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Characterization of shrimp Drosha in virus infection

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ABSTRACT

RNA interference (RNAi) mediated by microRNA (miRNA) is an evolutionarily conserved mechanism of posttranscriptional gene regulation in all eukaryotes, involving in natural antiviral immunity. The RNAase III Drosha is a key component for miRNA maturation. To date, however, the roles of Drosha in virus infection remain to be addressed. In this study, the Drosha was characterized in *Marsupenaeus japonicus* shrimp. The sequence analysis revealed that the shrimp Drosha gene encoded a 1081-amino-acid peptide, which comprised two tandem ribonuclease III C terminal domains and a double-stranded RNA binding motif. The shrimp Drosha was homologous with those of other animal species. The quantitative RT-PCR analysis revealed that the Drosha gene was highly expressed in lymphoid organ and was significantly up-regulated in response to WSSV challenge, suggesting that the Drosha was involved in the antiviral immunity of shrimp. The results showed that the knock down of Drosha gene led to the defect of miRNA maturation, and subsequent higher virus loads in shrimp. Our study presented that Drosha played important roles in the antiviral defense of shrimp.

1. Introduction

RNA interference (RNAi) is an important strategy of post-transcriptional gene regulation in all eukaryotes, involving two main classes of small noncoding RNAs that are 21–24 nucleotides in length: small interfering RNAs (siRNAs) and microRNAs (miRNAs) [1]. The siRNAs are processed as duplexes from perfectly base-paired dsRNA precursors by a cytoplasmic RNaseIII enzyme named Dicer [2]. In contrast, miRNAs are processed from the hairpin primary transcripts called primary miRNAs (pri-miRNAs), which are transcribed by RNA polymerase II or III within the nucleus [3]. In animals, the pri-miRNAs are processed in the nucleus by RNaseIII enzyme Drosha to form hairpin structures known as pre-microRNAs (pre-miRNAs), of ~70 bases. These pre-miRNAs are exported to the cytoplasm by exportin-5/Ran-GTP complex and processed into miRNA/miRNA* duplexes by Dicer. Thereafter, one strand of siRNA or miRNA/miRNA*duplex is selected as 'guide strand', and assembled into the RNA-induced silencing complex (RISC), leading to either mRNA cleavage or translational repression depending on the degree of sequence complementarity with the targets [4].

The miRNAs, an extension class of evolutionarily conserved small non-coding RNAs purposefully expressed from an organism's own genome, have emerged as important regulators in diverse pathways and biological processes in animals [4]. At the first step of miRNA maturation, the pri-miRNA is cleaved into pre-miRNAs by a microprocessor complex containing the RNaseIII enzyme Drosha and its binding partner DGCR8 (Pasha in invertebrates) [5,6]. It is revealed that Pasha/DGCR8 interacts with pri-miRNAs through the single-stranded (ss) flanking segment and the double stranded (ds) RNA of ~33 bp, and assists Drosha to cut the pri-miRNA (~11 bp) away from the ssRNA–dsRNA junction [7]. As reported [8], a terminal loop greater than 10 nucleotides is required for the processing of pri-miRNA into pre-miRNA. The Drosha measures the distance of about 22 bp from the loop and cuts the pri-miRNA away from the terminal loop. Recently, some studies showed that the processing of a small number of pre-miRNAs embedded in short introns, did not require Drosha [9,10]. There may exist multiple mechanisms of pri-miRNA processing in animals. In addition to the processing of pri-miRNA, Drosha can destabilize the transcripts in a miRNA-independent manner [11]. Furthermore, the Drosha is postulated to participate in the regulation of host immune systems [12]. It was reported that the knock down of Drosha resulted in a decrease of most miRNAs, leading to an increased sensitivity of

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host to virus infection [12]. However, the roles of Drosha in virus infection remain to be addressed.

To address this issue, the shrimp Drosha was characterized in this study. Shrimp, one of the most important species in marine aquaculture, has been threatened by viral diseases during the last decades, especially white spot syndrome virus (WSSV) [13]. The economic value of shrimp has made it an increasingly important model for the research of crustacean immunity. Due to the lack of true adaptive immune response system, invertebrates including shrimp are thought to rely on the ancient RNAi immune system against virus invasion [14]. Recent studies revealed that administration of dsRNA/siRNA into shrimp could provide an effective protection against virus invasion, suggesting that the RNAi pathways played very important roles in invertebrate immune response against virus infection [13,15–18]. To date, some key RNAi-related genes have been cloned from shrimp including Dcr1 (GenBank accession no. GU265733), Dcr2 (GenBank accession no. JQ349041), Ago1 (GenBank accession no. GU265732.1) and Ago2 (GenBank accession no. HM234690). However, the Drosha, a core component of the RNAi pathway, has not yet been identified. In this investigation, the full-length cDNA sequence encoding shrimp Drosha was obtained. The results showed that Drosha was essential for the biogenesis of miRNA and played an important role in host response to WSSV infection.

2. Materials and methods

2.1. Shrimp culture and WSSV challenging

For each treatment, *Marsupenaeus japonicus* shrimp of about 15 g body weight were reared in a group of 20 individuals, and maintained temporarily for 2–3 days. Three shrimp of each group were randomly selected for WSSV detection with WSSV-specific primers (5′-TATTGTCTCTCCTGACGTAC-3′ and 5′-CACATTCTTCACGAGTCTAC-3′) to ensure that the shrimp were virus-free before experiments. Then the WSSV-free shrimp were infected with 100 μl WSSV solution at 10^4 virions/ml by intramuscular injection using a syringe with a 29-gauge needle [13]. The WSSV virions were heat-inactivated for 20 min at 100 °C. Shrimps treated with inactivated WSSV were used as a control. After WSSV or inactivated WSSV challenge, four shrimps were randomly selected for each treatment at different time post-infection. Then the shrimp organs or tissues (heart, hemolymph, lymphoid organ, gill, muscle and hepatopancreas) were collected and immediately stored in liquid nitrogen for later use.

2.2. RNA extraction and cDNA synthesis

Total RNAs were extracted from shrimp using mirVanaP^{TMP} RNA isolation kit according to the manufacturer's instructions (Ambion, USA), followed by removal of DNA contamination with RNase-free DNase I (Takara, Japan) at 37 °C for 30 min. The concentrations of the total RNAs were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then 1 μg of the total RNAs was used for the first strand cDNA template synthesis according to the manufacturer's guideline of PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan).

2.3. Cloning of the full-length cDNA of shrimp Drosha gene

Based on amino acid alignments of Drosha proteins from *Drosophila melanogaster* (GenBank accession no. AAF59169.1) and *Apis mellifera* (GenBank accession no. XP_394444.3), the degenerate primers 5′-GDYTDGARTWYYTVGGHGATGCTGT-3′ and 5′-TCCMAR-ATCATCRCADACVACMGCYYGWGT-3′ (R = A/G; S = C/G; W = A/T; H = A/T/C; M = A/C; Y=C/T; D = A/G/T; V = A/G/C) were used for PCR amplification of shrimp Drosha gene. The PCR condition was conducted as follows: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. To get the full-length sequence of Drosha cDNA, the rapid amplification of cDNA ends (RACE) was performed using a 5′/3′ RACE kit (Roche, USA). Based on the sequences generated by degenerated primers, the sequence-specific primers (5′-CGATACTGCGGTCAGTAAAAGCTC-3′, 5′-TGCGGTCTCCTTGTGGCTCC-TGC-3′ or 5′-GTGG ATCGAGGTTAAGAACTC-3′ for 5′ RACE and 5′-TCTTGGGAGACACAGTCCTCCAGC-3′ for 3′ RACE) were employed for the subsequent 5′-end and 3′-end RACE PCR. The system and conditions for RACE PCR was carried out according to the manufacturer's instructions. The PCR products were cloned into pMD-18 vector (TaKaRa, Japan) and subjected to sequencing. After assembling the overlapping fragments, the full-length cDNA of Drosha was obtained (GenBank accession no. JQ918355).

2.4. Sequence analysis

The sequences of Drosha were obtained from The National Center for Biotechnology Information (NCBI) and analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The neighbour-joining phylogenetic tree was constructed using the MEGA 5.05 program. Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions. The identification of Drosha domains was conducted using the SMART program (http://smart.embl-heidelberg.de/).

2.5. RNAi assays

To silence the expression of shrimp Drosha, the sequence-specific siRNA (Drosha-siRNA, 5′-GCATCAGAATACTTATATA-3′) was synthesized according to the design rule for RNAi [13]. As control, one nucleotide of Drosha-siRNA sequence was randomly mutated, generating the Drosha-mutation-siRNA (5′-GCATCAGAAGACT TATATA-3′). The siRNAs were synthesized in vitro using the In vitro Transcription T7 Kit for siRNA Synthesis (Takara, Japan) according to the manufacturer's protocol. The synthesized siRNAs were dissolved in PBS solution (0.1 M, pH 7.4) and quantified by spectrophotometry. The Drosha-siRNA or Drosha-mutation-siRNA was injected into shrimp at the lateral area of the fourth abdominal segment at 30 μg/shrimp. As a negative control, the PBS solution was included in the injections. At various time points after siRNA injection, the gill tissues from three shrimp specimens were collected and subjected to subsequent analyses. All the assays were biologically repeated for three times.

2.6. Quantitative real-time PCR

Quantitative real-time PCR was conducted to assess the gene expressions with sequence-specific primers and TaqMan fluorogenic probes. The shrimp β-actin was used as a control. The primers were 5′-CTCTTGCAGCTGGCCCTTAC-3′ and 5′-GCTGCCGTATTCCA-CAATTTG-3′ for Drosha and 5′-CGAGCACGGCATCGTT ACTA-3′ and 5′-TTGTAGAAAGTGTGATGCCAGATCT-3′ for β-actin. The TaqMan probes were 5′-FAM-TTTGGCACGAATCCCGAT-TAMRA-3′ (Drosha) and 5′-FAM-CTGGGACGACATGGA-TAMRA-3′ (β-actin). Reactions were prepared in a total volume of 25 μl containing 12.5 μl of Premix Ex Taq (Takara, Japan), 1 μl of cDNA template, 0.5 μl of 10 μM forward and reverse primers and 0.5 μl of 10 μM TaqMan fluorogenic probes at a final concentration of 0.2 μM. Amplification profiles consisted of 95 °C for 1 min, and 40 cycles of 95 °C for 15 s and 55 °C for 45 s.

To quantify the copies of WSSV virions in shrimp, the real-time PCR was performed using WSSV-specific primers (5′-TTGGTTTCATGCCC-GAGATT-3′ and 5′-CCTTGGTCAGCCCCTTGA-3′) and TaqMan fluorogenic probe (5′-FAM-TGCT GCCGTCTCCAA-TAMRA-3′). The linearized plasmid contained a 1400-bp DNA fragment from the WSSV genome

was used as the internal standard of real-time PCR [19]. About 20 mg of shrimp gills were collected and subjected to genome DNA extraction using SQ Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instruction [19]. The PCR reaction mixture (25 μl) contained 12.5 μl of Premix Ex Taq (Takara, Japan), 1 μl of DNA template, 0.5 μl of 10 μM forward and reverse primers and 0.5 μl of 10 μM TaqMan fluorogenic probe at a final concentration of 0.2 μM. The reaction profile was 95 °C for 1 min, followed by 45 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C.

2.7. Northern blot

RNAs were extracted from gill tissues of shrimp at 48 h after injection of siRNAs. Then they were separated on a denaturing 15% polyacrylamide gel containing 7 M urea, followed by being transferred to Hybond-N+ membrane (Amersham Biosciences, Buckinghamshire, UK). After ultraviolet crosslink (Ultra-Violet Products Ltd., USA), the membrane was rinsed in DIG Easy Hyb granules buffer (Roche, Grenzacherstrasse, Basel, Switzerland) for 0.5 h. Subsequently the membrane was hybridized with DIG-labeled DNA probes complementary to miR-1 (5′-UGGAAUG UAAAGAAGUAUGGAG-3′), miR-let7 (5′-UGAGGUAGUAGGUU-GUAUAGUU-3′) or miR-100 (5′-AACCCGUAGAUCCGAACUUGUG-3′) for 20 h, respectively. As control, U6 was detected using the DIG-labeled U6 probe (5′-GGGCCATGCTAATCTTCTCTGTATCGTT-3′). The detection was conducted following the manual of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland).

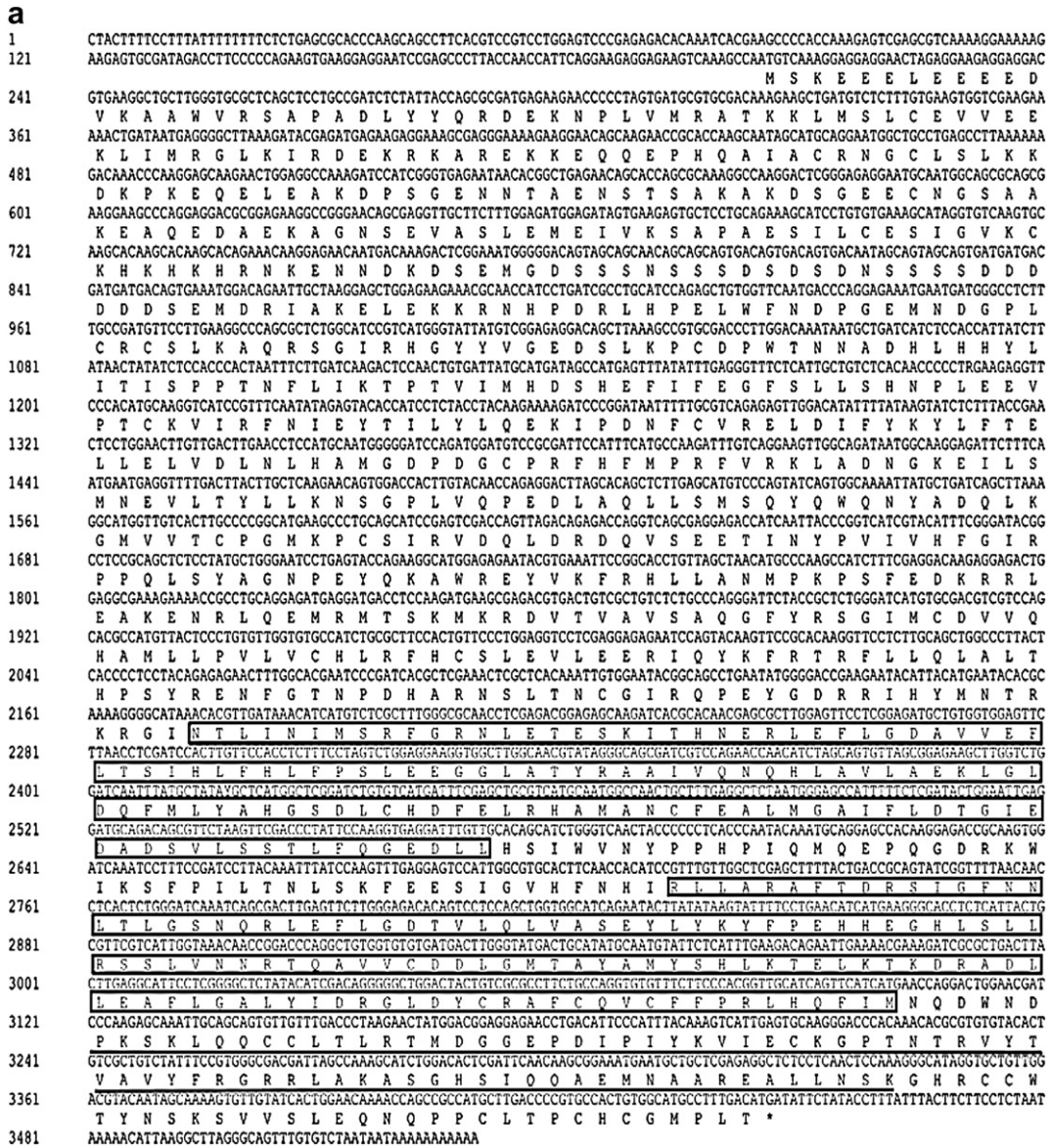

Fig. 1. Characterizations of shrimp Drosha. (a) Nucleotide sequence and deduced amino acid sequence of shrimp Drosha cDNA. The two RIBOc domains were boxed and the dsRNA binding domain was underlined. (b) Multiple sequence alignments of RIBOc and DRBM domains of Drosha proteins. Identical amino acids were indicated in black and similar amino acids were shown in dark grey. Hs, *Homo sapiens*; Mm, *Mus musculus*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Am, *Apis mellifera*; Mj, *Marsupenaeus japonicus*. (c) Phylogenetic analysis of Drosha proteins. The neighbor-joining phylogenetic tree was generated using MEGA 5.05 program. Bootstrap values were indicated. The bar represented the distance. The GenBank accession numbers were as follows: Hs-Drosha, Q9NRR4.2; Mm-Drosha, NP_001123621.1; Ce-Drosha, NP_001122460.1; Dm-Drosha, AAF59169; Am-Drosha, XP_394444.3; Mj-Drosha, JQ918355; Af (*Apis florae*)-Drosha, XP_003698687.1; Mr (*Megachile rotundata*)-Drosha, XP_003699917.1; Ae (*Acromyrmex echinatior*)-Drosha, EGI58626.1; Xt (*Xenopus tropicalis*)-Drosha, NP_001107152.1; Od (*Oikopleura dioica*)-Drosha, CAP07635.1; As (*Ascaris suum*)-Drosha, AEF32762.1. Bootstrap values were indicated for each branch from 1000 replicates.

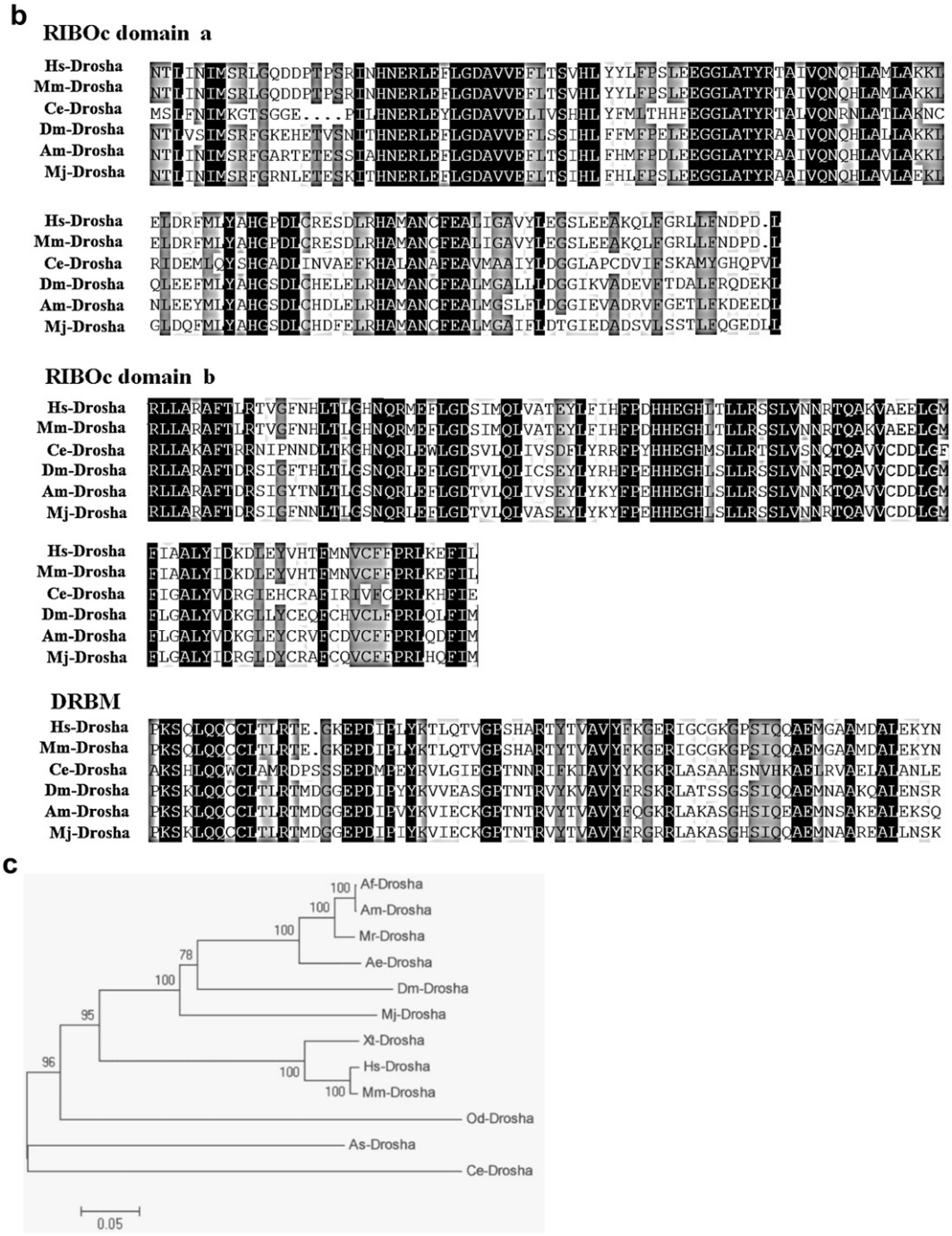

Fig. 1. (*continued*).

2.8. Statistical analysis

The numerical data from three independent experiments was analyzed by one-way ANOVA to calculate the mean and standard deviation of triplicate assays. Statistical significances between treatments were carried out using Student's *t*-test.

3. Results

3.1. The cDNA cloning and sequence analysis of shrimp Drosha gene

Based on PCR amplification with degenerated primers and RACE, a full-length cDNA of shrimp Drosha gene was obtained (Fig. 1a). The sequence analysis indicated that the 3529-bp cDNA contained an open reading frame (ORF) of 3243 bp, encoding a 1081-amino-acid peptide (Fig. 1a), which was highly homologous with Droshas of other animals. The deduced Drosha protein comprised three characteristic domains, including two tandem ribonuclease III C terminal domains (RIBOc) and a double-stranded RNA binding motif (DRBM). The multiple sequence alignments revealed that the three domains of Drosha protein were conserved in different species of animals (Fig. 1b).

To reveal the molecular evolution of shrimp Drosha and its homologs of other species, the full-length protein sequences from mammalians and invertebrates were used to construct phylogenetic tree by MEGA 5.05 program using Neighbor-joining

algorithm. The phylogenetic analysis presented that the shrimp Drosha was more closely related to insect Droshas than to those of vertebrates (Fig. 1c).

3.2. The expression profiles of Drosha gene

The expressions of Drosha were examined in different shrimp tissues or organs including heart, hemolymph, stomach, lymphoid organ, gill, muscle and hepatopancreas. The quantitative RT-PCR results showed that Drosha was detected in all the tissues or organs examined (Fig. 2a). It was found that the Drosha gene was significantly up-regulated in the lymphoid organ and hemolymph (Fig. 2a), which were the immune organ and issue of shrimp. The data suggested that the Drosha, as a core component of RNAi, played important roles in shrimp immunity.

To reveal the expression profiles of Drosha in response to virus infection, the lymphoid organs and gills of shrimp challenged with WSSV or inactivated WSSV were collected and subjected to quantitative real-time PCR. The time-course results showed that the virus loads of shrimp challenged with WSSV were increased, whereas no virus was detected for shrimp treated with inactivated WSSV (Fig. 2b). The data indicated that the shrimps were successfully infected by WSSV. It was found that the WSSV infection led to the increase of Drosha gene expression level (Fig. 2c). At 24 h post-infection, the Drosha gene was significantly up-regulated by comparison with the control (0 h post-infection) ($p < 0.05$) (Fig. 2c). However, the expression profile of Drosha gene in shrimp treated with inactivated WSSV did not change at time points examined (Fig. 2c). The results presented that the WSSV infection led to the increase of Drosha gene expression level, and that the host Drosha might be involved in shrimp immune response against virus infection.

3.3. Roles of Drosha in the biogenesis of miRNA and the host defense against virus infection

To assess the role of Drosha in the biogenesis of miRNA, the virus-free shrimp were injected with the Drosha-specific siRNA (Drosha-siRNA) or the control siRNA (Drosha-mutation-siRNA). The real-time PCR results revealed that the expression of Drosha was significantly down-regulated by Drosha-siRNA from 12 to 72 h after siRNA injection (Fig. 3a), whereas the Drosha-mutation-siRNA had negligible effect on the Drosha expression by comparison with the control PBS (Fig. 3a), indicating that the shrimp Drosha gene was silenced and that the siRNA was highly specific. At 48 h after siRNA injection, the expression level of Drosha was the lowest (Fig. 3a). Under the condition that the Drosha gene expression was silenced by Drosha-siRNA, the endogenous miRNAs (miR-let7, miR-1 and miR-100) of shrimp were examined. The three miRNAs were the most abundant in shrimp [20]. As revealed by Northern blots, all the three miRNAs could not be detected at 48 h after the Drosha-siRNAs injection (Fig. 3b). The results showed that the Drosha-mutation-siRNA took no effect on the miRNA generation. These data presented that the Drosha was required for the miRNA maturation.

In an attempt to characterize the role of Drosha in host responses to virus infection, the shrimp Drosha gene was silenced by Drosha-siRNA, followed by the evaluation of WSSV infection in shrimp. The results showed that the knock down of Drosha gene by Drosha-siRNA led to a statistically significant increase of WSSV

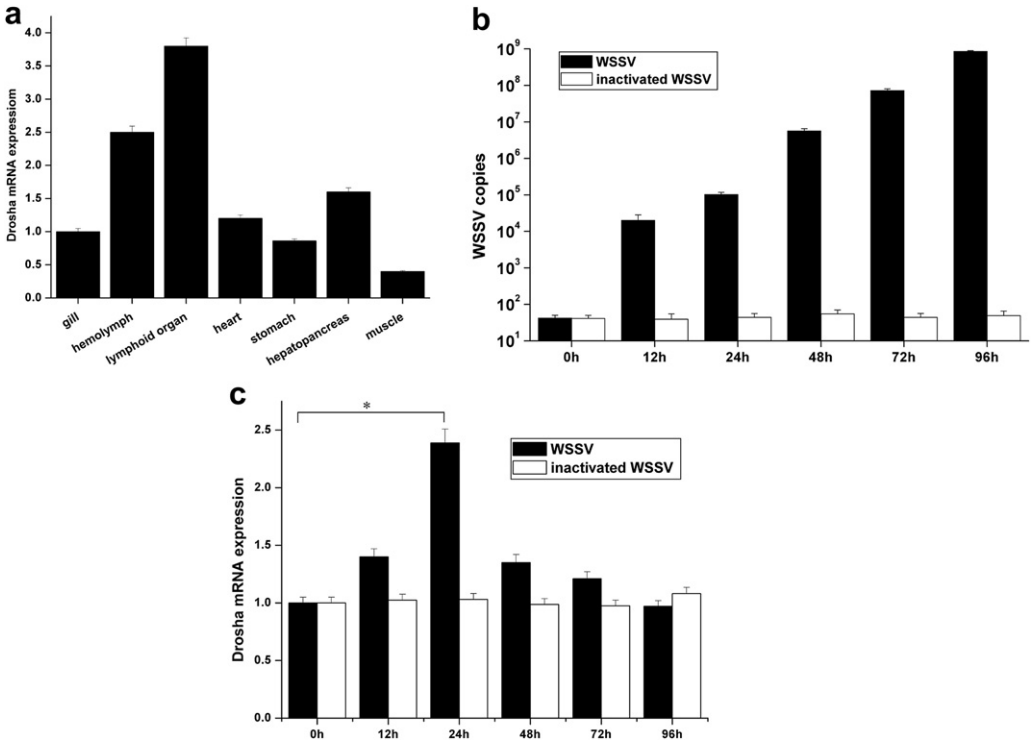

Fig. 2. The expression profiles of Drosha gene in shrimp. (a) The expression of Drosha in various shrimp tissues or organs. The expression levels of Drosha mRNA in various tissues or organs were normalized with shrimp β-actin mRNA. Each column represented the mean of triplicate assays within 1% standard deviation. (b) Quantification of WSSV copies by real-time PCR. The shrimps were injected with WSSV or heat-inactivated WSSV as a control. At different time after injection, the shrimp gills were collected and subjected to real-time PCR. All the assays were biologically repeated for three times. The numbers indicated the time points after injection. The solutions used for injections were shown on the top. (c) The time-course expression of Drosha in lymphoid organ of shrimp challenged with WSSV. The shrimp challenged with heat-inactivated WSSV was included in the analysis as a control. The numbers indicated the time after the injection of WSSV or inactivated WSSV. Each column represented the mean of triplicate assays within 1% standard deviation. The statistically significant difference between treatments was represented with an asterisk ($^*P < 0.05$).

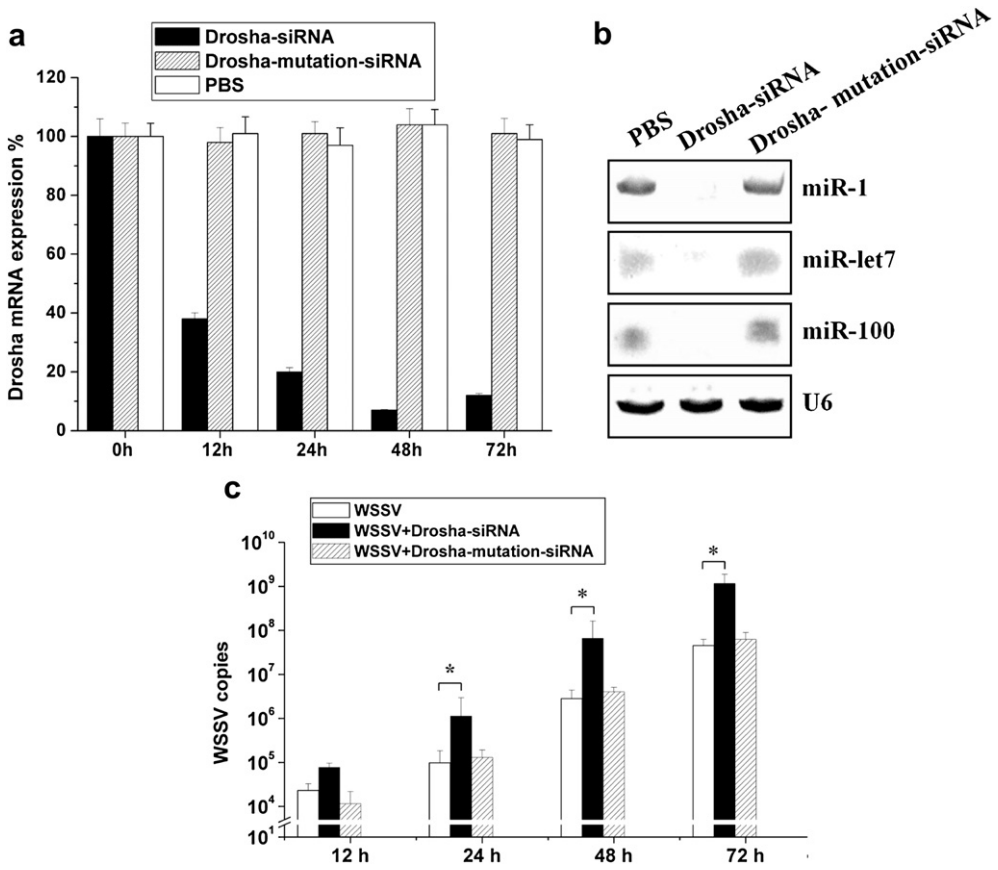

Fig. 3. Roles of Drosha in miRNA biogenesis and host antiviral response. (a) Silencing of shrimp Drosha gene by siRNA. The shrimp were injected with Drosha-siRNA, Drosha-mutation-siRNA or PBS, respectively. At different time after injection, the Drosha gene expression was examined by quantitative real-time PCR with Drosha-specific primers and probe. The numbers indicated the time after injection. (b) Detection of miRNA in response to Drosha gene silencing. The shrimp were injected with Drosha-siRNA, Drosha-mutation-siRNA or PBS. At 48 h after injection, the shrimp lymphoid organ tissues were subjected to Northern blots with the miR-1, miR-let7, miR-100 or U6 probes. The shrimp U6 was used as control. Lane headings indicated the solutions used for injections. The probes were shown at the right. (c) Effects of Drosha gene silencing on virus infection. The shrimp were injected with Drosha-siRNA or Drosha-mutation-siRNA. At 48 h after siRNA injection, the siRNAs and WSSV were simultaneously injected to the same shrimp. The WSSV only was used as positive control. The solutions used for injections were shown at the top. At different time post-infection, the shrimp gill tissues subjected to quantitative real-time PCR to monitor the WSSV replication. The numbers indicated the time points post-infection with WSSV. The statistically significant differences between treatments were represented with asterisk ($^*P < 0.05$).

copies from 24 to 72 h post-infection by comparison with the positive control WSSV only ($P < 0.05$) (Fig. 3c). However the Drosha-mutation-siRNA had no effect on WSSV replication (Fig. 3c). These results indicated that the Drosha protein played very important roles in the host antiviral responses.

4. Discussion

RNAi, mediated by siRNAs or miRNAs, is a mechanism of post-transcriptional gene regulation that functions as a natural defensive response to viral infection from plants to mammals [21–23]. In shrimp, several key components of RNAi pathway have been characterized except for Drosha. It is documented that Dicer-1 and Argonaute-1 (Ago-1) are involved in the antiviral defense of shrimp [24,25]. As reported, Drosha, a member of the ribonuclease III family, plays essential roles in the initial processing of miRNA maturation, which can cut the pri-miRNA into pre-miRNA [4]. Drosha enzymes have been identified in a wide range of animals, but not in plants. Up to date, however, the Drosha has not yet characterized in crustaceans. Our study revealed that the Drosha protein was required for the miRNA maturation and played important roles in the antiviral response of shrimp. Therefore our study presented the first characterization of Drosha in crustaceans. In this context, the present and previous studies provided strong evidence that the RNAi pathway took great effects in the crustacean antiviral immunity.

In this study, it was shown that the shrimp Drosha was up-regulated in the lymphoid organ and hemolymph. As documented, the lymphoid organ and hemolymph are the important immune organs of shrimp. The shrimp lymphoid organ serves as a filter for foreign materials encountered in the hemolymph, which plays a major role in the clearance of bacteria and viruses [26]. Due to its important in shrimp immunity, the shrimp lymphoid organ becomes one of the main target organs of many viruses including WSSV [26]. The up-regulation of Drosha in immune organs revealed in this investigation suggested the involvement of Drosha in shrimp immune responses. Our study presented that the Drosha was up-regulated in response to WSSV infection. It was further revealed that the Drosha took great effects on WSSV replication. Due to the requirement of Drosha in the miRNA biogenesis in shrimp, it was inferred that the involvement of Drosha in the shrimp antiviral response resulted from its complex effects on the miRNA maturation pathways. It is reported that the expression profiles of host miRNAs can be altered by infecting viruses [27–29]. Some host miRNAs represent a part of the host innate antiviral defense, inhibiting virus replication through directly targeting viral mRNAs or indirectly targeting host transcripts beneficial to the viruses [27–29]. The studies by Han et al. show that Drosha can

cleave the hairpin structures embedded in the DGCR8 mRNA and thereby mediate the direct cis-regulation of DGCR8 mRNA levels to auto-regulate its own miRNA biogenesis [11]. Possibly there are some additional mRNAs (containing pre-miRNA-like structures) are also directly controlled by Drosha-mediated cleavage. However, these transcripts have not been well-defined [11]. It is documented that the viral transcripts of Kaposi's sarcoma associated herpesvirus (KSHV), murine cytomegalovirus, Epstein Barr virus (EBV) and Marek's disease viruses 1 and 2 contain pre-miRNA structures that can be subjected to process by host Drosha [30]. In Kaposin B (KapB), a transcript encoded by KSHV is directly regulated by Drosha cleavage and the differential expression levels of Drosha contribute to low levels of KapB expression in latency and marked increases in expression during lytic replication [31]. However, it is unknown whether transcripts of WSSV are also directly regulated by Drosha-mediated cleavage, a mechanism which may be employed by host to destroy viral transcripts, leading to the inhibition of WSSV replication. This issue merited to be further investigated in future.

Acknowledgements

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