2. Effects of miRNAs on HIV-1 replication.



Relative inhibition of HIV-1 replication in human T-lymphocytic cell lines stably transfected with three highly homologous miRNAs, two least homologous miRNAs compared to untransfected cell line (□NC).

partially occupying the translational machinery of the infected cells. As shown in Figure 2 all three cell lines expressing highly homologous miRNAs significantly inhibited HIV-1 ($P<0.001$) as measured by HIV-1 p24 ELISA, when compared to the un-transfected Jurkat cell line and to the cell lines with empty vectors and two non-homologous cell lines. The inhibition assays were carried out for 48 days and the degree of inhibition ranged from 67% to 90% during the period of evaluation. In contrast, the empty vector and the two non-homologous miRNA-expressing cell lines exhibited a certain degree of inhibition, varying from 11% to 34%; but they were statistically not significant ($P<0.83$). Comparisons between the degree of homology and percentage of HIV-1 inhibition is shown in Figure 3, and suggests a direct correlation between the degree of homology of miRNAs to HIV-1 genetic motifs and percentage viral inhibition.

Figure 3. MiRNA-based inhibition of HIV-1.



miRNA-directed inhibition of HIV-1 replication is directly proportional to the percent of sequence mutual homologies to Flaviviridae/HIV-1 genomes.

Discussion

Members of the Flaviviridae, such as Pegiviruses (i.e. GBV-A, B and C) and HCV, cause persistent chronic infections. GBV-C, a non-pathogenic member of the *Pegivirus*, which is commonly found in HIV-1 infected patients, has received a great deal of attention since coinfection with this virus is known to impart beneficial effects in HIV-1 infected individuals [3,25,26]. HIV-1/GBV-C co-infected individuals display higher CD4+ T cell counts, lower mortality rate, slower progression to AIDS and survive three times longer than HIV-1 monoinfected individuals [3,26,27]. Researchers debate whether the beneficial effects of co-infection of GBV-C in HIV-1-infected individuals are due to GBV-C viremia, or rather the presence of GBV-C anti-E2 antibodies or GBV-C NS5 antigen [3,5,27]. Previously, we demonstrated that the observed beneficial effects are due to modulation of the miRNAs in the coinfected host cells influencing the intracellular defense system [3,27]. We forwarded the hypothesis that infection with a number of flaviviruses (i.e. GBV-C, DV, WNV, etc.) might result in a cascade of miRNA modulations creating dramatic changes in the intracellular profile of miRNA [3,27]. Some of the upregulated miRNAs share homologies to HIV-1 and the HIV-1 infected individual fortuitously benefits from such a coinfection. We also have shown that the major beneficial effects are achieved when the GBV-C infection precedes HIV-1 infection [27]. Here, by utilizing computational analyses and gene alignment tools, we have expanded our observations to suggest that besides GBV-C, co-infections with other members of the Flaviviridae may also impart beneficial effects, including DV, YFV and WNV; all of these exhibit mutually homologous miRNAs. Numerous clinical reports and *in vitro* data support the notion that the most likely mechanisms of HIV-1 silencing observed with the Flaviviridae co-infection are mainly due to miRNAs.

It must be noted that DV, YFV and WNV constitute significant global human health problems [7]. However, our understanding of the molecular interaction of WNV, DV and YFV with mammalian host cells is limited. Generally, *Flaviviridae* infection encodes only 10 proteins, implying that the virus uses many cellular proteins for infection and that a large degree of cellular manipulation is required to take over the host's transcription and translational systems [7]. *Flaviviridae* enters the cytoplasm either via the CD4-receptor or through pH-dependent endocytosis, undergoes cycles of translation and replication, assembles progeny viral particles in association with endoplasmic reticulum, and exits along the secretory pathway [3,12].

MiRNAs Silence Numerous Viruses in Susceptible Hosts

miRNA provides intracellular immune defense when the body is faced with challenges from transgenes, viruses, transposons, and aberrant mRNAs [16-18,24]. MiRNA molecules trigger gene silencing in eukaryotic cells [24,28,29]. More than 3,000 different human miRNAs (*hsa-miRs*) have been identified thus far, and it is generally agreed that cellular gene regulation is significantly impacted by cellular miRNAs [16,28]. Recent findings demonstrate that some viruses can actually encode miRNAs if these are processed

through cellular miRNAs [29]. During ontogenesis, viral or intracellular parasitic infections, miRNAs are differentially expressed [33]. A single miRNA has the complex capacity to target multiple genes simultaneously [16,28,29].

Sustained research from various laboratories suggests how miRNAs control RNA and DNA viral replication; invading viruses can be recognized by cellular miRNAs, which can target specific genetic material [30]. A substantial part of eukaryotic genomes is made up of retroelements (RE) (e.g., lentiviruses, retroviruses, retrotransposons, and transposons). Given genetic mutational capacity, these particular mobile elements consistently threaten host genomic integrity. RE mutagenic ability may be silenced by sophisticated molecular mechanisms [18,24,27,30]. This apparently occurs via a process of strategic RE expression involving genetic entities that have become incorporated during past evolutionary periods [18]. These RE pieces exert a fundamental influence on the silencing of exogenous retroviruses (IERVs), as well as human endogenous retroviruses [18,30].

The role that miRNAs play in quelling infectious agents cannot be underestimated. Lecellier *et al*. [31] were one of the first investigators to directly show the anti-PFV activities of *miR-32*. Knock out of *miR-32* doubled the rate of viral replication. MiRNAs play key regulatory roles in diverse biological processes and are frequently modulated in human diseases. Thus, miRNAs have emerged as a class of promising targets for therapeutic intervention. For example, many miRNAs have key roles in viral pathogenesis of HCV infection across multiple tissue compartments [32,33]. HCV utilizes host cellular miRNAs and modulates expression of miRNAs in infected hepatocytes for its own propagation and infection. Moreover, such miRNAs directly or indirectly alter HCV replication efficiency and induce liver diseases including liver fibrosis, cirrhosis, or HCC. HCV is critically dependent on the cellular miRNA *miR-122* for translation of its RNA genome [7] and studies have shown that *miR-122* directly modulates the HCV life cycle by increasing HCV translation and genomic RNA stability. Recently, a phase IIa clinical trial with *Miravirsen*, a locked nucleic acid (LNA) form of anti-*miR-122* oligonucleotides, showed significant reduction in serum HCV levels in patients chronically infected with HCV with no detectible evidence of resistance [32,33]. In addition to *miR-122*, other miRNAs, involved in the regulation of HCV propagation, have been identified and could be targeted in strategies to modulate HCV replication and pathogenesis.

miRNAs present a powerful forward genetics approach to dissect virus-host cell interactions [4–6]. By using human genome-wide miRNA multi-genome alignments we identified a total of 76 human miRNAs that can potentially silence HIV-1 at different stages of the viral life cycle. We maintain that cellular miRNAs not only play a pivotal role in HIV-1, but also regulate its latency and suppression of the integrated HIV-1 provirus. Cellular miRNAs may allow the establishment of latency and escape from immune surveillance [3, 34, 35].

Numerous clinical reports have confirmed that infections with various Flaviviruses results in serendipitous benefits to the human host, even in cases of flaviviral infections that are known to cause significant morbidity and mortality such as DV, YFV and WNV. For example, Gonzalez *et al.* [36] have reported that coinfection with DV in HIV-1 infected patients resulted in stable CD4+ T cell counts and no acceleration in progression to HIV-1 to AIDS. López-Lemus *et al.* [9] and McLinden *et al*. [10] have shown DV NS5 expression interference with HIV-1 replication *in vitro*.

Seven members of Flaviviridae homologous host miRNAs target HIV-1

We have been concentrating on mechanisms by which the members of the *Flaviviridae* inhibit HIV-1 replication. These mechanisms include CD4+ receptor modulations by NS4B, NS3 or NS5 proteins [8,10]. In order to determine whether HIV inhibition induced by flavivirus NS5 protein expression is conserved among additional members of the *Flaviviridae*, Xiang *et al*. [12] characterized the effect of NS5 protein expression for five members of the virus family in $CD4^+$ T cells by plasmids containing the genetic sequences of NS5 DNAs. In addition, they examined the YF 17D vaccine strain of T lymphocytes and macrophages to determine whether the effects of NS5 protein expression were recapitulated during viral replication. These studies identified a mechanism by which flavivirus NS5 protein modulates T cell homeostasis and that may contribute to HIV replication inhibition. They demonstrated that expression of the NS5 protein of the five different members of the *Flaviviridae* decreased CD4 expression in a CD4+ T cell line and in primary CD4+ T cells with resultant inhibition of HIV replication. Although CD4+ T cells are not the primary target for DV, WNV, YFV or HCV, their results suggested that all of these viruses infect these cells. In contrast, human T and B cells are

permissive for pegiviruses and appear to be the primary site of viral replication [3]. In the above-mentioned studies, the key point that needs to be made is that in each of the cases, the observed protective effects of the NS5 from the *Flaviviridae* were due to the presence of NS5 expressing nucleic acid sequences (DNA or RNA vectors) [12]. We maintain that in all these cases the actual protective mechanism may primarily be due to stimulation of anti-HIV-1 homologous miRNAs that results from the presence of the specific nucleic acid expressions of the *Flaviviridae* associated homologous miRNAs in the host cells and not the presence of NS5 protein.

From various reports, (*vide supra*), it appears that the *Flaviviridae* in general, and GBV-C infection in particular, imparts beneficial effects *vis-à-vis* HIV-1 replication [3,4,25]. However, the issue that appears to be of importance is whether NS5 [12] protein, or other viral proteins [10], or the antibodies (Abs) to the *Flaviviridae*, envelops anti-E2 Abs that the host produces are those that actually quell HIV-1 replication? At this point, it is reasonable to explore why the *Flaviviridae* viremia would quell HIV-1 replication. HIV-1 replication involves several events divided into at least seven steps [3]. Step 1 starts with the attachment of an HIV-1 viral particle to the CD4+ T lymphocyte or macrophage/monocyte membrane. With the help of a co-receptor (CCR5 or CXCR4), the virion's envelope fuses with the host membrane, and the virion's core gains access to the cellular cytoplasm. In step 2, which takes place within the cytoplasm, one of the virion's RNAs is reverse transcribed into cDNA (step 3), after which dsDNA (step 4) is formed. The dsDNA, known as provirus, forms complexes with intracellular miRNAs and cellular proteins, now known as a pre-integration complex (PIC) [34,35]. The most rate-limiting step is step 5, where an HIV-1 PIC can remain dormant for a long time in a resting CD4+ T cell [34,35]. Upon activation, the HIV-1 provirus can secure entry into the host genome, integrate, and begin to reproduce (step 6). In the last step, the integrated HIV-1 viral genes begin to transcribe and new HIV-1 particles are produced from the host cells [35]. Therefore, if HIV-1 replication inhibition by GBV-C is somehow connected to any of the intracellular events, then it is most likely associated with one or two intracellular events in which GBV-C replication might cause interference in one or more of the HIV-1 replication steps [3,27].

On the other hand, if HIV-1 entry is blocked by the extracellular event, as has been proposed by various groups [12,37], whereby the *Flaviviridae* NS5-protein or E2 Abs may be interfering with HIV-1 entry, then it is likely interfering with step 1; either by blocking the HIV-1 binding sites (i.e., CD4, CCR5, or CXCR4 molecules) by structural mimicry, or through indirect interference at the entry sites [14]. Obviously, this would not be a logical explanation since only pegiviruses infect T- and B- lymphocytes and other members of the *Flaviviridae* do not infect CD4+ T lymphocytes [3]. We believe that the *Flaviviridae* viral-based quelling of HIV-1 replication should be viewed through the lens of well-established immunological principles. We maintain that the onset of the viremia, and its later clearance by anti-E2 Abs (or other Abs), may not be mutually exclusive events. We realize that the *Flaviviridae* antigens are viewed by the host's immune system as "foreign", and that a classical immune response to antigens is a normal physiological response [3,35]. Therefore, the immune system of any individual exposed to the virus will eventually respond to the *Flaviviridae* viral antigens by producing either humoral immunity (i.e., Abs) or cell-mediated immunity (CMI). In other words, all the *Flaviviridae* infected individuals will produce Abs or CMI to the *Flaviviridae* antigens. A crucial question arises: At what stage during the *Flaviviridae* infection do the beneficial effects of the virus become apparent? Does it occur at the early stage of infection when there is elevated viremia? or, later when viral antigens have disappeared and the anti-viral Abs can be measured by immunological means? Previously, we established that pre-exposure to the GBV-C appears to be more important for the antiviral effects when the host cells are primed with anti-HIV-1 homologous miRNAs in cell lines stably transfected with particular miRNAs [3,27]. It is also theoretically possible that despite the presence of anti-*Flaviviridae* Abs, the viral RNA may still be present in the CD4+ T lymphocytes and B-lymphocytes providing continuous protection against pegivirus/HIV-1 co-infected individuals [3].

The Flaviviridae and HIV-1 Homologous miRNAs

Overall, these findings provide an illustration of how the *Flaviviridae*-exposed cellular miRNAs have evolved to control HIV-1 infection in co-infected individuals. Clinical reports indicate that GBV-C and some other members of the *Flaviviridae* infections are able to quell HIV-1 replication [3-6,8-12,25,27]. Our computational analyses, which show that a large number of mutually homologous miRNAs are present that may quell HIV-1, suggest that the mechanisms of the beneficial effects of the *Flaviviridae* co-infection against HIV-1 likely is via miRNAs and other

mechanisms reported by various investigators may be of secondary nature, as has been previously reviewed [5-6,8-12,25,27]. If the hypothesis of extracellular structural mimicry is correct, then HIV-1 and GBV-C protein structures must show some degree of mutual homology. Several groups have shown neutralization of diverse isolates of HIV-1 by GBV-C E2 Abs or synthetic peptides, but have found no structural homology [9,12,38-39].

We have approached the homology issue at the molecular (intracellular) level: one at which miRNAs have been documented to interfere in viral replication [3,20,21]. Do the *Flaviviridae* and HIV-1 share miRNAs? Do the *Flaviviridae* homologous miRNAs also share homologies to HIV-1? If so, then the mutually homologous miRNAs would be able to quell HIV-1 replication. In order to evaluate whether the beneficial effects of the *Flaviviridae* may be due to mutually homologous human miRNAs that are upregulated due to the *Flaviviridae* infection we transfected human cells lines with the selected miRNAs as a proof of concept. We evaluated three randomly selected miRNAs exhibiting high homologies to HIV-1 and one with low homologous miRNA and determined that the expressions of these high homology miRNAs down-regulates HIV-1 infection whereas, non-homologous miRNAs did not (Figures 2, 3). From the clinical evidence it appears that pre-exposure to the *Flaviviridae*, and most importantly GBV-C results in some degree of protection.

Conclusion

In summary, we show evidence that infection with the *Flaviviridae* may modulate numerous anti-HIV-1 miRNAs in the host cells and block HIV-1 replication. We also propose that incorporation of the *Flaviviridae* genetic fragments into a non-pathogenic GBV-C vector that are complementary to anti-HIV-1 genetic motif identified in our study, may serve as a powerful HIV-1 preventive vaccine without any adverse effects. Currently, our laboratory is evaluating HIV-1 inhibitory effects of miRNA expression cloned in CD4+ T cell lines and the effects of recombinant pegiviruses containing crucial genetic motifs.

Acknowledgements

This research was supported from funds from Claflin University.

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Conflict of interests: No conflict of interests is declared.