RNA editing of microRNA prevents RNA-induced silencing complex recognition of target mRNA

Yalei Cui†, Tianzhi Huang† and Xiaobo Zhang

College of Life Sciences, Zhejiang University, Hangzhou 310058, People’s Republic of China

MicroRNAs (miRNAs) integrate with Argonaut (Ago) to create the RNA-induced silencing complex, and regulate gene expression by silencing target mRNAs. RNA editing of miRNA may affect miRNA processing, assembly of the Ago complex and target mRNA binding. However, the function of edited miRNA, assembled within the Ago complex, has not been extensively investigated. In this study, sequence analysis of the Ago complex of Marsupenaeus japonicus shrimp infected with white spot syndrome virus (WSSV) revealed that host ADAR (adenosine deaminase acting on RNA) catalysed A-to-I RNA editing of a viral miRNA (WSSV-miR-N12) at the +16 site. This editing of the non-seed sequence did not affect association of the edited miRNA with the Ago protein, but inhibited interaction between the miRNA and its target gene (wsv399). The WSSV early gene wsv399 inhibited WSSV infection. As a result, the RNA editing of miRNA caused virus latency. Our results highlight a novel example of miRNA editing in the miRNA-induced silencing complex.

1. Introduction

Post-transcriptional mechanisms play important roles in the regulation of gene expression. RNA editing is one of the most important mechanisms of post-transcriptional genetic modification and generates a variety of cellular RNA signatures by base substitutions, insertions and deletions. The best characterized form of RNA editing found in mammals is base substitution of C to U (cytosine to uracil) and A to I (adenosine to inosine) [1]. The hydrolytic deamination of adenosine to inosine is catalysed by ADAR (adenosine deaminase acting on RNA) proteins [2]. A-to-I RNA editing is conserved from sea anemones to Homo sapiens and represents an irreversible RNA modification [3]. The targets of ADARs are double-stranded regions of at least 15–20 base pairs [4], and following A-to-I RNA editing, the translational machinery recognizes inosine (I) as guanosine (G), producing different protein isoforms. The RNA editing involved in forming the coding region of the glutamate receptor subunit GluR-B is a well-known example [5]. Alternative ADAR2-mediated editing of GluR-B alters the gene-encoded glutamine (Q) codon CAG to the arginine (R) codon CIG, producing an ion channel that is impermeable to Ca2+ [5]. Additionally, RNA editing of the serotonin (5-HT) receptor 2C (5-HT2cR) affects its G-protein-binding affinity [6]. However, bioinformatic analyses report that the majority of A→I RNA editing sites exist in noncoding sequences, 5' and 3' untranslated regions (UTRs), intronic retrotransposon elements and repetitive sequences [7–9], and the role of the RNA editing in these regions is largely unknown.

MicroRNAs (miRNAs) also undergo RNA editing [10–12]. MiRNAs, generated from primary genome transcripts, integrate with Argonaute (Ago) to produce miRNA-induced silencing complexes (miRISC) that suppress expression of their target genes [13]. Mature miRNA ‘seed sequences’ recognize target sites in mRNA, and miRNAs predominantly target sequences in the 3' UTR of mRNAs. Binding of the miRISC can cause mRNA destabilization and/or
inhibition of translation [14,15]. RNA editing can shield miRNA from recognition and processing by Drosha and Dicer, and even alter the seed sequence, and thus mRNA target. A-to-I editing of the primary transcript of miR-142 shields this miRNA from processing by Drosha, reducing expression of mature miR-142 [12]. Editing of the fold-back double-stranded RNA (dsRNA) structure of primary miR-151 inhibits its cleavage by Dicer, causing accumulation of edited pre-miR-151 intermediate RNA [16]. Editing of primary miR-376 at the +4 and +44 sites within the seed sequences of miRNA-376-5p and -3p strands alters the miRNA’s seed sequence, and thus miRNA target [11]. At present, however, the functional consequences of these miRNA editing processes remain unclear.

As virus life cycles are short and can be completed within a single cell, a virus may represent a useful model in which to explore the mechanism of RNA editing. A-to-I RNA editing of viral mRNAs has been reported to be crucial for replication of hepatitis D virus (HDV) [17]. And although DNA viruses are reported to encode miRNAs [18,19], the role of viral miRNA editing in virus–host interactions has not been intensively explored. In this study, we characterized miRNA–mRNA interactions in Marsupenaeus japonicus shrimp haemocytes infected with white spot syndrome virus (WSSV). Based on the morphology and genomic composition, WSSV is assigned to a distinct virus family, Nimaviridae [20]. WSSV with 305-kb circular double-stranded genomic DNA has the capacity to encode 180 viral proteins and 89 viral miRNAs [18,19,21]. During WSSV infection, early genes, transcribed before 6 h post-infection, encode the viral regulatory proteins [22,23]. As reported, most of the WSSV miRNAs are transcribed at the early stage of virus infection [18,24]. The viral miRNAs regulate the expressions of the virus and/or host target genes in the WSSV–shrimp interactions [19,24,25].

In the present investigation, it was found that one viral miRNA (WSSV-miRNA-N12) underwent A-to-I RNA editing at its non-seed sequence, and this editing was dependent on the host ADAR. The edited miRNA no longer recognized its target gene, WSSV early gene wssv399, leading to accumulation of off-target miRNA in the Ago complex, thus preventing target gene silencing. At present, the function of wssv399 has not been characterized. The results of this study revealed that the silencing of wssv399 promoted the WSSV infection. In this context, the RNA editing of viral miRNA played an important role in viral latency. Thus, we have characterized a novel example of miRNA editing and miRNA–mRNA interactions in virus-infected animals.

2. Results

2.1. Characterization of miRNAs and mRNAs in the Ago1 complex of shrimp in response to virus infection

To investigate the miRNAs involved in antiviral immunity, shrimp were infected with WSSV, and the RNA contained within shrimp haemocyte Ago1 complexes was sequenced. We confirmed WSSV infection of shrimp (figure 1a), and co-immunoprecipitated the Ago1 complexes of infected haemocytes with an Ago1-specific antibody at 0, 24 and 48 h post-infection. The RNAs (mRNA and miRNA) contained in these complexes were extracted and subjected to deep sequencing. For example, the RNAs extracted from the Ago1 complex of WSSV-infected haemocytes 24 h post-infection were illustrated in figure 1b.

High-throughput small RNA sequencing yielded an average of 2,318,149 high-quality reads. Most small RNA reads were 20–25 nucleotides (nt) in length, which was typical for products generated by the enzyme Dicer (figure 1c). A total of 1,629,369 high-quality small RNA reads were mapped to known animal miRNAs or WSSV miRNAs, and 55 known shrimp miRNAs [26] and 45 known WSSV miRNAs [18,19] were identified (tables 1 and 2). The remaining nine candidate miRNAs, with no homologue, included seven shrimp putative miRNAs (mja-miR-27, mja-miR-28, mja-miR-29, mja-miR-30, mja-miR-31, mja-miR-32 and mja-miR-33) and two putative WSSV miRNAs (WSSV-miR-N50 and WSSV-miR-N51; tables 1 and 2). The WSSV miRNAs accumulated in shrimp at 24 and 48 h post-infection, and accounted for over 12% of the total small RNAs sequenced at 48 h post-infection (figure 1d). Some shrimp miRNAs were upregulated and some downregulated in response to virus infection, while viral miRNAs were detected only after WSSV infection (figure 1e). To confirm the involvement of these shrimp and viral miRNAs in WSSV infection, the expression of 11 randomly selected miRNAs was detected by northern blotting. The expression patterns of these miRNAs revealed by northern blots were similar to those revealed by sequencing (figure 1f).

To investigate potential target genes of shrimp and WSSV miRNAs, the mRNAs in the Ago1 complexes of shrimp haemocytes challenged with WSSV were sequenced at 0, 24 and 48 h post-infection. After removal of repetitive and low-quality reads, a total of 5,212 million high-quality reads were aligned to the assembled expressed sequence tags (ESTs) of shrimp or to the WSSV genome sequence. The results included 265,400 unigenes. Among them, only a small proportion (0.033%) of the reads originated from WSSV transcripts. Cluster analysis revealed that the pattern of mRNA expression changed throughout the time course of WSSV infection (figure 1g). In comparison to uninfected cells (0 h post-infection), 984 genes were significantly differentially expressed (p < 0.01), of which 513 genes were upregulated and 471 were downregulated. To confirm the gene expression profiles of shrimp in response to WSSV infection, three genes were randomly selected for quantitative real-time PCR. The gene expression patterns revealed by quantitative real-time PCR were similar to those revealed by sequencing (figure 1h).

2.2. Interactions between miRNAs and mRNAs in the Ago1 complex

In order to evaluate the roles of these miRNAs, their mRNA targets were analysed. Approximately 40% of target RNA tags were aligned to 3' UTRs and 20% to coding sequences, while only 3–4% were aligned to 5' UTRs (figure 2a). Both shrimp genes and WSSV genes were targeted by shrimp miRNAs and WSSV miRNAs (figure 2b). During virus infection, the number of shrimp transcripts targeted by WSSV miRNAs increased from 77,276 (24 h) to 90,835 (48 h) (figure 2b), and the number of WSSV transcripts...
**Figure 1.** Host and virus miRNAs and mRNAs in the Ago1 complex. (a) The copy number of WSSV genomic DNA in the haemocytes of virus-infected shrimp pre-infection (0), and 24 and 48 h post-infection was quantified using quantitative real-time PCR. (b) The Ago1 complex of virus-infected shrimp haemocytes was co-immunoprecipitated with Ago1-specific antibody. Then the complex was analysed by western blot analysis (i). The anti-GST antibody was used as a control. (c) Size distribution of small RNAs in the Ago1 complex pre-infection (0), and 24 and 48 h post-infection. (d) Percentages of sequence reads of shrimp and viral miRNAs in the Ago1 complex of shrimp pre-infection (0), and 24 and 48 h post-infection. (e) The shrimp miRNA (i) and WSSV-miRNA (ii) expression profiles of WSSV-challenged shrimp pre-infection (0), and 24 and 48 h post-infection. The copy number of WSSV genomic DNA in the haemocytes of virus-infected shrimp. (f) Northern blots of selected shrimp and WSSV miRNAs. Total RNAs extracted from the Ago1 complexes of virus-free or WSSV-infected shrimp haemocytes were blotted with the DIG-labelled oligodeoxynucleotide probes pre-infection (0), and 24 and 48 h post-infection. (g) Heat map of differentially expressed shrimp and WSSV genes in the Ago1 complex of shrimp haemocytes. The shrimp were infected with WSSV. mRNAs in the Ago1 complex of shrimp haemocytes were sequenced pre-infection (0), and 24 and 48 h post-infection. The colours indicate the Z-values of genes. (h) Real-time PCR detection of gene expression profiles of shrimp in response to WSSV pre-infection (0), and 24 and 48 h post-infection.
| name       | sequence (5’–3’)                      | length (nt) | references of reported microRNAs |
|------------|---------------------------------------|-------------|----------------------------------|
| miR-1      | UGGAAUGUAAGAAGAUAGGAG                 | 22          | Huang et al. [26]                |
| miR-10a    | UACCCGUAGAUCGGAAGUG                   | 22          | Huang et al. [26]                |
| miR-100    | AACCGGUAGAUCGGAACUGU                  | 22          | Huang et al. [26]                |
| miR-1000   | AUUUGCCGCCUGAGACAGAUGA               | 22          | Huang et al. [26]                |
| miR-12     | UAGAGAAUCUACUAGACUCUGU               | 23          | Huang et al. [26]                |
| miR-122    | GGAGUGUGACUAGACUGUGU                  | 21          |                                  |
| miR-133    | UUGGUCUCUCCUCUCACCAUUGGUGG           | 22          | Huang et al. [26]                |
| miR-184    | UGACGGAGACGAGAGAAGGCC                | 22          | Huang et al. [26]                |
| miR-190    | AGAIAUGUGUAAGUAIUCAGUUGG             | 24          | Huang et al. [26]                |
| miR-193    | UACUGGCCUGCUAGAGGCCCA                | 21          | Huang et al. [26]                |
| miR-2a     | UAUCAGCGAGCGAGGAGAUGUGG              | 24          | Huang et al. [26]                |
| miR-2001   | UUGUGACCUGUAIAUAGGGA                 | 21          | Huang et al. [26]                |
| miR-235    | UUGCCAUUUUCCCGGCU                    | 18          |                                  |
| miR-252    | CUAAGUACUAGUUCGUCGUGG                | 22          | Huang et al. [26]                |
| miR-252b   | CUAAGUACUAGUUCGUCGUGU                | 23          | Huang et al. [26]                |
| miR-263a   | AAUGGCACUGGAAGAAUACGG                | 23          | Huang et al. [26]                |
| miR-263b   | CUGGACUGAGGAAAGAAUACAGU             | 26          |                                  |
| miR-275    | UACGGUACUAGUAGUCGCGG                | 21          | Huang et al. [26]                |
| miR-276    | UAGGACUUCAUACUGCUGUCUU             | 22          | Huang et al. [26]                |
| miR-276s   | UUGAAACUCCUCACCCGUGGA               | 22          |                                  |
| miR-278    | UCGUGCGACUCCUCUGGCUUU               | 22          | Huang et al. [26]                |
| miR-278s   | CACUGCCCUUGAUAUCCCAAA              | 22          |                                  |
| miR-279    | UGACUCAGUCACACUCACACCA             | 22          | Huang et al. [26]                |
| miR-281    | CUGUCAGGAGUUGGCUCUCUU               | 22          | Huang et al. [26]                |
| miR-282    | UAGCUCUCUCCUUGGCUCUUGU             | 22          | Huang et al. [26]                |
| miR-30a    | UGUAACCCUCUCUGAGCUAGG               | 23          |                                  |
| miR-3017b  | GACCGGAGCGGCCGGGAA                   | 19          |                                  |
| miR-305    | AUUGUAUCUACUCAGGUGGUGG             | 23          | Huang et al. [26]                |
| miR-306a   | UCAGGACUACAGGUAGCUCU                | 20          |                                  |
| miR-315    | UUUGUGAUGUGUGUACGAGG               | 21          | Huang et al. [26]                |
| miR-316    | UGUCUUCUUCUUGCUCUUGCC              | 22          |                                  |
| miR-317    | UGAAACACCUCCGGUAGUAUCAGU            | 25          | Huang et al. [26]                |
| miR-320a   | AACUGCGGUGAGAGGCGAAUA               | 23          |                                  |
| miR-33     | AUGCAUUGUGUGUGUGCAUGCA              | 21          | Huang et al. [26]                |
| miR-34     | UGCGAGUGGGUUGAUGCUGUGU             | 23          | Huang et al. [26]                |
| miR-378a   | ACUGGACUAGGAGUGAAAG                 | 20          |                                  |
| miR-5097   | GUGAUCUGCCUCUGUUUGU                 | 17          |                                  |
| miR-5112   | UGGGUGUGAGCCUGAGU                  | 18          |                                  |
| miR-5592   | UUCGCGGUGAUGACCCA                    | 18          |                                  |
| miR-7      | UGGAAAGACUAGUAGUUGUUGUGU            | 24          | Huang et al. [26]                |
| miR-71     | UGAAAGACUGGGUAGUGAGAU              | 22          | Huang et al. [26]                |
| miR-745    | CGACUGCCCAUGAGAGGCGGUGS            | 22          |                                  |
| miR-750    | CACAGACUACUACUCUCUCUCGA             | 23          | Huang et al. [26]                |
| miR-8      | UAAUGCUUCGAGUAAAGAUGUA            | 23          | Huang et al. [26]                |
| miR-9      | UCUUGUGUGCUACUGCUGUAGU             | 23          | Huang et al. [26]                |
| miR-92b    | AUUGGACUAGUGCCGGCGGUGU             | 21          | Huang et al. [26]                |

(Continued.)
targeted exclusively by shrimp miRNAs increased from 62 (24 h) to 72 (48 h), while the number of WSSV transcripts targeted by WSSV miRNAs increased from 65 (24 h) to 79 (48 h) (figure 2b). Together, shrimp and WSSV miRNAs targeted 3038–4024 shrimp transcripts, and two WSSV genes (figure 2b). These findings suggested that both host and viral gene expression was regulated by shrimp miRNAs and/or WSSV miRNAs during virus infection.

To investigate the functions and pathways of miRNA target genes, the coding sequences of Ago1 complex transcripts were analysed with the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG). The GO analysis revealed that most identified genes were associated with membrane-enclosed lumen, organelles, macromolecular complexes and membranes, while many genes were involved in viral reproduction and immune system processes (figure 2c). The miRNA target genes were classified into 239 KEGG pathways. Some were involved in pathogen–host interactions, the NF-κB cascade, RNA interference, the Toll-like signalling pathway, the JAK–STAT cascade, the Wnt signalling pathway and the Notch signalling pathway (figure 2c). The 20 most highly expressed miRNAs targeted genes involved in host immunity including phagocytosis, endocytosis, virus–host interaction, apoptosis, autophagy, NF-κB and RNAi (figure 2d). These data suggest that these miRNAs played important roles in immunity.

2.3. Viral miRNA editing mediated by host adenosine deaminase acting on RNA

To further characterize these viral miRNAs, their sequences were compared to the WSSV genomic DNA sequence. Sequence analysis identified one A-to-G editing site in WSSV-miRNA-N12 (figure 3a). It was the only case of WSSV-miRNA editing based on sequence analysis. The sequencing data showed that the percentage of the edited WSSV-miR-N12 accounted for 31% (24 h post-infection) and 5.8% (48 h post-infection) of the total WSSV-miRNA-N12 sequences. A-to-G editing is prevalent in human miRNAs. Analysis of the predicted hairpin structure revealed that the precursor of WSSV-miRNA-N12 could form a fold-back structure (figure 3b) which can be edited by ADAR, which catalyses A-to-I RNA editing in humans [4]. To investigate the mechanism of viral miRNA (WSSV-miRNA-N12) editing, the shrimp ADAR gene was cloned (GenBank accession no. AHK28065.1). The shrimp ADAR contained two dsRNA-binding domains and one dsRNA adenosine deaminase domain. Neighbour-joining phylogenetic tree analysis indicated that ADAR was highly conserved in animals (figure 3c).

As previously reported, a primary miRNA or a precursor miRNA can be the substrate of ADAR–RNA editing [12,16]. To explore the involvement of shrimp ADAR in viral miRNA editing, a construct expressing the shrimp ADAR protein and a synthetic precursor of WSSV-miRNA-N12 were cotransfected into insect cells, then WSSV-miRNA-N12 was sequenced. Western blots revealed that ADAR was expressed differentially in insect cells (figure 3d). The total insect cell RNAs were extracted and the WSSV-miRNA-N12 precursor was cloned. Sequencing revealed that 17 of 120 clones isolated from cotransfected cells possessed copies of WSSV-miRNA-N12 that had undergone A-to-G RNA editing, while no edited WSSV-miRNA-N12 was detected in 60 clones isolated from cells transfected with WSSV-miRNA-N12 precursor alone (figure 3c). These findings indicated that the shrimp ADAR protein could edit viral miRNAs.

2.4. The role of viral miRNA editing in virus latency

To explore the role of viral miRNA editing in viral replication, the time-course of expression of unedited and edited WSSV-miR-N12 s was investigated in more detail. Northern blotting
Table 2. WSSV miRNAs in Ago1 complex.

| name       | sequence (5’ – 3’) | length (nt) | references of reported microRNAs |
|------------|--------------------|-------------|----------------------------------|
| WSSV-miR-N4 | GGAGCAACAGUGGUUCUGG | 20          | Huang et al. [19]                |
| WSSV-miR-N5 | CACUGGGCGUGGUGUUGGAA | 23          | Huang et al. [19]                |
| WSSV-miR-N6 | GAAACGACUUGCUCAGAAC | 23          | Huang et al. [19]                |
| WSSV-miR-N7 | GAUGAUUGGAGAAGAGGCAGU | 21          | Huang et al. [19]                |
| WSSV-miR-N8 | GAUGUUGGAAGAAUAAACAUUCAAUCUU | 25          | Huang et al. [19]                |
| WSSV-miR-N11 | AUCAUUUCUCUCUCUCUCU | 23          | Huang et al. [19]                |
| WSSV-miR-N12 | UUUUAAUGGAGAAGAAUAGAAUA | 22          | Huang et al. [19]                |
| WSSV-miR-N13 | AACAUGUGGAGAAUGGGGAGACU | 23          | Huang et al. [19]                |
| WSSV-miR-N14 | GAGAGAGAAGGAGAAGAAA | 21          | Huang et al. [19]                |
| WSSV-miR-N16 | AUCCGAUGAGAUGAUAUGAU | 25          | Huang et al. [19]                |
| WSSV-miR-N17 | CUGAAUUGACAUGAAGAUAUGAUGA | 24          | Huang et al. [19]                |
| WSSV-miR-N18 | GAAGAAUUGGUCUUCUAGGAA | 22          | Huang et al. [19]                |
| WSSV-miR-N19 | UGGAGCAUAUCUGUUGAUGGA | 22          | Huang et al. [19]                |
| WSSV-miR-N20 | GUUGUCUAGUAAAGUAGGAA | 23          | Huang et al. [19]                |
| WSSV-miR-N22 | GGGGCGUAAAAAGACUGUAGG | 21          | Huang et al. [19]                |
| WSSV-miR-N23 | GUGGUCUAAACGAAGGGCAAU | 21          | Huang et al. [19]                |
| WSSV-miR-N24 | GUAUGAGAUGAAGAAGAUAAGAAC | 23          | Huang et al. [19]                |
| WSSV-miR-N26 | UAUUUGUCUUGAGAAGAUAUCUU | 21          | Huang et al. [19]                |
| WSSV-miR-N28 | UUCGAGUGCCGAAUAUCUGGUC | 24          | Huang et al. [19]                |
| WSSV-miR-N29 | AAAGAGACAAAAACACAGGGU | 21          | Huang et al. [19]                |
| WSSV-miR-N30 | AUUGCCUAGAUCUCUCUAGUAAU | 25          | Huang et al. [19]                |
| WSSV-miR-N33 | GACGUGGCAUUUUCUGCCUU | 21          | Huang et al. [19]                |
| WSSV-miR-N34 | CGCGCAGUGUUUUAUAUGCA | 22          | Huang et al. [19]                |
| WSSV-miR-N36 | AGUUCUCUGAUUGAAGAUA | 20          | Huang et al. [19]                |
| WSSV-miR-N37 | UUUAAGACAAUUUCUGCCUCAG | 22          | Huang et al. [19]                |
| WSSV-miR-N38 | UUAGUGCCUGAUCUGAGAUCUG | 22          | Huang et al. [19]                |
| WSSV-miR-N39 | UGGAGAUAGUAGGCUAGUAAU | 22          | Huang et al. [19]                |
| WSSV-miR-N40 | AUCCGAUGAACAUGAAGA | 23          | Huang et al. [19]                |
| WSSV-miR-N41 | GAAAGAAUUGGGGAGGGAUC | 22          | Huang et al. [19]                |
| WSSV-miR-N44 | CGACGACGCUUUCUACUACUC | 21          | Huang et al. [19]                |
| WSSV-miR-N45 | GAGACCUUUCUACAGAUGAAGA | 23          | Huang et al. [19]                |
| WSSV-miR-N46 | AGUGCCAGAUAACCGGUAAG | 22          | Huang et al. [19]                |
| WSSV-miR-N48 | AGAGAGAGAUGGUGGGGACUCU | 21          | Huang et al. [19]                |
| WSSV-miR-N49 | GUGAGAUAUGUGGCUAUCU | 21          | Huang et al. [19]                |
| WSSV-miR-N73 | UAGUGAUAUAAAGAAGAGGAA | 22          | He et al. [18]                   |
| WSSV-miR-N82 | CUCAGAUUUCUGGUUGJUAU | 22          | He et al. [18]                   |
| WSSV-miR-N105 | CACACAGACUUCUCUCUCU | 22          | He et al. [18]                   |
| WSSV-miR-N110 | CCAAAAAAUAACCCGCUAUAU | 22          | He et al. [18]                   |
| WSSV-miR-N134 | ACCAGAUUCAUAAACAGGUAU | 22          | He et al. [18]                   |
| WSSV-miR-N142 | UGGUGAUAUCUGCUUCUCUCG | 22          | He et al. [18]                   |
| WSSV-miR-N156 | UUUUAGAUAACAAUAAGA | 22          | He et al. [18]                   |
| WSSV-miR-N180 | CCAUAAAACAUACUUCUCUCU | 22          | He et al. [18]                   |
| WSSV-miR-N195 | AAAAAAACAAGUGGAAGAUAUU | 22          | He et al. [18]                   |
| WSSV-miR-N201 | CGUGUGCCGGCUUCUGAAGAAC | 22          | He et al. [18]                   |
| WSSV-miR-N214 | AAGAGAAGAAGAAGAAGAAGAAGA | 22          | He et al. [18]                   |
| WSSV-miR-N50 | GAGCGACCGCGCCUGUAGGGA | 23          | He et al. [18]                   |
| WSSV-miR-N51 | GAGGUUGGCAUUCUGGCUUCU | 22          | He et al. [18]                   |
Figure 2. Interactions between miRNAs and mRNAs in Ago1 complex of shrimp. (a) Distribution of target RNA-annotated clusters across transcripts from Ago1 complex of virus-free and virus-infected shrimp pre-infection (0), and 24 and 48 h post-infection. The numbers indicated the time points of virus infection. (b) Numbers of genes targeted by host and virus miRNAs detected at 0, 24 and 48 h post-infection. (c) Function and pathway analyses of genes targeted by miRNAs. GO was performed by comparing the coding sequences of the transcripts in the Ago1 complex with the GO database with the blast E-value of less than $1 \times 10^{-5}$. KEGG classifications of the genes were simultaneously characterized. (d) Heat map generated from GO analysis of transcripts targeted by the top 20 miRNAs with high expression level. Tree showed the hierarchical clustering of miRNAs based on GO analysis. The colours indicated the significant differences between clusters.
indicated that both the unedited and edited WSSV-miR-N12s were detected in WSSV-infected shrimp at 6 h post-infection (figure 4a), suggesting that this viral miRNA was expressed and the miRNA editing occurred at the early stage of WSSV infection. The results showed that the frequency of viral miRNA editing increased before 18 h post-infection and decreased after 24 h post-infection (figure 4a). To evaluate the effects of this miRNA on WSSV infection...
Figure 4. (Caption overleaf.)
replication in shrimp, its expression was silenced using anti-miRNA oligonucleotide (AMO)-WSSV-miR-N12 (figure 4b). Silencing of WSSV-miR-N12 expression significantly reduced WSSV copy number (figure 4c) and significantly decreased shrimp mortality (figure 4d), indicating that expression of WSSV-miR-N12 contributed to viral replication.

To investigate the influence of viral miRNA editing on virus infection, the edited and unedited WSSV-miR-N12 were overexpressed in shrimp, which were then infected with WSSV. Northern blotting confirmed that the edited and unedited WSSV-miR-N12 were overexpressed in shrimp (figure 4e). Significantly more copies of the WSSV genome were detected in shrimp in which unedited WSSV-miR-N12 was overexpressed than shrimp infected with WSSV alone. However, the number of copies of the WSSV genome detected in shrimp in which edited WSSV-miR-N12 was overexpressed did not differ significantly from shrimp infected with WSSV alone (figure 4f), indicating that WSSV-miR-N12 promotes WSSV infection and that WSSV-miR-N12 editing could cause WSSV latency in shrimp. Shrimp mortality analysis generated similar results (figure 4g).

These findings suggest that A-to-I editing of viral miRNA (WSSV-miR-N12) could reduce viral replication in the shrimp, and cause virus latency.

2.5. The mechanism of viral miRNA editing in the miRNA-induced silencing complex

To investigate the mechanism of viral miRNA editing in the miRISC, WSSV-miR-N12 target genes were predicted. The prediction results indicated that the WSSV wsv399 gene could be targeted by WSSV-miR-N12 (figure 5a). To evaluate the direct interaction between WSSV-miR-N12 and wsv399, the EGFP gene and the 3’UTR of the wsv399 gene or its mutant were cloned, generating the EGFP-wsv399-3’UTR construct and the EGFP-wsv399-3’UTR-mutation construct (figure 5b). Co-transfection of WSSV-miR-N12 mimic and the EGFP-wsv399-3’UTR or EGFP-wsv399-3’UTR-mutation revealed that the fluorescence intensity of insect cells cotransfected with WSSV-miR-N12 and EGFP-wsv399-3’UTR was significantly weaker than that of controls (figure 5c), indicating that WSSV-miR-N12 directly targeted the wsv399 gene. To illustrate the effect of viral miRNA editing on the direct interaction of viral miRNA with its target gene, the edited WSSV-miR-N12 and the EGFP-wsv399-3’UTR were cotransfected into insect cells. We found that the wsv399 gene was not targeted by the edited WSSV-miR-N12 (figure 5d), indicating that the base change at the miRNA +16 site inhibited target mRNA recognition.

To explore the capacity of unedited or edited WSSV-miR-N12 to be loaded onto the host Ago1 protein during the assembly of miRISC, the viral miRNA was incubated with the shrimp Ago1 protein. Electrophoretic mobility shift assay (EMSA) results revealed that both the unedited and edited WSSV-miR-N12 mimics were co-injected with WSSV into shrimp, respectively. Edited and unedited WSSV-miR-N12 were detected pre-infection (0), and 12, 24, 36 and 48 h post-infection by northern blots. U6 was used as a control. (f) The shrimp were simultaneously injected with WSSV and unedited or edited WSSV-miR-N12, and the WSSV copies in shrimp were monitored by quantitative real-time PCR at 12, 24, 36 and 48 h post-infection (g) The cumulative mortalities of WSSV-infected shrimp treated with the unedited or edited WSSV-miR-N12 were monitored 1–5 days post-infection. All the assays were repeated three times (*p < 0.05; **p < 0.01).
Figure 5. (Caption overleaf.)
Figure 5. (Overleaf) The mechanism of viral miRNA editing in virus infection. (a) The region of the viral gene wsv399 3'UTR targeted by WSSV-miR-N12. The seed sequence of WSSV-miR-N12 is underlined. (b) Constructs of EGFP-wsv399-3'UTR and EGFP-wsv399-3'UTR–mutation. The sequence targeted by WSSV-miR-N12 is underlined. (c) Direct interaction between WSSV-miR-N12 and wsv399 gene in insect cells. Insect High Five cells were cotransfected with the WSSV-miR-N12 mimic or WSSV-miR-N12–mimic-scrambled and EGFP, EGFP-wsv399-3'UTR or EGFP-wsv399-3'UTR–mutation. At 36 h after cotransfection, the fluorescence of cells was examined. (d) The interaction between the edited WSSV-miR-N12 and wsv399 gene in insect cells. Insect High Five cells were cotransfected with the edited WSSV-miR-N12 mimic and EGFP-wsv399-3'UTR. WSSV-miR-N12–mimic-scrambled, EGFP and EGFP-wsv399-3'UTR–mutation were used as controls. At 36 h after cotransfection, the fluorescence of cells was evaluated. (e) The interaction between edited or unedited viral miRNA and host Ago1 protein. The unedited or edited WSSV-miRNA-N12 was incubated with recombinant shrimp Ago1 protein, then separated by native polyacrylamide gel and stained with ethidium bromide to visualize the miRNA (top), followed by staining with Coomassie blue (bottom). The wedges indicate the concentration gradient of recombinant protein used. (f) The interaction between viral miRNA and its target gene in the miRISC. The unedited or edited WSSV-miRNA-N12 and the 3'UTR of the wsv399 gene were incubated with shrimp Ago1 protein. Subsequently, the mixture was separated by agarose gel and stained with ethidium bromide to show the miRNA and target gene, followed by staining with Coomassie blue (bottom). The wedges indicate concentrations of recombinant protein used. (g) Northern blot analysis of expression profiles of the wsv399 gene in WSSV-infected shrimp. The shrimp were challenged with WSSV. Numbers indicated the time points post-infection. Shrimp β-actin was used as a control. (h) Silencing of wsv399 expression in WSSV-infected shrimp. The wsv399-siRNA and WSSV was co-injected into WSSV-infected shrimp. Then the shrimp haemolymph was subjected to northern blot analysis to detect the expression level of the wsv399 gene. Numbers indicate the time points post-infection. Shrimp β-actin was used as a control. (i) Effect of wsv399 gene silencing on WSSV copies in shrimp. The shrimp were simultaneously injected with WSSV and wsv399-siRNA, followed by detections of virus copies with quantitative real-time PCR. As controls, WSSV alone and wsv-399-siRNA-3'-UTR or wsv399-siRNA-3'-UTR–scrambled were included in the injections. (j) Accumulative mortalities of WSSV-infected shrimp. At different times post-infection, the accumulative mortalities of shrimp were monitored daily. Numbers showed the time points post-infection. (k) The detection of ADAR expression in virus-challenged shrimp. Shrimp were infected with WSSV. At different times post-infection, the ADAR mRNA level was evaluated with quantitative real-time PCR. (l) The examination of wsv399 expression. The wsv399 mRNA in shrimp treated with WSSV and unedited or edited WSSV-miRNA-N12 was quantified with quantitative real-time PCR. In all panels, the statistically significant differences between treatments were represented with asterisks (*p < 0.05; **p < 0.01).

3. Discussion

Virus replication, one of the most key steps in the virus life cycle, is elaborately regulated by virus. During the virus replication process, miRNAs are required [19,24,25]. In this investigation, the results revealed that the editing of viral miRNA played an important role in the virus replication, showing the elaborate mechanism of virus replication regulated by miRNA. miRNAs have been reported to influence both virus replication and pathogenicity, and host innate antiviral immune responses [27]. Studying the regulation of miRNA expression, including RNA editing of miRNA, can further reveal the function of miRNA. A-to-I RNA editing, catalysed by the ADAR enzyme, can generate RNA diversity post-transcriptionally [28,29]. Our study indicated that a viral miRNA, WSSV-miR-N12, underwent A-to-I RNA editing at the +16 site, and that RNA editing of WSSV-miR-N12 influenced virus latency. WSSV-miR-N12 could promote virus replication by targeting the WSSV early gene, wsv399. The role of wsv399 in the WSSV–shrimp interaction has not been identified. Our study revealed that wsv399 expression inhibited WSSV infection. Although the edited WSSV-miR-N12 bound Ago just like the unedited WSSV-miR-N12, the edited WSSV-miR-N12-coupled Ago could not interact with the target gene wsv399. As a result, WSSV-miR-N12 editing at the early stage of virus infection promoted the expression of viral wsv399 gene, leading to the inhibition of WSSV replication and subsequently affecting virus latency. In this context, our study presented a novel aspect of viral miRNA editing which could act as a mechanism to promote virus latency.

miRNAs, loaded into the miRISC, are thought to target multiple miRNAs, affecting the translation or stability of several target genes [30]. Although computer algorithms that constrain searches for mRNA sites complementary to the miRNA ‘seed’ region to the 3'UTR of mRNA can identify potential matches, accurately predicting the miRNA target of miRNAs remains difficult [31,32]. Different computer algorithms generate divergent target sets with high false-positive rates, and usually contradict one another [33,34]. Recently, miRNA overexpression or knockdown studies have been combined with proteome analysis to identify miRNA targets [31,35]. However, these studies have identified a relatively small number of proteins, and the results of miRNA overexpression or knockdown studies do not distinguish between primary miRNA effects and secondary effects. Also, it is known that not all miRNAs load onto Ago proteins and bind target genes. Therefore, it has become important to use a combination of target identification methods to reveal the spectrum of miRNA targets. With an aim to understand the role of miRNAs in virus–host interactions, in this study we characterized the miRNAs and miRNAs loaded into the Ago complex of WSSV-infected shrimp haemocytes. Many miRNAs and their target genes were identified based on the miRNA–miRNA interactions facilitated by the Ago complex. Therefore, we presented an efficient strategy for the comprehensive analysis of miRNA-mediated regulation of gene expression in virus–host interactions.

Our findings reveal that while A-to-I editing of WSSV-miR-N12 did not affect binding to shrimp Ago, edited WSSV-miR-N12 could not recognize the target gene via A:U Watson–Crick pairing. The change to G:U pairing at the +16 site weakened the interaction between miRNA and its target gene. Previous studies have reported that even a single G:U base-pair change in the seed region can reduce the efficiency of target recognition, and miRNA 3’ pairing with the target favours contiguous Watson–Crick pairs.
uninterrupted by wobbles, bulges or other mismatches at 12–17 sites [36,37]. Our findings reveal a novel regulatory aspect of miRNA editing in the miRNA–target interaction. The mechanism merits further study.

4. Material and methods

4.1. Shrimp culture and white spot syndrome virus infection

*Marsupenaeus japonicus* shrimp (about 15 g each) were reared in groups of 20 individuals in air-pumped circulating seawater at 25°C. Then three shrimp were randomly chosen for the WSSV detection using PCR with the WSSV-specific primers (5'-TTGGTTTCATGCCCGAGATT-3' and 5'-CCCTGGTCAGCCCC TTGA-3'). Virus-free shrimp were infected with WSSV as previously described [26]. Before infection, and 24 and 48 h post-infection, shrimp haemocytes were collected. WSSV copies were quantified by quantitative real-time PCR, as previously described [26].

4.2. Recombinant expression of shrimp Ago1 in *Escherichia coli* and antibody preparation

The *Ago1* gene of *M. japonicus* was amplified with primers 5'-AAGGATCCATGTACCCTGTC GGGCAGCCACC-3' (BamHI, underlined) and 5'-AATTCGAGTTAAGCAAAGTACATGACT CT GTTCGT-3' (XhoI, underlined) and cloned into the pGEX-4T-2 vector downstream of glutathione S-transferase (GST). Gene expression and protein purification were achieved by following the manufacturer's protocols (Amersham Biosciences, USA). Purified GST or GST–Ago1 proteins were used as antigen to immunize mice to prepare antibody. Antiserum titres were evaluated by enzyme-linked immunosorbent assay, the immunoglobulin fraction was purified with protein A–Sepharose (Bio-Rad, USA) and antibody specificity was evaluated by western blot.

4.3. Co-immunoprecipitation of shrimp Ago1

Shrimp haemocytes were collected and UV (ultraviolet) irradiated at 254 nm, then lysed in lysis buffer (20 mM Tris–Cl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, pH 7.5). The cross-linked lysate was treated with 15 μl of RNAsin (Promega, USA) and incubated on ice for 10 min, then 30 μl of RQ1 DNAse was added (Promega). After 5 min at 37°C the Ago1–RNA complex was immunoprecipitated using the polyclonal antibody against GST or GST–Ago1 for 16 h at 4°C, followed by incubation with protein A–Sepharose (GE Healthcare, USA) for 30 min at 4°C. After washing in lysis buffer, the Ago1–RNA complex was eluted with elution buffer (50 mM glycine, pH 2.8) and subjected to western blot. RNAs (including mRNA and miRNA) were extracted from the complex using a mirVanaPTMP miRNA isolation kit according to the manufacturer's instructions (Ambion, USA). After separation on a 15% polyacrylamide–8 M urea gel, RNAs of 16–30 nt and RNAs of more than 100 nt were recovered, using the RNA gel extraction kit (Takara, Japan) for sequencing.

4.4. Western blot

Protein samples were analysed on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, USA). The membrane was immersed in blocking buffer (3% bovine serum albumin (BSA)) at 4°C overnight, followed by incubation with anti-GST or anti-GST–Ago1 antibody. Then the membrane was incubated in AP-conjugated goat anti-mouse IgG (Sigma, USA) for 1 h and binding was visualized with NBT and BCIP solutions (BBI, Canada).

4.5. Sequencing and sequence analysis of Ago1-associated RNAs

Small RNAs and longer RNAs were sequenced with a GA-I genome analyser (Illumina, San Diego, CA, USA) according to the manufacturer’s protocols. Small RNAs were also analysed by searching the ACGT V3.1 program developed by LC Sciences (Houston, TX, USA). After the removal of adaptor sequences, mRNA, rRNA, tRNA, snRNA, snoRNA and other non-coding RNA sequences available in Rfam (http://www.sanger.ac.uk/software/Rfam), the high-quality sequences were compared with known animal miRNAs in miRBase 19.0 and the known WSSV miRNAs, as previously described [18,19,26]. The miRNAs with no homologue were further analysed using BLASTN against the shrimp EST database (National Center for Biotechnology Information, NCBI).
http://www.ncbi.nlm.nih.gov) and the WSSV genome sequence (GenBank accession no. AF332093.1), allowing one or two mismatches between each pair of sequences. Hairpin structure of novel miRNA candidates was predicted using MFOLD online software (http://frontend.bioinfo.rpi.edu/applications/mfold/) with default parameters. The longer RNAs were analysed using Trinity software and the Illumina paired end method developed by LC Sciences (Houston, USA). After the removal of repetitive and low-quality reads, the remaining high-quality reads were aligned to the shrimp assembled ESTs in the National Center for Biotechnology Information (as the M. japonicus genome was not listed) or to the WSSV genome (GenBank accession no. AF332093.1).

### 4.6. Northern blot

Total RNAs were extracted from the Ago1 complex of shrimp haemoocytes using a mirVanaPTMP miRNA isolation kit according to the manufacturer’s instructions (Ambion, USA). The NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the concentration of the extracted RNAs. Total RNAs (5 μg) were separated on a denaturing 15% polyacrylamide gel containing 8 M urea. Then the separated RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences, Buckinghamshire, UK). The membrane was washed with DIG Easy Hyb granules buffer (Roche, Basel, Switzerland) for 0.5 h at 42 °C after cross-linking by UV irradiation and subsequently hybridized with a DIG-labelled DNA probe of a miRNA for 20 h at 42 °C. To identify the unedited and edited WSSV-miR-N12, locked nucleic acid (LNA)-modified oligonucleotide probes were used in northern blot [38]. The LNA-modified oligonucleotide probes (unedited WSSV-miR-N12, 5'-DIG-TATTCATTCTCTCGATAA-3'; edited WSSV-miR-N12, 5'-DIG-TATTCATTCTCTC GATAA-3') were modified with LNA at the fifth and seventh bases. The expression level of wsv399 mRNA or ADAR mRNA was measured using quantitative real-time PCR with wsv399-specific primers (5'-TGCAGCTACGCCATGC- TACA-3' and 5'-TGTGAAAGTGTTGATG CCGATCT-3') or ADAR-specific primers (5'-GACGGCCCTGATG ATG-3' and 5'-CTGACACACTCCAAACTTG-3'). Shrimp β-actin was used as a control.

### 4.8. Prediction of genes targeted by microRNAs

miRNA target genes were predicted by employing the transcriptome sequence of the shrimp Ago1 complex using two independent computational algorithms, including TargetScan 5.1 (http://www.targetscan.org) and miRanda (http://www.microrna.org/). Based on the sequence analysis of the transcriptomic sequencing data of the shrimp Ago1 complex, the shrimp genes and WSSV genes in the Ago1 complex were obtained and used for the prediction of genes targeted by miRNAs. TargetScan was used to search for miRNA seed matches (nucleotides 2–8 from the 5'-end of miRNA), while miRanda was used to match the entire miRNA sequence with the parameters free energy less than −20 kcal mol⁻¹ and score greater than 50. Finally, the data predicted by both algorithms were combined and overlaps were calculated.

### 4.9. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analysis

GO analysis was performed as previously described [26]. Briefly, the coding sequences of transcripts retrieved from the Ago1 complex were extracted and used as queries to search the protein sequences collected in the GO database with the blast E-value of less than 1 × 10⁻⁵ [39]. The best hit GO identities were assigned to the transcripts. Then the
4.10. Analysis of white spot syndrome virus-miR-N12 RNA editing site

The sequence of mature WSSV-miR-N12 was aligned with the WSSV genomic DNA sequence using DNAMAN. The hairpin structure of WSSV-miRNA-N12 was predicted using mFold (http://frontend.bioinfo.rpi.edu/applications/mfold/) with the default parameters.

4.11. Cloning and sequence analysis of shrimp adenosine deaminase acting on RNA gene

Total RNAs were extracted from shrimp haemocytes using a mirVanaTM miRNA isolation kit according to the manufacturer’s instructions (Ambion, USA). The first-strand cDNA template was synthesized using Prime Script 1st Strand cDNA Synthesis Kit (Takara, Japan). Rapid amplification of cDNA ends (RACEs) was performed for the full-length ADAR gene sequence using a 5'/3’ RACE kit (Roche, USA). The full-length sequence of shrimp ADAR gene was obtained and deposited in GenBank (accession no. AHH23065.1).

The ADAR domains were analysed using the SMART program (http://smart.embl-heidelberg.de/). Neighbour-joining phylogenetic tree analysis of ADAR proteins was performed with the MEGA5.1 program. The GenBank accession numbers of the ADAR proteins were indicated as follows: Xenopus (Sihara, tropicalis), XP_002943575.2; Gallus gallus, XP_001231262.2; Danio rerio, NP_571671.1; Drosophila melanogaster, NP_001254761.6; Apis mellifera, NP_001941684.1; Homo sapiens, NP_0565562.2; Mus musculus, CAJ18531.1; Ciona intestinalis, AAC25097.1; Zootermopsis nevadensis, DQ224469.1. Bootstrap analysis was conducted for 1000 replicates to define the confidence of tree branch positions.

4.12. Identification of white spot syndrome virus-miR-N12 RNA editing site in insect cells

The shrimp ADAR gene was cloned into a pIIZ/V5-His vector (Invitrogen, USA) using primers 5′-ATCGAATTCGAGATCTCGAAG-3′ and 5′-AGGCGGCGGATGAAAAAGCTGGTCGC-3′ to express the V5-ADAR fusion protein. The WSSV-miR-N12 precursor was cloned from the WSSV genome using the in vitro Transcription T7 Kit (Takara, Japan) with specific primers (5′-GGATCCACTACGTTATTACGTTATTATGTAATGGTACTATATGTTGCG-3′). The purified RNAs were folded in the folding buffer as previously described [40]. Subsequently different quantities (0.5, 1, 5, 12 μg) of plasmid expressing V5-ADAR and the synthesized WSSV-miR-N12 precursor were cotransfected into insect High Five cells (3 x 10⁶ cells ml⁻¹) (Invitrogen) and cultured at 27°C in Express Five serum-free medium (SFM) (Invitrogen) containing l-glutamine (Invitrogen). Two days later, the cells were collected and subjected to western blot analysis and RNA sequencing.

4.13. The silencing and overexpression of white spot syndrome virus-miR-N12 in shrimp

To evaluate the effects of WSSV-miR-N12 on virus infection, the expression of WSSV-miR-N12 was knocked down in shrimp by injection of AMO. AMO-WSSV-miR-N12 (5′-ATTCTCTCGAATCTTAAA-3′) targeting WSSV-miR-N12 was synthesized (Sangon Biotech, Shanghai, China) with a phosphorothiate backbone and a 2′-O-methyl modification at the 6th and 12th nucleotides. The sequence of AMO-WSSV-miR-N12 was randomly scrambled generating AMO-WSSV-miR-N12-scrambled (5′-ATTCTCTGCTCTATTAG-3′) as a control. Subsequently, 10 nM of AMOs was co-injected with WSSV (10⁶ copies ml⁻¹) into virus-free shrimp (100 μl per shrimp).

In order to explore the role of WSSV-miR-N12 editing in virus infection, the unedited WSSV-miR-N12 (5′-UUUAUCUGAGAAUAAGAAAUUU-3′ and 5′-UAUUCUUAUUUCUCUGAUAUAAA-3′) and the edited WSSV-miR-N12 (5′-UUUAUCUGAGAAUAAGAAAUUU-3′ and 5′-UAUUCUAAUUCUCUGAUAUAAA-3′) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China), generating WSSV-miR-N12 mimics. Virus-free shrimp were injected with 30 nM of the unedited or edited WSSV-miR-N12 mimic in addition to WSSV (10⁶ copies ml⁻¹). The sequence of WSSV-miR-N12 mimic was randomly scrambled to yield WSSV-miR-N12-mimic-scrambled (5′-UUUCCUGGAACGUGUCAUGTT-3′ and 5′-ACGUAGCGUUCCAGAAATTT-3′) as a control. WSSV (10⁶ virus copies ml⁻¹) was used as a positive control.

At different time points (0, 6, 12, 24, 36, 48 h) after the injection of AMOs or mimics, the shrimp haemolymph was collected and subjected to northern blotting.

4.14. Shrimp mortality assay

To analyse the shrimp mortality, 20 shrimp were collected from each experimental condition in three independent experiments. Shrimp were injected with AMOs (10 nM) or mimics (30 nM). WSSV (10⁵ virus copies ml⁻¹) was used as a positive control. Shrimp mortality was monitored daily over a 5-day period.

4.15. The direct interaction between white spot syndrome virus-miR-N12 and its target gene wsv399

To explore the direct interaction between WSSV-miR-N12 and its target gene wsv399, the enhanced green fluorescent protein (EGFP) gene was cloned into a pIIZ/V5-His vector (Invitrogen) with EGFP-specific primers (5′-AAGAGCTCGAGATCCCGGACACGTTGAG-3′ and 5′-AATCTAGAGTCGCGGCCGCCTTTA-3′). To generate the EGFP-wsv399-3′UTR (3′ untranslated region) construct, the 3′UTR of wsv399 gene was cloned into the pIIZ/V5-His vector downstream of EGFP gene using sequence-specific primers (5′-GATCTGAAATATGCCTGGAAT-3′ and 5′-ATACCCCGGGTTCTCCATGATGTTG-3′). The sequence of wsv399 3′UTR was randomly mutated by PCR with sequence-specific primers (5′-AGTTCCACTATTTGCGAUC
GGCGAACTCC and 5′-TTAGGACCGTGGAGTTCGCC
GTGCGCAA-3′) generating the EGF-vas399-3′UTR
mutation construct. Recombinant plasmids were confirmed by sequencing.

Insect High Five cells (Invitrogen) were cultured at 27°C in Express Five SFM medium (Invitrogen) supplemented with l-glutamine (Invitrogen). After culture at about 70% confluence (3 × 10⁴/well) in a 96-well plate, 200 ng of EGF, EGFP-vas399-3′UTR or EGF-vas399-3′UTR-mutation construct was transfected into High Five cells. At the same time, the cells were transfected with 50 nM of either a synthesized WSSV-miR-N12 mimic, the edited WSSV-miR-N12 mimic or a synthesized WSSV-miR-N12-mimic-scrambled. The mimics were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). At 36 h after transfection, fluorescence was determined by a Flex Station II microplate reader (Molecular Devices, USA; excitation/emission at 480/520 nm, respectively), and normalized to cells not transfected with EGF, in three independent experiments.

4.16. Electrophoretic mobility shift assay

The recombinant glutathione S-transferases (GST)-Ago1 was purified. To investigate the ability of WSSV-miRNA-N12 to load onto shrimp Ago1 protein, 40 μM of the unedited or edited WSSV-miRNA-N12 was incubated with 12.5, 25, 50 or 100 μM Ago1 protein. Unedited or edited WSSV-miRNA-N12 was synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). After incubation in the reaction buffer (0.1 M KCl, 1 mM DTT, 1 mM MgCl₂, 10 mM HEPES, pH 7.6) for 30 min at 37°C, the mixture was separated on a 5% native polyacrylamide gel at 120 V for 1 h. Then the RNA bands were stained with ethidium bromide and subsequently the proteins were stained with Coomassie blue.

To illustrate the interaction between the viral miRNA and its target gene in the miRISC, 40 μM of the unedited or edited WSSV-miRNA-N12 and the 3′UTR of vas399 gene were incubated with shrimp Ago1 protein. The 3′UTR of vas399 was cloned with primers 5′-TAAATACGACTCACTATAGGGAT GCTGAGTAATC-3′ and 5′-GTAACACTTTCCATGAT GTG-3′. Then the 3′UTR of vas399 was synthesized using an *in vitro* T7 transcription kit (TaKaRa, Japan) according to the manufacturer’s instructions. After incubation in the reaction buffer, the mixture was electrophoresed on a 1% agarose gel at 120 V for 30 min. Then the gel was stained as described above.

4.17. Synthesis of siRNAs and RNAi assay in shrimp

Small interfering RNAs (siRNAs) specifically targeting the vas399 gene were synthesized using an *in vitro* T7 transcription kit for siRNA synthesis (TaKaRa, Japan) according to the manufacturer’s instructions. The vas399-siRNA sequence was 5′-CCGACCTAGATATGATAGCGACA-3′. As a control, the sequence of vas399-siRNA was scrambled, generating vas399-siRNA-scrambled (5′-ATTCATGCTCCCGACATCC GATGAC-3′). The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5). Then the synthesized dsRNAs were assessed by agarose gel electrophoresis and quantified by spectrophotometry. The RNA interference (RNAi) assay was conducted by co-injection of siRNA (15 μg) and WSSV (10⁵ copies ml⁻¹) into virus-free shrimp. Twenty virus-free shrimp were used for each treatment. At 12 h after the co-injection, these shrimp were injected with the siRNA (15 μg). The injection of WSSV alone served as a positive control. At different times post-infection (0, 12, 24, 36 and 48 h), the shrimp haemocytes were collected and subjected to northern blot analysis and levels of WSSV copies were measured. Mortality of WSSV-infected shrimp was also monitored daily. All the experiments were biologically repeated three times.

4.18. Statistical analysis

Numerical data were processed using one-way analysis of variation (ANOVA), and Student’s *t*-test was employed to assess the significant difference.

**Competing interests.** We declare we have no competing interests.

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