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RNA Association, RNA Interference, and microRNA Pathways in Dengue Fever Virus-Host Interaction

Imran Shahid

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Abstract

Dengue fever is a fatal vector transmitted disease and is one of the most significant health problems which have magnified its impact globally by afflicting 390 million people across 110 countries. The causative agent of this life-threatening disease is a positive single-stranded RNA arbovirus known as dengue virus (DENV), which uses *Aedes aegypti* mosquito as an intermediate host. It has been well demonstrated that virus evades mosquito's RNA interference (RNAi)-mediated antiviral defense and manipulates host microRNA (miRNA) profile to its own benefit. However, the exact mechanisms are still not exclusively elucidated. The molecular mechanisms which characterize the role of novel DENV-encoded small RNAs and other viral proteins in host miRNA modulation and evasion of RNA interference are still elusive. Furthermore, the possibility of small activating RNAs-(RNAa)-mediated activation in mosquitoes in conjunction with dengue virus genes is not fully explored. This book chapter pragmatically overviews intricate interplay between virus-host interactions, how virus invades host antiviral defense mechanisms, and possibly the potential emerging therapeutic role of RNA activation (RNAa) and RNAi for the infections, which can be cured by specific gene activation and gene silencing, respectively.

Keywords: dengue fever, dengue fever virus, *Aedes aegypti*, RNA interference, microRNAs, RNA association, host-virus interaction, DENV therapy

1. Introduction

Dengue virus (DENV) is a major arbovirus that uses *Aedes aegypti* mosquitoes as an intermediate host to be transmitted to human and is the causative agent of most serious mosquito-borne viral disease (i.e., dengue fever) afflicting around more than 390 million people worldwide [1]. Despite substantial efforts to control dengue virus vector, it is still emerging rapidly [2]. Therapeutic

options are limited as the available treatments are just supportive, and there is no specific therapy or approved vaccine available. DENV belongs to *Flaviviridae* family, which comprises lipid-enveloped, positive-sense single-stranded RNA viruses [3]. DENV is classified into four closely related serotypes based on antigen distinction and represented as DENV-1 to DENV-4. The length of DENV genome is 10.7 kilobases comprising a single open reading frame (ORF) flanked by highly conserved 5' and 3' nontranslated regions. Single ORF encodes a polyprotein of approximately 3391 amino acids, which is further processed by host (furin and signalase) and viral proteases (NS2B/NS3 protease complex) to generate three structural (C, prM, and E) and seven nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4, 5].

RNA interference (RNAi) is evolutionarily conserved phenomenon, constituting a major component of mosquito innate immune response to virus infections [6]. It has been demonstrated that dengue virus elicits RNAi response in *Ae. aegypti* but is unable to completely repress viral replication instead it may just modulate virus replication to maintain persistent viral infection to ensure long-term survival of infected mosquitoes [7]. It has been explored that DENV employs two different mechanisms to inhibit RNAi pathway, involving RNAi suppression protein NS4B and subgenomic flavivirus RNA (sfRNA). Dengue virus encoded nonstructural protein, NS4B, has been reported to suppress RNAi pathway in both human Huh-7 cells by the mechanism still not fully understood [8]. Furthermore, dengue virus infection produces 3' NTR degradation product sfRNA that is thought to incapacitate RNAi pathway by inhibiting dicer-mediated cleavage of dsRNA [9]. Recently, it has been ferreted out that in *Culex* mosquitoes, West Nile virus (WNV) replication is repressed by cytokine-like secreted antiviral protein "vago" in a dicer-2-dependent manner [10]. However, the possible effect of vago protein against dengue virus replication in *Ae. aegypti* research area is not explored yet. Such evidence clearly indicates that dengue virus opts variety of mechanisms to evade mosquito immune response to successfully replicate in the mosquitoes.

Apart from small interference RNAs (siRNA) reputation to inhibit gene expression through a phenomenon called RNAi, the target gene can also be induced by a mechanism called RNA activation (RNAa). The term RNAa was first coined by Li, who named them after he surprisingly found up regulation of human E-cadherin, p21, and VEGF genes by synthetic dsRNAs designed to target promoters of these genes [11]. He further named those promoters targeting small RNAs as short activating RNAs (saRNAs). Recent studies in which authors evaluated RNAa of above-said genes in monkey, rat, and mouse found that RNAa is highly conserved among all mammals [12]. However, the term RNA activation is not necessarily limited to transcriptional activation by saRNA, targeting promoter region only, rather it should be used to describe all small RNA-mediated gene and epigenome activation mechanisms, including transcriptional activation by targeting 3' terminal regions of genes with saRNAs [13], piwi-interacting RNA (piRNAs)-mediated epigenetic activation [14], and microRNA (miRNA)-mediated translation activation [15]. One of the key differences between RNAi and RNAa is their kinetics. RNAi effect is known to occur within a couple of hours and disappears 5–7 days following siRNA transfection, while RNAa effect does not appear until about 48 h posttransfection and lasts much longer (at least 2 weeks) [16]. This delay may be due to the more complex mechanism of RNAa involving nuclear access and chromatin restructuring as compared to RNAi [17].

microRNAs (miRNAs) represent one of the shortest functional classes of noncoding RNAs, depicting hottest spot of gene regulation research. They were first reported in 1993 in small round worms *Caenorhabditis elegans* [18]. Since then, miRNAs have been implicated in regulating all cellular pathways in eukaryotes ranging from development to oncogenesis. These 20–24 nucleotide-long noncoding RNAs have been demonstrated to be encoded by a diverse range of organisms including plants, humans, insects, and even viruses [19]. The miRNA shows a very high degree of evolutionary sequence conservation among species [20] proved by the presence of miRNA biogenesis complex in archaea and eubacteria [21]. Historically, these microRNAs were thought to regulate different gene expressions either by degradation of particular mRNA or inhibition of translation [22, 23]. However, recent studies have revealed that these small noncoding RNAs can also activate the target gene expression through mRNA stabilization [15, 24]. As the molecular biology, bioinformatics approaches and protocols of small RNA sequencing continue to grow and sophisticate, so is the number of miRNAs discovered in different species. It is becoming more and more apparent that miRNAs not only constitute a fundamental part of regulatory machinery but also may act as molecular decoys to interfere with the function of regulatory RNA-binding proteins [25]. miRNA is involved in fundamental physiological and pathological processes like cancer development and host-pathogen interactions [26–29]. The pathogens have evolved to use to host machinery by altering the expression level of host miRNAs for their benefit [30]. Furthermore, microorganism-encoded miRNAs have also been reported to vary the host gene expression to their advantage [30]. Despite recent advances, there are still significant gaps in our understanding of miRNAs role in host-pathogen interactions, which is probably due to lack of miRNA characterization.

This pragmatic book chapter overviews the possibility of RNAi suppressor activity of DENV in the context of different viral genes. By characterizing the role of different DENV genes in RNAi suppression, we will review to gather valuable lead into the mechanism that governs DENV RNAi evasion in mosquitoes and the viral genes that are responsible for it. We will also explicit the possibility of RNA activation (RNAa) in insect, especially mosquitoes. It is interesting to characterize whether RNAa has any role in host pathogen interaction because, despite the difference in their mode of action, RNAa use same RNAi machinery to induce gene activation. Although very few virus-encoded miRNAs have been fully characterized, it is understandable that they can target both viral and host genes. The information will produce a better understanding of microRNAs role in DENV replication and further deeper insight into characterization of DENV-mediated differential expression of *Ae. aegypti* miRNAs.

2. Dengue virus genome organization

The dengue virus (DENV) genome comprises a single open reading frame (ORF) of approximately 10.7 kilobases flanked by 5' and 3' nontranslated regions (NTRs) [30]. The nontranslated regions contain highly conserved secondary structures and coding regions in ORF, which play integral roles in viral life cycle. The composition of secondary structures is based on the sequencing of DENV-2 (a southeast Asian strain) strain, which is similar to other

Structural proteins	Protein functions
C	Viral RNA packaging
prM	Prevention of premature fusion
E	Envelope glycoprotein, receptor binding and fusion
Non-structural proteins	Protein functions
NS1	Signal transduction
NS2B	Co-factor for NS3 serine protease
NS3	Serine protease/helicase; NTPase
NS4B	Inhibition of interferon signal transduction
NS5	RNA-dependent RNA polymerase (RdRP); methyltransferase
RNA elements	Functions
5' UTR	Viral RNA synthesis and non-canonical translation
3' UTR	Cap-dependent and non-canonical translation; viral RNA synthesis

Table 1. Functions of DENV genome structural and nonstructural proteins.

different viral strains and serotypes. Although most of the DENV proteins are with known functions but some remains to be explored (**Table 1**) [31].

3. Viral entry

DENV is mostly introduced in human by infected *Ae. aegypti* mosquitoes. After the introduction, DENV particularly targets mononuclear phagocyte lineage cells including the skin resistant Langerhans cells [31, 32]. However, in mosquitoes, DENV is thought to initially target midgut and then extends and replicate in other peripheral tissues [33]. DENV can infect a very diverse range of cell lines including human, mosquito, monkey, hamster, and murine cell lineages. This suggests that either DENV uses a ubiquitous receptor or it uses multiple receptors for its entry [33]. In mosquito cells, different independent groups have reported many potential DENV receptors like heat shock protein 70 (HSP), R80, R60, and 45 kDa protein [33]. In human beings, heparan sulfate [34], Hsp90 [35], CD14 [36], GRP78/BiP [37], and a 37/67-kDa high-affinity laminin receptor have been reported [38]. DENV particles interact with human myeloid cells by C-type lectin receptors (CLR) including DC-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing no integrin (DC-SIGN, CD209) [39, 40], mannose receptor (MR), and C-type lectin domain family 5, member A (CLEC5, MDL-1) [41].

4. Viral replication

DENV like other flaviviruses, after adsorption to receptors, is endocytosed by cells in clathrin-dependent manner and then is further transported to endosomes [42]. However, according to one study, DENV can adopt clathrin-independent pathways as well to gain entry into mammalian cells in particular [43]. DENV nucleocapsid is released into cytosol after acidic pH triggered rearrangements of envelope protein in late endosomes leading to fusion of viral envelope and cellular endosome membrane [44, 45]. The nucleocapsid disassembles, and viral genome is translated by endoplasmic reticulum (ER)-located ribosomes generating numerous copies of viral proteins. NS5 and other viral nonstructural proteins along with various host proteins establish replication complexes [46]. At replication complex, the viral polymerase (NS5) transcribes negative viral RNA, which serves as a template for synthesis of subsequent positive viral RNA copies. Viral replication is regulated by 5'-3' UTR sequences (upstream of AUG region) present in 5'-3' UTRs, which are actively involved in the circularization of DENV RNA [47]. These newly synthesized positive DENV RNAs interact with core proteins to assemble into nucleocapsids [48]. The nucleocapsid buds into ER lumen, thereby getting enveloped in a membrane bilayer carrying the viral prM and E proteins [46]. From ER lumen, immature viral particles are transported through cellular secretory pathways, furin protease cleaves prM, and results in the formation of mature viral particles capable of infecting naïve cells [49].

5. Virus-host interactions

In nature, almost every organism engages in ecological or molecular interactions whether antagonistic or mutualistic with other organisms of different species to thrive and excel. These interactions are major drivers of diversification and adaptive evolution. Among these interactions, most fascinating examples are those involved in invertebrate susceptibility to pathogens [50]. According to a recent study, the susceptibility of *Ae. aegypti* to dengue virus has a genetic basis for genotype-by-genotype interactions [51]. It is evident by the fact that despite pathogenic nature of dengue virus in human, its interaction with *Ae. aegypti* mosquito is somehow mutualistic. DENV has somehow improvised to overcome mosquito defenses and manipulate its cellular machinery in a way still not well known that emancipates its replication without interfering too much with normal growth of mosquitos.

6. RNA interference (RNAi)

RNA interference (RNAi) is a major component of innate defense of invertebrates against pathogens. Most arboviruses cause persistent infections within their arthropod vectors. However, how viruses maintain persistent infection in the face of a robust RNAi response is still not fully understood. Many plants and animal viruses have evolved molecular mechanisms for subverting the host RNAi response. For example, Flock house virus (FHV) encodes B2 protein, which

directly binds to dsRNA to inhibit DCR2 activity, to inert siRNA pathway [52]. In another study, researchers have found that plasmid-expressed La Crosse virus (LACV) NSs protein successfully inhibits interferon (IFN) and RNAi pathway in mammalian cells and mice, while fails to show any RNAi-suppressive effect in transfected U4.4 cells and C6/36 cells infected with LACV [53]. A recent publication has revealed that DENV NS4b functions as an RNAi suppressor in human Huh-7 cells via inhibition of dsRNA processing by Dicer [8]. However, whether NS4b behaves similarly in mosquitoes has not been investigated, and to date, no RNAi suppressor activity has been described for an arbovirus protein during mosquito infection. Given that DENV successfully develops persistent infection in mosquitoes despite their RNAi response, the precise mechanism of RNAi evasion is definitely a significant point of interest.

7. RNA activation (RNAa)

RNA activation (RNAa) is one of the nascent phenomena around the scientific field. RNAa is considered to be a potent emerging therapeutic strategy for the disease that can be cured by particular gene activation [54]. However, most of the studies relating to RNAa research have been carried out in human cell line. Till date, proper mechanism of RNAa is still not clear. However, it has been demonstrated that it is mostly a nuclear event that leads to chromatin restructuring [17]. RNA activation is still in its infancy, so its exact mechanism is still elusive. Recently, Portnoy et al. has proposed a model for the promoter-directed-small-activating-RNA (saRNAs)-mediated RNA activation. In this model, authors proposed that an exogenously introduced saRNA or endogenously produced small noncoding RNA, (e.g., miRNA) is loaded into an Ago protein in the cytoplasm. The Ago protein processes the saRNA by discarding its passenger strand to form an active Ago-RNA complex. Ago-RNA complex enters the nucleus through active transport/passive diffusion cell division. Guide strand directs Ago-RNA complex to complementary genomic DNA sequences usually in promoter region or to noncoding RNA (ncRNA) sequences, which tethers to the DNA. After that, the Ago-RNA complex initiates a process that differs from RNAi, which alter chromatin structure and epigenetic states of target gene via two different potential mechanistic models. In model A, the saRNA guide strand leads the Ago protein to its DNA target by constituting an RNA–DNA duplex or triplex structure, while Ago protein then serves as a docking platform to attract histone-modifying activities, like opening of chromatin structure and active transcription. On the other hand, in model B, the RNA guide strand binds to cognate promoter transcripts making saRNA-RNA complex and then Ago protein recruits histone modifiers to introduce active chromatin marks on local chromosome, resulting in activation of transcription [17].

8. MicroRNAs (miRNAs)

As the molecular biology, bioinformatics approaches and protocols of small RNA sequencing continue to grow and sophisticate, so is the number of miRNAs discovered in different species. Virus-encoded miRNAs have been known to be crucial for the viral replication. miRNAs encoded by DNA viruses have been well studied in comparison with those by RNA viruses because RNA

viruses lack nuclear access [55]. However, recent discovery of noncanonical miRNA biogenesis pathways that utilize Argonaute (Ago) 2 for processing of pre-miRNA to mature miRNA has highlighted the fact that RNA viruses may opt similar pathway to generate functional miRNAs. Similar noncanonical pathways might be used to generate functional miRNAs derived from RNA viruses that replicate in the cytoplasm. Several examples up to now have been accumulated like cellular miRNAs, miR-124, is expressed from a cytoplasmic RNA virus, Sindbis virus (SINV) [56]. Several miRNAs were found to be produced by a retrovirus, Bovine leukemia virus (BLV), based on DNA polymerase III transcription *in vivo* as well as *in vitro* [57]. Recently, Hussain et al. have identified six DENV-encoded miRNA like small RNAs in which one of miRNAs directly targets DENV NS1 gene during late infection to autoregulate its replication. Functional analysis has revealed that viral miRNAs can target both cellular and viral mRNAs to regulate viral replication leading to a successful infection [58]. Although, very few virus-encoded miRNAs have been fully characterized, it is understandable that they can target both viral and host genes.

Flaviviruses are well reported to manipulate host miRNA machinery to facilitate their replication. It has been well reported that DENV induce differential expression of miRNAs in human peripheral blood mononuclear cells (PBMCs) [59]. It has been previously reported that miR-124a, -128a, -218, and -let-7c may be important for neurological symptoms caused by a chimeric tickborne encephalitis/dengue virus [60]. Additional studies have shown that miR-122 and miR-142 of the host cells are involved in restricting dengue virus [61, 62]. However, most of the above work has been done in human. Very little is known about the differential miRNA expression of *Ae. aegypti* influenced by DENV infection, leaving a significant gap in our understanding of DENV-*Aedes aegypti* interaction. Although a recent study has shown aberrant host small RNA (sRNA) profiles in *Ae. aegypti* during DENV infection, it mainly focused on piRNAs [63] leaving a significant gap in understanding the differential expression of *Ae. aegypti* miRNAs during DENV replication.

9. miRNA biogenesis

9.1. miRNAs genomic arrangement and transcription

miRNAs are usually encoded by different regions of genome ranging from coding as well as noncoding [64]. Seventy percent of mammalian miRNAs are the intron products of protein coding genes, while rest comes from the noncoding transcription units. miRNAs have been found to be originated from both senses and antisense strands of DNA [65]. Studies have shown that about 30% of *C. elegans* miRNA genes are on the antisense strand overlapping protein coding region [66]. In *Drosophila* too, many miRNAs are originated from antisense strand overlapping protein coding region [67]. Multiple miRNAs can be transcribed as one long transcript named clusters [18].

Majority of miRNAs are transcribed by RNA polymerase II; however, some miRNAs usually located near Alu repeats are transcribed by RNA polymerase III, from independent genes having their own promoter or represent introns of protein-coding genes [18]. Nearly, all the independent miRNA promoters have particular features of Pol II promoters, including initiator elements and TATA boxes [18].

9.1.1. Canonical pathway of miRNA biogenesis

So far, a vast majority of reported miRNAs are produced through an RNase III enzyme-controlled canonical pathway. Transcription of primary transcript (i.e., pri-miRNA) harboring one or several stem loop structures by RNA polymerase II or otherwise RNA polymerase III kick start the biogenesis process in nucleus [68, 69]. pri-miRNAs are then further processed at stem loop sites by two RNase III proteins Drosha and DGCR8/Pasha (DiGeorge critical region 8) to produce about 70 nucleotide pre-miRNA [70]. This pre-miRNA is then exported to cytoplasm with the help of exportin-5 and RanGTP proteins [71]. Once in cytoplasm, pre-miRNA is chopped down into 22 nucleotide miRNA duplexes. Apart from above-mentioned proteins, many other proteins also work as cofactors to influence the outcome of miRNA biogenesis [65, 72].

9.1.2. Noncanonical pathway of miRNA biogenesis

Although, it was thought earlier that there is only one universal mechanism of all mature miRNAs biogenesis; however, multiple recent discoveries have led us to the conclusion that there may be several other miRNA biogenesis pathways as well. Biogenesis of quite a few miRNAs has been demonstrated to be not only Drosha independent, but also Dicer dependent [73]. It has been reported that in *Drosophila* and *C. elegans* along with some other vertebrates, mature miRNAs called “mirtrons” can be produced after splicing from intron hairpins independently of Drosha processing [74–76]. Recent studies have revealed that mature functional miRNAs can arise from highly conserved small nucleolar RNA (snoRNAs) in human and *Giardia lamblia* [77, 78] as well as DicerII-processed endogenous siRNAs mostly derived from transposable elements and load into AgoI in *Drosophila* [76]. Furthermore, researchers have identified miRNAs derived from transfer RNA (tRNA) in mouse embryonic stem cells [79].

Recent studies have led to the discovery of other types of miRNA noncanonical biogenesis pathway that is Dicer independent. One prime example of this is miR-451 biogenesis, which is Dicer independent as pre miR-451 has ~18 nucleotide stem duplex that is too short for Dicer activity. However, pre miR-451 requires AgoII for its maturation [80]. A few examples of other types of noncanonical biogenesis come from virus-encoded miRNAs. For example, several miRNAs are produced by a retrovirus, Bovine leukemia virus (BLV), based on DNA polymerase III transcription *in vivo* as well as *in vitro* [57]. The products of these transcripts were too small to be recognized by Drosha; therefore, these were directly processed by Dicer-1 to mature miRNAs.

10. Mode of action of miRNAs

After the production of mature miRNA by either canonical or noncanonical pathway, the resulting mature miRNA duplex is loaded into miRNA inducing silencing complex (miRISC) having Ago as core component [81]. After degradation of one strand, other strands of duplex, representing mature miRNA, then guide miRISC complex to target mRNA to determine its

fate [82]. The stability of the base pairs at the ends of the strands of miRNA duplex usually determines which strand will be degraded. Usually, less stable strand avoids degradation [82]. There are different modes of action of miRNAs that influence the expression of target gene ranging from mRNA degradation, translational repression to activation of transcription [16, 83, 84]. Whatever the outcome is, the general way of miRNA-mRNA interaction relies on the sufficient sequence complementarity between mRNA and 5' seed region (usually 2–8 nucleotides) of miRNA [85]. Perfect complementarity between mRNA and miRNA is likely to down-regulate the expression of target gene through its mRNA degradation by endonuclease activity of miRISC complex. This type of miRNA-mediated gene regulation is mostly evident in plants [86]. However, in animals, there is mostly imperfect complementarity between mRNA and miRNA, which usually leads to translational repression of target gene [86]. Interestingly, there are solid experimental evidences that miRNAs are also involved in upregulation of target genes as well. One of its well-characterized examples is host miR-122-mediated upregulation of HCV RNA replication by stabilizing HCV RNA and preventing its decay [87].

11. Virus-encoded miRNAs

As most viruses need host genes to facilitate their replication, one cannot rule out the possibility of viruses encoding miRNAs in order to manipulate host gene expression or keep viral copy number under check to ensure persistent infection. Possible benefits of virus-encoded miRNAs are that they usually do not elicit immune response, require less coding ability, and they have ability to constantly evolve to target new transcripts. Till date, more than 200 viral miRNAs have been identified. Most of the well-characterized viral-encoded miRNA comes from DNA viruses as compared to RNA viruses, with herpes viruses as a major contributor having average copy number of more than 10/genome [58]. Different recent studies have identified novel microRNAs from different RNA viruses. Bovine leukemia virus (BLV), a retrovirus, also encodes microRNAs [57]. Hussain et al. has reported microRNA like small RNAs in both West Nile virus and Dengue virus [88]. These viral-encoded miRNAs are produced through canonical as well as noncanonical biogenesis pathways. Based on their function, viral encoded miRNAs can be classified into two broad classes:

- i. Virus-encoded miRNA regulating host genes
- ii. Virus-encoded miRNAs regulating virus copy number

Important functions of viral miRNAs, which target host genes, include promoting cell survival through downregulation of apoptotic factors, thus promoting infected cells survival and proliferation, as well as modulating the immune response of the host cell [89]. It is becoming increasingly evident that one mechanism the viruses have evolved to facilitate regulatory control over their hosts is by generating transcripts, which outmatch cellular miRNAs. Interestingly, Human Kaposi's sarcoma-associated herpes virus (KSHV) and chicken oncogenic Marek's disease virus (MDV) transcribe miR-K12-11 and miR-M4, respectively; these miRNAs appear to be an ortholog of miR-155 to identical seed region [90, 91]. miR-155 has been reported to be involved in many malignancies [92], so exploitation of its targets by

viruses may contribute to viral oncogenesis. Recently, it has been reported that a flavivirus named West Nile virus encode an miRNA KUN-miR-1 that specifically induces the expression of GATA4 in *Ae. albopictus* that in turn facilitates the WNV replication [88].

Interestingly, virus-encoded miRNAs can regulate their own replication as well their own benefit. For example, simian virus 40 (SV40)-encoded miRNA miR-S1, expressed during late infection, has been shown to downregulate viral T-antigen, which is crucial to evade cytotoxic T cell response [93]. Another example is HvAV-miR-1 encoded by *Heliothis virescens* ascovirus (HvAV), an insect with dsDNA virus that downregulate viral replication by targeting viral DNA polymerase I [94]. Recently, it has been reported that Dengue virus (DENV)-encoded miRNA like viral small RNA DENV-vsRNA-5 plays an important role in the autoregulation of DENV replication by directly targeting dengue virus NS1 protein during late infection [30].

12. miRNA role in host-viral interactions

Regulative control of miRNAs over gene expression in every organism is the core reason why pathogens try to hijack them. This takeover may include disruption of miRNA biogenesis pathway, inhibiting a specific host miRNA that hinders pathogen propagation or differential miRNA expression. In case of human, it has been well documented that miR-146 and miR-155 are involved in upregulating immune function in response to various bacterial infections including *H. pylori* [95] and *Salmonella enterica* [96]. Many viruses encode miRNAs, having the potential to not only the viral genomes, which encode them, but also to target host transcripts, which facilitate their replication [97, 98]. Functions of viral miRNA targeting host genes include cell survival through downregulation of apoptotic factors as well as modulation of host immune response [89]. One example is upregulation of GATA4 by WNV-encoded miRNA, which has been found to be very important in virus replication in mosquito cells [88]. Recently, it has been demonstrated that viruses have evolved to encode miRNAs that mimic host miRNAs. Kaposi's sarcoma-associated herpes virus (KSHV) encoded miR-K12-11 that is an ortholog of human miR-155 sharing same seed region homology that will likely be able to regulate all targets of miR-155 [90].

12.1. miRNA role in *Aedes aegypti* with an insect-specific flavivirus

Previous studies demonstrated that mosquito miRNAs could be affected by different viral infections. However, those studies were restricted to pathogenic viruses, mainly restricted to the role of differently expressed miRNAs with limited characterization. Limited knowledge is available about the modulation of host miRNAs with respect to insect-specific flaviviruses (ISFs, e.g., Palm Creek virus (PCV)). ISFs share the similar genome organization with other flaviviruses; however, their rate of vertical transmission between insect hosts is higher than other vertebrate-infecting flaviviruses. Although it is still unclear that why ISFs fail to replicate in mammalian cells, it is assumed that ISFs potentially lack to antagonize the host interferon system. Similarly, ISFs express a high adaptation level in insect hosts, which may further limit their replication in vertebrate hosts. Recently, Lee et al. explicated the potential role

of miRNAs in Palm Creek virus (PCV) infection using the *Ae. aegypti* mosquito model [99]. They proposed differently expressed miRNAs after combining small-RNA sequencing and bioinformatics tools although the results hardly predicted the potential involvement of PCV infection to alter host miRNAs. They revealed that only one miRNA (i.e., aae-miR-2940-5p) out of 101 reported miRNAs of *Ae. aegypti* that had significantly altered expression over the course of PCV infection. The level of aae-miR-2940-5p was induced within 2 days p.i. and suppressed at 12 days p.i.; however, a different pattern of miRNA screening was demonstrated in *Ae. aegypti* Aa20 cell line without displaying any significant change upon PCV infection. Further *in vitro* miRNA inhibition experiments while using aae-miR-2940-5p inhibitors demonstrated that this miRNA did not directly impact on PCV replication and has no significant role in PCV-*Ae. aegypti* interaction. This variable response to virus infection in cell lines and host mosquito might be due to tissue tropism of the virus. At mRNA target level, the study reported the inconsistent expression level of MetP with miR-2940-5p expression although previously it was reported a potential target of aae-miR-2940-5p with positive interaction. It means that there was no correlation of MetP expression to PCV infection because silencing the gene did not significantly affect the virus replication. The pair MetP--aae-miR-2940-5p also positively regulate West Nile virus (WNV) virion production where aae-miR-2940-5p was selectively downregulated upon WNV infection in infected cells. The MetP human ortholog like M41 ftsh, YME1L, plays an important role as an antiapoptotic factor. It is assumed that the induction of aae-miR-2940-5p in PCV-infected cells during infection boosts MetP transcript levels and thus protects infected cells undergoing apoptosis under stress responses. However, the MetP physiological role is still uncertain in insects and requires further investigation [99].

13. RNA interference in *Aedes aegypti* with an insect-specific flavivirus

RNA interference is considered a vital antiviral defense response in mosquitoes as lot of studies reported the production of viral-specific small RNAs for different viruses. However, several reports also confirm the establishment of persistent viral infection only in mosquitoes either infected by pathogenic mosquito-borne viruses or insect-specific viruses that produce these small RNAs. While doing so, viral-derived DNA (vDNA) is produced by reverse transcription during persistent infection and may survive in extrachromosomal or integrated forms. Such vDNAs increase RNAi-mediated antiviral response in mosquitoes to boost mosquito tolerance to arbovirus infection while establishing persistent infection. In one study, Lee et al. detected PCV-specific 21 nucleotide small-RNAs in mosquitoes at different time points of infection (2, 6, and 12 days p.i.) as a potential indicator of active viral replication in the mosquitoes [99]. These findings complied with other studies where the researchers demonstrated the similar phenomenon for other pathogenic mosquito-borne flaviviruses (e.g., DENV) and insect-specific flaviviruses (e.g., cell fusing agent viruses). Their presence as hot spots suggests that either those are the potential targets of Dicer-2, or are more stable or can be reverse-transcribed into vDNA. All these might be the potential cause to enhance RNAi antiviral response. However, the authors are also afraid of bias, which could be due to

technical issues such as library preparation. In addition to RNAi, piRNAs (piwi RNAs) have been reported in mosquitoes and knockdown of piRNAs-related specific proteins indicates their endogenous antiviral activity instead of well-established exogenous RNAi activities in mosquitoes. Similarly, virus-specific piRNAs with typical A₁₀ bias in sense RNA and U₁ bias in antisense RNA have been reported to be produced by Bunya viruses and alphaviruses. However, the piRNAs expressed by dengue virus and cell fusing agent virus have only A₁₀ bias in sense RNA. Interestingly, the PCV-specific small RNAs do not show either A10 or U1 bias features. In this scenario, it is uncertain that either they are piRNAs or just viral degradation products. This situation is very similar seen in other flavivirus-specific piRNAs, which map to very small number of sequences in the genome where the presence of one copy of genome indicates more specific targeting than random RNA degradation. The production of virus small RNAs and piRNAs through vDNA synthesis is also documented in mosquitoes or in their derived cell lines. It is worth mentioning to note the contribution of vDNA in the production of small RNAs of virus and piRNAs in mosquitoes, although insect-specific viruses like PCV establish persistent infection in their hosts [99].

14. Long intergenic noncoding RNAs (lincRNAs) and their association with DENV-host interaction

Considering as another important class of regulatory RNAs, lincRNAs (sometimes known as dark matter) have various biological functions including genomic imprinting and cell differentiation specifically in host-pathogen interaction [100]. A growing number of evidences also reflect their possible role in gene regulation either epigenetics or nonepigenetics. However, their role in immune cell differentiation and activation is poorly understood, but the recent discoveries show their potential role in defense system as well as rapid responses to various stimuli and stress factors. Some studies also predict their active role to enhance viral replication or decrease antiviral immunity. It was also depicted that some lincRNAs also interact with other noncoding RNAs like miRNAs. The *Ae. aegypti*-linked lincRNAs are shorter in length (approximately 3000 nucleotide bases) than their protein-coding genes. Similarly, their GC content was also lower (mean:40.1%) than their protein-coding gene sequences (mean:47.8%). However, the AT enrichment or lower GC content is a typical characteristic of lincRNAs and congruent with other lincRNAs in other species [100].

Etebari et al. identified lincRNAs in dengue fever vector *Ae. aegypti* and demonstrated their potential role in host antiviral defense [100]. They evaluated lincRNA's expression in DENV serotype 2 (DENV-2) and *Wolbachia*-infected and noninfected adult mosquitoes as well as in Aa20 cells. The findings revealed the increased number of host lincRNAs under the circumstances of DENV-2 infection, some of which inhibit viral replication in mosquito cells. Furthermore, the silencing of only one lincRNA_1317 by RNA interference enhanced the viral replication in host cells, which clearly indicates their possible role in host antiviral defense. The lincRNA_1317 suppression was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). It was found that the lincRNA_1317 expression was increased substantially upon the progression of infection, indicating the possible role of this

lincRNA in antiviral defense. The findings might be consistent as the highly overexpressed lincRNA_1317 expression (2.33 fold) was reported in *Wolbachia*-infected mosquitoes as compared to noninfected [100].

The authors also described lincRNAs potential involvement in mosquito-pathogen interaction by determining its association with host-endogenous small RNAs and its direct interaction with DENV-2 infection. lincRNA_1317 was not found to be located in any of the known piRNA clusters; however, no differences were found in the mapping pattern and mapped read length distribution when reads from DENV-infected and noninfected small RNA libraries were mapped to lincRNA_1317. Gene silencing like role of piRNAs on lincRNA_1317 transcriptome was also speculated. However, a little information is available about piRNA-mediated lincRNA although some recent studies predict piRNA-mediated degradation of lincRNAs in mouse's late spermatocytes [100].

The researchers also tested the hypothesis that *Ae. aegypti* lincRNA_1317 response to microbial challenge could be due to cross regulation between miRNAs and the lincRNAs. For this purpose, the normalized minimum free energy (mfe) of hybridization for each *Ae. aegypti* miRNA and lincRNA_1317 was calculated by using RNAhybrid core script. The basic objective was to identify *Ae. aegypti* miRNA recognition elements on lincRNA_1317. The results were quite interesting as binding sites enrichment for a few miRNAs with more than two recognition elements were detected on lincRNA_1317 (e.g., more than four recognition sites for miR-278-5p and miR-252-3p were predicted on lincRNA_1317). Furthermore, some hot spots for miRNA recognition sites on lincRNA_1317 were also identified, which may facilitate multiple miRNAs to bind the same regions. microRNAs contain the capability to shake lincRNA stability by targeting their transcripts similar to targeted mRNAs. Similarly, lincRNAs possessing multiple recognition sites might act as a competitive inhibitor of miRNA by eliminating them to bind their genuine targets by sequestration. The mfe values of hybridization for miRNA-lincRNA recognition sites could be a strong predictor of a binding event between two; however, further investigations and trials are still needed to validate this interface [100].

15. IsomiRs and their impact on miRNAs in dengue fever vector

Ae. aegypti

microRNAs may exist in various lengths and sequence variations, which are known as isomiRs [101]. Earlier, those were considered as sequencing errors; however, some studies predicted to be physiologically relevant and posttranscriptionally modified miRNA variants. IsomiRs may express affinities for different targets than their canonical miRNA counterparts. Furthermore, nucleotide heterogeneity could be found at both ends of miRNA sequence in the form of nucleotide substitutions despite the variations are more frequent at 3' end. The molecular biology, genome structure, and epigenetics of isomiRs are still poorly understood, and the mechanisms of their biogenesis are also considered very complex and even cell-type specific. Some variations in miRNA sequences are supposed to be the product of template variations, which might be brought by the exonuclease activity of Drosha and Dicer [101].

It is a well-documented fact that miRNAs may play a vital role to modulate the capability of vectors to propagate the infection for widely damaging arboviruses (e.g., dengue virus) [101]. It has been demonstrated that miRNA could be modified in mosquitoes upon the induction of DENV infection. As genetic variations in isomiR prevalence have significant impact on gene regulation, a clear understanding of the role of isomiR profile of mosquitoes in DENV and other arboviruses infection propagation is urgently needed. Etebari et al. in one study found this altered posttranscriptional modification role of miRNAs in *Ae. aegypti* mosquitoes after DENV infection in comparison with uninfected mosquitoes. For this purpose, they utilized already published RNA-seq data from 2-, 4-, and 9-day DENV-infected and uninfected mosquitoes. The findings showed significant variations in miRNA prevalence in response to dengue virus infection although the effects were not ubiquitous, and no remarkable alteration in overall pattern of isomiR expression was noted upon DENV infection. They calculated the exact/all read count ratio as an index for isomiR production rate for all known *Ae. aegypti* miRNAs. DENV-infected mosquitoes increased the isomiR production of 3 miRNAs (miR-2c, miR-210, and miR-34) with notable impact on two miRNAs (miR-276 and miR-10) with less read count by DENV infection. The data also demonstrated that 3 isomiRs of miR-34-5p were also significantly altered by dengue virus infection. Collectively, those alterations might have net benefit to determine the mosquito's role upon DENV infection propagation; however, potential biological significances of these modifications are still unclear except to infer that some evolutionary function of miRNAs. Similarly, it is also ambiguous that why some specific isomiRs potentially modify more in response to DENV infection in *Ae. aegypti*. Furthermore, it was also noted that one particular miRNA with significant increase in one specific isomiR variants upon DENV infection also contained a common variant in at least one other isomiR as well. The authors speculated that establishment and persistence of DENV infection might cause significant changes in activity levels of some enzymes involved in the production of isomiRs, which ultimately modify the isomiR production frequencies of some specific miRNAs. The isomiR production power against different *Ae. aegypti* miRNAs was also demonstrated differently as observed by the read count. The "true" miRNAs were considered as those which had an exact match to the canonical sequence (already reported and available in miRbase), while the "false" ones were classified as those which significantly differed to that reported in miRbase. The authors explicated that in *Ae. aegypti*, most abundant miRNA sequences matched to true miRNAs were just 45%, while 55% miRNAs produced by *Ae. aegypti* miRNAs were false miRNAs, and interestingly, this overall trend was not altered by DENV infection. Although the findings are striking and indicate that little variations in miRNA sequences may significantly impact target affinities in DENV-host interaction as well as in infection propagation, still extensive studies are required in the field to validate such hypothesis [101].

16. Conclusions

An over-growing number of reports and continuous publication of journals and books illustrate an intricate interplay between virus-host interactions in dengue fever virus infection. Similarly, an increase in morbidity and mortality rate with DENV infection and still unavailability of standard care to treat the infection have diverted the DENV-research to explore new paradigms in treatment and to map/identify cellular/molecular pathways to better understanding of disease progression in infected vectors, either mosquitoes or humans. Small interference

RNA and microRNA as anti-mRNA-based treatment strategies with strong evidences are evolutionary, but unfortunately still not a successful and reliable treatment line in viral infections with certain issues of drug delivery and long-term therapeutic effects. The involvement of lincRNAs and variations in miRNA epigenetic profile in disease-specific arboviruses (e.g., dengue virus) during infection also indicate complex genome interactions between virus and hosts. RNA association as a novel therapeutic approach to treat infection by gene activation, to find out some specific modulators of gene regulation in viral replication as well as in disease progression is still in an explanatory phase specifically for disease-specific or insect-specific flaviviruses. In conjunction, the molecular approaches to find out disease progression pathways in disease-specific arboviruses are still investigative and antiviral treatment approaches in the form of siRNAs, microRNAs as well as RNA association still need to be explored fully before their practical implementations in diagnostics and as some antiviral therapeutics.

Conflict of interest

The author declare that there is no conflict of interest.

Author details

Imran Shahid^{1,2*}

*Address all correspondence to: iyshahid@uqu.edu.sa

1 Department of Pharmacology and Toxicology, College of Pharmacy, Umm-Al-Qura University, Makkah, Saudi Arabia

2 Applied and Functional Genomics Laboratory, Center of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan

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