Identification and characterization of pyrethroid-resistant miRNAs in the genome of malaria vector *Anopheles Sinensis*

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Research Article

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Abstract

Background: microRNAs (miRNAs) are small endogenous non-coding RNAs that play important roles in many developments, differentiation, apoptosis, and innate immunity in insects. Pyrethroid resistance has become the unique largest obstacle for the mosquito control, and *An. sinensis* is the main malaria vector mosquito in Eastern and other Asian countries.

Methods: We investigated miRNA expression profiles associated with pyrethroid-resistant in *An. sinensis* by Illumina Hiseq sequencing. Meanwhile, we applied bioinformatics methods to identify miRNAs and their target genes associated with pyrethroid resistance in. miRNAs selected in the bioinformatics approach were confirmed by RT-qPCR. In addition, dual luciferase reporter was also performed to verify the interaction of miRNA and its target genes.

Results: We obtained 7,120,612- 7,157,914 clean RNA sequencing reads from the different sample sequencing. A total of 329 miRNAs (included 246 novel ones) were identified by Illumina Hiseq sequencing. Thirty-nine miRNAs were significantly down-regulated in the pyrethroid-resistant populations in comparison of those in susceptible strain. Furthermore, 1909 and 6696 target genes were predicted for the dysregulated 20 known and 19 novel miRNAs respectively. GO annotation and KEGG pathway analyses for the predicted genes of dysregulated miRNAs revealed that they might be involved in a broad range of biological processes related with the pyrethroids, such as integral component of plasma membrane, intracellular binding, catalytic activity, signal transduction, protein transport and several key pathways including ABC transporters. Through miRNA-gene network analysis, 13 key miRNAs and 34 mRNAs of their target genes were predicted. Consistent with the RNA-seq results, nine miRNAs are significantly down-regulated by RT-qPCR in pyrethroid-resistant strains of *An. sinensis*. In addition, luciferase reporter assay demonstrated that miR-87 directly bound with the 3'UTR of target gene *Sinensis_GLEAN_10013226*.

Conclusion: This study provides the whole-genome information of miRNAs and their characteristics in *An. sinensis*, which lays an important foundation for further research on miRNAs associated with pyrethroid resistance. Targeting the miRNA-mRNA network may be a new strategy to overcome insecticide resistance in mosquito vectors.

Background

Chemical insecticides have been used to control vector mosquitoes since 1940. Up to now, pyrethroids have become the main insecticides for vector control due to their low toxicity to humans and effective killing and residual effects on mosquito vectors [1]. However, the wide use of pyrethroid has led to widespread pyrethroid resistance in *Anopheles sinensis* [2]. The reduced efficiency of pesticides cause a great threat to malaria control and may lead to increased transmission of various diseases [3], especially the pyrethroid resistance in *An. gambiae* [4]. *An. sinensis* is the main malaria vector mosquito in China, South Korea, Japan and other Asian countries [5, 6]. At present, the most important method of malaria prevention is to control the transmission vector, and thus the research on pyrethroid resistance in *An. sinensis* is extremely important. Recently, we sequenced and annotated the genome and transcriptome of *An. Sinensis* [7, 8]. The genome sequencing in *An. sinensis* facilitated identification and characterization of some families of genes involved in pyrethroid resistance, such as P450s [2, 9], GSTs [9], CCEs [9, 10], cuticular proteins [11], ionotropic glutamate receptors [12] and odorant-binding proteins [13]. Besides, previous report has shown the sodium channel-related genes also affected pyrethroid resistance [14].

MicroRNAs (miRNAs) are a class of non-coding RNAs with a length of 18-24 nucleotides, and they are single-stranded and highly abundant in cell. miRNAs can regulate gene expression through translational repression or mRNA degradation with specific base pairing, often at 3'UTR of target mRNAs, and the translational repression is mediated by a guiding complex called the miRNA-induced silencing complex (miRISC) [15]. miRNA regulation in insects has been reported to involve in various of biological processes such as apoptosis, differentiation, and cell division [16, 17]. miRNA was first reported in *Drosophila melanogaster* for insects, and 258 miRNAs have been identified for the species [18]. Among them, the dme-miR-310 family is related to immunity [19], and dme-miR-184, dme-miR-125, and dme-miR-100 is involved in metabolism and longevity [20]. For mosquitoes, there are 173 miRNA recognized in *An. gambiae* [21], and inhibition of aga-miR-305 increased resistance to *Plasmodium falciparum* infection and suppressed the midgut microbiota [22]. One hundred fifty-six miRNAs have been identified in *Aedes aegypti*, aae-miR-2940 affects the expression of dengue virus, and aae-miR-375 is related to developmental immunity [23, 24]. One hundred forty-two miRNAs have been identified in *Culex pipiens pallens* [25], and cpi-miR-2, cpi-miR-13, cpi-miR-71, cpi-miR-92 have been

reported to regulate the expression of pyrethroid resistance-related genes[26, 27]. However, there are only 164 miRNAs identified in different developmental stages of *An. sinensis* [28]. To date, little is known about the miRNAs in the whole genome of *An. sinensis*. Furthermore, the research of miRNAs on pyrethroid insecticides resistance genes is still limited, and the molecular mechanism leading to insecticide resistance is quite unclear. As such, it is of great significance to identify and characterize pyrethroid-resistant miRNAs in the genome of *An. sinensis*.

In this study, we identified and classified the miRNA of *An. sinensis* using RNA-seq and bioinformatics in the whole genome of *An. sinensis*. More importantly, we investigate association of miRNA with pyrethroid resistance in this species. To explore potential gene regulatory networks, 13 down-regulated miRNAs were defined as key miRNAs in the process of pyrethroid resistance. Furthermore, miRNA-mRNA network was analyzed based on the analysis of pyrethroid resistance pathways. miRNA-mRNA network of *An. sinensis* in pyrethroid resistance is beneficial for further research to overcome insecticide resistance in mosquito vectors.

Methods

Mosquito samples and genome sequence resource

Resistant populations (AH-FR, CQ-FR and YN-FR) and susceptible populations (AH-FS, CQ-FS and YN-FS) of pyrethroids were collected from three regions in Anhui, Yunnan and Chongqing. A laboratory pyrethroid susceptible strain (WX-LS) were collected from Wuxi, Jiangsu. Through continuous drug screening, we established three laboratory pyrethroid resistant populations(AH-LR, CQ-LR and YN-LR) based on pyrethroid resistant populations. The three laboratory resistant pyrethroid populations were identified as *An. sinensis*[29] through morphological and molecular techniques,.

The sensitivity of adult *An. sinensis* to deltamethrin was tested by female *An. sinensis* without blood feeding for three days after adulthood. Unfed female *An. sinensis* adults three days post emergence were tested to determine their susceptibility to deltamethrin using the standard World Health Organization (WHO) tube bioassay with 0.05% deltamethrin test papers, with paraffin oil-treated papers without insecticide used as the control [2]. After 60 minutes of exposure to 0.05% deltamethrin, mosquitoes that died during the exposure were collected immediately. If they remained motionless after mechanical stimulation, they were recorded as a pyrethroid susceptible populations. After 60 minutes of exposure, the living mosquito was transferred to a recovered cup and kept in a 6% sucrose solution for 24 hours. Mosquitoes that survive 24 hours later are considered to have pyrethroid resistance. The mosquitoes of each strain were collected and placed in RNeasy (Shanghai, China), and stored at -80°C until further processing. Total RNA was extracted from samples for miRNAs (Takara, Dalian, China) followed manufacturer's protocol.

Small Rna Sequencing And Annotation

Small RNA library construction and Illumina sequencing: seven samples (AH-FR, CQ-FR, YN-FR, WX-LS, AH-FS, CQ-FS and YN-FS) of RNA libraries were established. Each sample was a pool of individuals, and there were biological replicates for each sample for the small RNA sequencing. A total of seven RNA libraries of samples were sequenced using Illumina HiSeq™ 2000.

Read quality check and mapping: The initial readings obtained by Illumina sequencing were processed. After the removal of the linkers at both ends of the sequence, low-quality sequences (including sequences greater than 30 nucleotides in length and less than 15 nucleotides in length) and polyA fragments, the data is initially filtered to obtain clean sequences. The clean reads were assembled to RNA sequences using an online bioinformatics tool SOAP [30] and small RNAs were identified and analyzed for their small RNA diversity.

Small non-coding RNAs recognition: The small RNAs were annotated, mapped and searched against small RNAs database using Rfam11 (<http://rfam.xfam.org/>). They were divided into six categories: miRNA, rRNA, snRNA, tRNA, repeat-associated RNA and unannotated small RNA. In the alignment and annotation, some small RNA tags may be mapped more than one category. The

frequency of each unique microRNA mapped to different annotation is GenBank > Rfam > known miRNA > repeat > exon > intron3 with tag2 annotation.

Characteristics And Identification Analysis Of Mirnas

The known miRNAs were identified using miRBase22 [31], and the novel miRNAs were predicted using miRDeep2 [32]. For the identification of known miRNAs, the miRbase from different insect species was also searched with identified miRNAs in comparison. A number of stringent criteria were used for evaluating whether novel miRNA candidates were genuine miRNAs, such as exploring the secondary structure by RNAfold, calculating minimum free energy (MFE), and avoiding isolated base pairs. All miRNAs identified were named rendering to their most similar species in miRbase. Custom scripts adapted from quantitative script of miRDeep2 were used to obtain the miRNA counts. The miRNA read count is standardized, using (RPKM) Reads per kilobase per million mapped Reads $RPKM = (\text{mapped reads per miRNA} / (\text{sequence length} / 1000 \times \text{total mapped reads} / 106))$ RPKM. The miRNAs of seven libraries (AH-FR, CQ-FR, YN-FR, AH-FS, CQ-FS, YN-FS, WX-LS) were statistically processed to obtain the overall description of the miRNAs of each library. Quantitative statistics and expression analysis were performed based on the seven libraries.

Identification and functional analysis of miRNAs and target gene related to pyrethroid resistance

To study the difference between miRNAs in resistant strains and susceptible strains, the differentially expressed miRNAs between field pyrethroid-resistant populations (AH-FR, CQ-FR and YN-FR) in three provinces and laboratory pyrethroid-sensitive strains (WX-LS) in Wuxi, Jiangsu were determined. The previous difference analysis method for miRNA ($\log_2 > 1$, $P \leq 0.05$, $FDR < 0.01$, count reads > 15) was used. This analysis uses the R language. To predict the potential target genes of miRNAs, miRanda [33] and RNAhybrid [34] were used to predict differentially expressed miRNA targeted mRNAs. mRNAs were from the genomes of *An. gambiae* (vectorbase download) and *An. sinensis* (ASDB: The *Anopheles sinensis* omics database). The predicted target genes were further subjected to GO[35] enrichment and KEGG[36] pathway analyses by DAVID [37].

Pyrethroid-resistant Mirna Validation By Rt-qpcr

miRNA expression validated by RT-qPCR was used to validate the presence and expression of the identified miRNAs, eight miRNAs of differential expression were selected for RT-qPCR as described previously [38]. The total RNA was extracted using TRIzol Reagent (Invitrogen). cDNA was synthesized using PrimeScript RT Master Mix kit (Takara). SYBR Prime Script miRNA RT-PCR kit (Takara, Dalian, China) was used for miRNA RT-qPCR. All reactions were assayed in three biological and technical replications and performed on CFX ConnectTM (Bio-Rad) using SYBR PrimeScript RT-qPCR kits (Takara, Dalian, China). The relative expression level of miRNA was normalized to the internal control of U6 small nuclear (U6). PCR conditions consisted of pre-denaturation and hot start Taq activation at 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 45 s, and the relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Luciferase Reporter Assay

TargetScan was applied to predict the potential binding site between miR-87 and Sinensis_GLEAN_10013226(TF). The 3'UTR sequence of TF containing the putative binding site of miR-87 was cloned into a GLO-ge 3'UTR luciferase vector (Promega, Madison, WI, USA) to construct TF-WT1'UTR and TF-MUT1-3'UTR. The Recombinant vector, miRNA mimic or negative control (GenePharma) were transfected into HEK293T cells by Lipofectamine 3000 (Thermo Fisher). 48 hours later, the luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) and analyzed using the GloMax 96 Microplate Luminometer (Promega).

Results

Small non-coding RNA in pyrethroid resistant and sensitive strains of *An. sinensis*

We performed small non-coding RNA sequencing on pyrethroid-resistant and sensitive strains of *An. sinensis*, and the raw data was accessible via PRJNA796374. we obtained a total of about 5.04×10^7 Raw Reads from seven libraries in Table 1. The clean reading sequence is 5.00×10^7 , and the effective ratio of each library is more than 97%. Map these clean readings to the genome. The WX-LS mapping is the most abundant (5 729 817) and largest proportion (80.44%), the other six strains have similar mapping results (5 490 514- 5 691 367), and the proportion is also similar (77.04%-79.95%). There are no obvious differences between strains in different regions. Fig. 1 showed that the number of miRNAs in YN-FS is the largest (1 456 158, 20.46%), while it is smallest in AH-FR (409 580, 5.73%). In WX-LS, rRNA had the most reads (403 844, 5.74%) and CQ-FS had the least reads (900 029, 1.26%). Among the 7 libraries, the Repeat sequence reads (1 034 068, 14.53%-1 562 869, 21.89%) and the unannotated reads (3 623 491, 51.52%-4 290 905, 60.00%) were similar. Resistant comparison with sensitive to libraries of pyrethroids in the three regions was show that miRNAs accounted for a larger proportion in sensitive types than that in resistant ones.

Table 1

Sequencing and mapping results of seven different field populations of small RNA libraries in *Anopheles sinensis*. AH, CQ and YN indicate Anhui, Chongqing and Yunnan province, respectively; FR and FS indicate the pyrethroid-resistant and -susceptible field populations, respectively; WX-LS presents the pyrethroid-susceptible laboratory strain from Wuxi, Jiangsu province.

	AH-FR	AH-FS	CQ-FR	CQ-FS	YN-FR	YN-FS	WX-LS
Total reads	7,200,000	7,200,000	7,200,000	7,200,000	7,200,000	7,200,000	7,200,000
Clean reads	7,157,914	7,149,576	7,121,311	7,120,612	7,141,200	7,147,605	7,132,914
Total sRNAs	7,152,065	7,122,704	7,116,887	7,118,781	7,117,617	7,118,716	7,032,828
Mapped reads to the genome	5,583,335 (78.07%)	5,729,817 (80.44%)	5,586,931 (78.50%)	5,513,319 (77.45%)	5,490,514 (77.14%)	5,691,367 (79.95%)	5,418,030 (77.04%)

Diversity and characteristics of *An. sinensis* miRNAs

A total of 83 known miRNAs and 246 new miRNAs were identified from the small RNA library obtained from these seven samples (Table 2). 79 known miRNAs were found in AN-FS, while 73 ones were found in AH-FR. Moreover, Fig. 2a clearly showed that there is no significant difference between the known and novel identified miRNAs in the above 7 libraries, Fig. 2b showed that there is no significant difference of the number between resistant and susceptible samples. Fig. 2c and Supplementary Table 1 further showed that asi-miR-1, asi-miR-184, asi-miR-989, asi-miR-8, asi-miR-275 in *An. sinensis* expressed relatively high levels. The expression of all the miRNAs in all the samples were shown in Fig. 2c.

Table 2

Number of known and novel miRNAs identified from seven different field populations of small RNA sequencing.

miRNA	AH-FR	AH-FS	CQ-FR	CQ-FS	YN-FR	YN-FS	WX-LS	Total
Known	73	79	74	74	75	76	76	83
New	75	103	52	43	74	97	78	246

Table 3

Significantly down-regulated miRNAs and their negatively-/positively-regulated mRNAs in *Anopheles sinensis*. Both miRNAs and mRNAs are commonly up/down-regulated in three pyrethroid-resistant field populations (AH-FR, CQ-FR and YN-FR) in comparison of the laboratory susceptible strain (WX-LS).

Significantly down-regulated miRNA			Target gene			
Name ^a	Sequence	Average ^b	Gene ID ^c	Gene symbol ^d	Average ^b	Up/down ^e
Negatively-regulated pairs						
miR-124	UAAGGCACGCGGUGAAUGCCAAG	-2.489	10016785	AGAP011458	1.743	Up
miR-124	UAAGGCACGCGGUGAAUGCCAAG	-2.489	10011945	AGAP006113	2.505	Up
miR-2	UAUCACAGCCAGCUUUGAUGAGC	-1.838	10011668	AGAP011092	2.221	Up
miR-278	UCGGUGGGACUUUCGUCCGUUU	-2.082	10011186	PPO2	5.428	Up
miR-278	UCGGUGGGACUUUCGUCCGUUU	-2.082	10017232	AGAP003099	2.009	Up
miR-87	GGUGAGCAAUAUUCAGGUGU	-1.339	10013226	AGAP000376	2.324	Up
miR-932a-5p	UCAAUUCCGUAGUGCAUUGCAG	-2.252	10006623	AGAP006348	2.767	Up
miR-981	UUCGUUGUCGACGAAACCUG	-1.836	10009751	AGAP008440	2.813	Up
miR-981	UUCGUUGUCGACGAAACCUG	-1.836	10006525	AGAP007254	1.903	Up
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10010088	hsp90	3.712	Up
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10011186	PPO2	5.428	Up
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10009751	AGAP008440	2.813	Up
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10014376	AGAP002198	2.797	Up
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10008251	AGAP001346	6.437	Up
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10005782	AGAP000879	2.423	Up
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10014903	Vg1	5.375	Up
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10011643	SIX4	2.581	Up
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10015344	AGAP001532	3.307	Up

^amiR-124 indicates asi-miR-124, and miR-52(n) indicates asi-miR-new52, which is newly identified in this study; ^bAverage: expression quantity calculated as LOG₂ (fold change); ^c10000375 is for gene "Sinensis_GLEAN_10000375" and so on; ^d Orthologous genes determined by annotation of combined KEGG, GO, Swissport, Trembl and IPR methods (see detail in Table S2); ^eSignificantly up- or down-regulated for the target gene.

Significantly down-regulated miRNA			Target gene			
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10014898	Vg1	6.367	Up
miR-34(n)	GCCCGGCGGGCGGUUGGACC	-10.179	10008819	AGAP010764	1.771	Up
miR-34(n)	GCCCGGCGGGCGGUUGGACC	-10.179	10008816	AGAP010766	2.311	Up
miR-34(n)	GCCCGGCGGGCGGUUGGACC	-10.179	10011912	AGAP012296	2.938	Up
miR-34(n)	GCCCGGCGGGCGGUUGGACC	-10.179	10016010	AGAP002851	2.358	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10000722	AGAP002754	2.332	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10001284	AGAP003430	1.666	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10006525	AGAP007254	1.903	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10004503	AAEL000200	2.236	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10012974	AAEL012964	2.210	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10016785	AGAP011458	1.743	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10015344	AGAP001532	3.307	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10017232	AGAP003099	2.009	Up
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10012903	AGAP010200	2.046	Up
miR-60(n)	GCGAGCAUCUGUAGCGUGCA	-5.285	10009751	AGAP008440	2.813	Up
Positively regulated pairs						
miR-10-5p	UACCCUGUAGAUCCGAAUUUGU	-1.279	10006910	AGAP009554	-2.786	Down
miR-124	UAAGGCACGCGGUGAAUGCCAAG	-2.489	10016076	AGAP003571	-2.444	Down
miR-210	UUGUGCGUGUGACAACGGCUA	-1.803	10001409	AGAP007548	-2.749	Down
miR-210	UUGUGCGUGUGACAACGGCUA	-1.803	10006910	AGAP009554	-2.786	Down
miR-278	UCGGUGGGACUUUCGUCCGUUU	-2.082	10013335	AGAP000037	-3.226	Down

^amiR-124 indicates asi-miR-124, and miR-52(n) indicates asi-miR-new52, which is newly identified in this study; ^bAverage: expression quantity calculated as LOG₂ (fold change); ^c10000375 is for gene "Sinensis_GLEAN_10000375" and so on; ^d Orthologous genes determined by annotation of combined KEGG, GO, Swissport, Trembl and IPR methods (see detail in Table S2); ^eSignificantly up- or down-regulated for the target gene.

Significantly down-regulated miRNA			Target gene			
miR-278	UCGGUGGGACUUUCGUCCGUUU	-2.082	10015043	AGAP003976	-1.683	Down
miR-932a-5p	UCAAUCCGUAGUGCAUUGCAG	-2.252	10015043	AGAP003976	-1.683	Down
miR-981	UUCGUUGUCGACGAAACCUG	-1.836	10001409	AGAP007548	-2.749	Down
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10006910	AGAP009554	-2.786	Down
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10001409	AGAP007548	-2.749	Down
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10017409	AGAP003442	-2.385	Down
miR-11(n)	UGUUGUCGUAGCUCACAAGCGCUG	-1.895	10013658	DSM2777_RS04415	-2.708	Down
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10006905	AGAP009553	-1.778	Down
miR-24(n)	CGUGGUACUCUUUUGGUACGGA	-2.086	10006910	AGAP009554	-2.786	Down
miR-34(n)	GCCCGGCGGGCGGUUGGACC	-10.179	10006910	AGAP009554	-2.786	Down
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10006910	AGAP009554	-2.786	Down
miR-52(n)	UCGGUUUCGUCGGAUGAUCGGCG	-5.358	10015043	AGAP003976	-1.683	Down
^a miR-124 indicates asi-miR-124, and miR-52(n) indicates asi-miR-new52, which is newly identified in this study; ^b Average: expression quantity calculated as LOG_2 (fold change); ^c 10000375 is for gene "Sinensis_GLEAN_10000375" and so on; ^d Orthologous genes determined by annotation of combined KEGG, GO, Swissport, Trembl and IPR methods (see detail in Table S2); ^e Significantly up- or down-regulated for the target gene.						

Screen of the miRNA associated with pyrethroid-resistant in different strains of *An. sinensis*

To identify the possible miRNAs involved in the pyrethroid-resistant in *An. sinensis*, we use R language to screen out the differentially expressed miRNAs (P value ≤ 0.05 , $\log_2 \geq 1$, expression ≥ 10 TPM) between the pyrethroid-resistant and pyrethroid-sensitive *An. sinensis* in the above regions, respectively. A total of 115 differential miRNAs were obtained (shown in Fig. 3a). Especially, 39 different expressed miRNAs were found in all the three paired groups. The hierarchical heat map showed the different expression patterns of the above 39 miRNAs in the 3 paired groups (Fig. 3b). These 39 differentially expressed miRNAs are all down-regulated in pyrethroid-resistant strains. Among them, asi-miR-new-34, asi-miR-new100, asi-miR-new52, asi-miR-new60, asi-miR-new67 and asi-miR-new77 are the extremely down-regulated ones.

To determine the possible role of the above 39 miRNAs in pyrethroid-resistance, different miRNA target gene prediction algorithms were applied to minimize the number of putative target genes in *An. sinensis*. A total of 7475 annotated transcripts were predicted to be target genes of 39 differentially expressed miRNAs. Subsequently, GO-term annotation and KEGG pathway analyses are used to search the biological functions and signaling pathways of these putative target genes. The detailed classification of their biological processes, cellular components and molecular functions was shown in Fig. 3c. For molecular functions, they were mainly concentrated on binding and catalytic activity. During biological processes, they were mainly concentrated on signal transduction and protein transport. For cellular components, they were mainly concentrated on integral component of plasma membrane, Intracellular, etc. Through the KEGG pathway (Fig. 3d) analysis, 7 enrichment pathways were

found. They were related with phosphatidylinositol signaling system, insulin resistance, wnt signaling, notch signaling, Jak-STAT signaling, glycerolipid metabolism and ABC transporters.

Screen for the miRNA-mRNA network associated with pyrethroid-resistant base on different-expressed target mRNA

To further narrow the miRNA-mRNA network associated with pyrethroid-resistant, the expression of predicted 7475 target genes obtained were analyzed in ASDb (<https://jungleran.com>) which built by our lab contained all the differentially expressed mRNAs involved in pyrethroid-resistant. Thirty-four from 7475 developmental genes were screened out. Since miRNAs usually have multiple mRNA targets, we use a network to show the relationship between these miRNAs and mRNA. Thirty-four target mRNAs (in round box) and their potentially upstream miRNAs (in diamond box) were showed in Fig. 4a. Fig. 4b showed that most of miRNA-mRNAs were negatively correlated, except a small part of positive-correlated miRNA-mRNAs. Most of the newly discovered miRNAs may have more differential target genes, while the known miRNAs have a smaller number of differential target genes. We found that some differentially expressed target genes have been identified, such as iron transport, p450, and HSP protein. Interestingly, the p450 gene has been reported to be involved in the metabolic process of pyrethroid-resistance[39].

Verification of differentially expressed miRNAs and target mRNAs in pyrethroid-resistance

To verify the results of small RNA sequencing, the above 11 differentially expressed miRNAs were analyzed by RT-qPCR. Fig. 5a showed eleven differentially expressed miRNAs by RNA-seq, while Fig. 5b showed the results of the RT-qPCR. The results showed that most miRNAs were downregulated in resistant strains, and nine miRNAs (asi-miR-124, asi-miR-87, asi-miR-new60 etc.) showed similar expression patterns to those revealed by the sequencing results, suggesting restoration of the miRNAs would be novel strategy to overcome pyrethroid-resistant. In addition, TF showed a significantly up-regulated expression in all resistant strains in our *An. sinensis* pyrethroid-related expression genome, and we annotated the gene and found that its homologous gene is AGAP000376, which transports irons and related with pyrethroid resistance.

To verify the binding of miR-87 with TF, we performed dual luciferase reporter assay. We designed a fragment deletion luciferase reporter (WT) and mutant for seed sequence (MUT) (shown in Fig. 5c). Fig. 5d clearly demonstrated that asi-miR-87 dramatically repressed the activity of the TF-WT-3'UTR, compared with TF-MUT-3'UTR control. Collectively, these data indicates that asi-miR-87 directly binds to TF to inhibit its expression, suggesting that targeting asi-miR-87-TF axis might be novel strategy to overcome pyrethroid- resistance.

Disssussion

The prevalence of pyrethroid resistance in *An. sinensis* greatly affected its prevention and control. It has been reported that the development of pyrethroid resistance in *An. sinensis* is quite complicated, especially the cytochrome P450 and ABC-related pathways. However, whether miRNAs affect pyrethroid resistance through these pathways has been rarely studied. In our study, we focused on the different expressed miRNAs of *An. sinensis* mosquitoes in resistant and sensitive strains. We identified 329 miRNAs, of which 246 are newly identified, consistent with other articles[5, 28]. The number of miRNAs in *An. sinensis* is conserved, and different expressed miRNAs may play an important role in the development of drug resistance. We identified 39 miRNAs differentially expressed in pyrethroid-resistant strains and sensitive strains. Among these 39 miRNAs, there are 20 known miRNAs, and 6 of 20 known miRNA have been reported to be related to pyrethroid resistance in other species. It has been reported that most abundant miRNAs related with pyrethroid resistance contains miR-2, miR-13, miR-285[40], miR-278[41], and miR-932[42], which have been confirmed to target P450 family genes. Among them, miR-285 targets P450 (CYP6N23) to affect the pyrethroid resistance [40]. Three miRNAs including miR-2, miR-13, and miR-71 are down-regulated, resulted in the increased target genes CYP9J35 and CYP325BG3, which lead to deltamethrin resistance[43]. In addition, miR-10 and miR-2 have an effect on *Bacillus thuringiensis*(Bt) Cry toxins in *Plutella xylostella*[44, 45]. Besides these miRNAs, little is to date revealed about the functions of miRNAs in pyrethroid resistance in *An. sinensis*. Previous reports mainly focused on the dysregulation of miRNAs in the regulation of P450 family [27, 46].

In this study, 39 miRNAs predicted 7475 mRNAs were enriched in many pathways related to pyrethroid resistance, including calcium ion binding function, protein serine/threonine kinase activity function, jak-stat signal pathway, insulin resistance and

ABC transport-related pathways. For calcium ion binding, it has been reported that calcium ion channels and sodium ions in mosquitoes synergistically affect pyrethroid resistance [47]. Protein serine/threonine kinase activity and signaling pathways have also been reported to regulate cytochrome p450 in *Culex quinquefasciatus* to participate in the expression of pyrethroid resistance [48, 49]. Jak-STAT up-regulated p450-4e3 (CYP4e3) lead to permethrin resistance in fruit flies [50]. In addition, insulin resistance was enriched in mice fed permethrin, which changed blood glucose and energy metabolism through intracellular signaling pathways and kinase action., most important ABC transport-related pathways, which have been widely reported to play an important role in the production of pyrethroid resistance [51, 52]. Based on KEGG pathway and go annotation analyses, these pathways are also related to pyrethroid resistance in *An. Sinensis*, and have an important impact on the metabolic process of pyrethroids. By the genome data of our pyrethroid resistant and sensitive strains of *An. Sinensis*, the predicted 34 target genes showed significant differential expression, and only 13 of 34 corresponding miRNAs. miR-2 and miR-932 have been reported to regulate cytochrome p450 family genes in *Culex pipiens pallens*. miR-10, miR-210, miR-124 have also been reported to be related to immunity and drug resistance, while asi-miR-87 related functions have been reported less. Dual luciferase reporter assay further confirmed that asi-miR-87 directed bound with its target gene TF. The function of this homologous gene in *An. gambiae* is to combine with metal ions, mainly iron ion transport. The key to the synthesis of cytochrome p450 is iron ions [53], thus miR-87 may regulate the metabolism of cytochrome p450 to pyrethroids through this gene. In our study, some miRNAs have a positive associated with target genes. Guo *et al* confirmed that the regulation of a target gene by multiple miRNAs does have an impact on the inhibitory effect of a single miRNA. Through the miR-2~13~71 cluster interacts in *Culex pipiens pallens*, high expression levels of miR-2 and miR-13 will inhibit miR-71 expression levels and vice versa [43].

Collectively, our study further analyzed the expression of these miRNA-mRNA network, which will certainly help us to understand the complex regulatory mechanism of resistance to pyrethroids produced by *An. sinensis*. *An. sinensis* is the most common and widely distributed vector of malaria in China, with various ecological behaviors and feeding habits. It is also important in medicine and veterinary medicine. Therefore, the identification of pyrethroids resistance to the expression of miRNAs in *An. sinensis* not only helps to better understand the effect of deltamethrin resistance on *An. sinensis*, but also benefits for the provide new ideas for the control of mosquito. However, the function and molecular mechanism of these miRNAs still need further study.

Conclusion

This study provides a comprehensive account of miRNA profile and its dysregulated expression patterns across pyrethroid resistance of *An. sinensis*. Some miRNAs show specific expression patterns. We also studied the targets of these dysregulated miRNAs and their related functions in important life activities in order to better understand the effect of pyrethroids on *An. sinensis*. we provide a comprehensive miRNA-mRNA network of pyrethroids resistance in *An. sinensis*. Conversely, this information may provide a new method or basis for envisioning the control of them in the future. Understanding their function in the development of anti-insecticide, may provide an innovative approach with regard to development of alternative control methods.

Abbreviations

An. sinensis: *Anopheles sinensis*; *An. gambiae*: *Anopheles gambiae*; GO: gene

ontology; KEGG: kyoto encyclopedia of genes and genomes; UTR: untranslated region; RT-qPCR: reverse transcription quantitative real-time polymerase chain reaction; RPKM: Reads Per Kilobase per Million mapped reads; FDR: false discovery rate; DAVID: The Database for Annotation, Visualization and Integrated Discovery; ABC: ATP-binding cassette transporter; TF: Sinensis_GLEAN_10013226 gene; RNA-seq: RNA sequencing.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are available in the

NCBI BioProject repository,

(PRJNA796374, <http://www.ncbi.nlm.nih.gov/bioproject/796374>).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BC and XX conceived and designed the study. XX and BC performed the experiments and data analysis, and drafted the manuscript. SA, CH, FLS and FT joined experiments. All authors read and approved the final version of the manuscript.

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References

1. Zhang HW, Liu Y, Hu T, Zhou RM, Chen JS, Qian D, Yang CY, Zhao YL, Li SH, Cui J, et al. Knockdown resistance of *Anopheles sinensis* in Henan province, China. *Malaria J.* 2015;14:137.
2. Yan ZW, He ZB, Yan ZT, Si FL, Zhou Y, Chen B. Genome-wide and expression-profiling analyses suggest the main cytochrome P450 genes related to pyrethroid resistance in the malaria vector, *Anopheles sinensis* (Diptera Culicidae). *Pest Manag Sci.* 2018;74(8):1810–20.
3. Alout H, Roche B, Dabire RK, Cohuet A. Consequences of insecticide resistance on malaria transmission. *PLoS Pathog.* 2017;13(9):e1006499.
4. Oumbouke WA, Rowland M, Koffi AA, Alou LPA, Camara S, N'Guessan R. Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN(R) LN against pyrethroid resistant *Anopheles gambiae* s.s.: an experimental hut trial in M'be, central Cote d'Ivoire. *Parasite Vector.* 2019;12(1):544.
5. Feng X, Zhou X, Zhou S, Wang J, Hu W. Analysis of microRNA profile of *Anopheles sinensis* by deep sequencing and bioinformatic approaches. *Parasite Vector.* 2018;11(1):172.
6. Feng X, Zhang S, Huang F, Zhang L, Feng J, Xia Z, Zhou H, Hu W, Zhou S. Biology, Bionomics and Molecular Biology of *Anopheles sinensis* Wiedemann 1828 (Diptera: Culicidae), Main Malaria Vector in China. *Front Microbiol.* 2017;8:1473.

7. Zhang YJ, Lan Y, Chen B. ASDB: A comprehensive omics database for *Anopheles sinensis*. *Genomics*. 2021;113(3):976–82.
8. Chen B, Zhang YJ, He ZB, Li WS, Si FL, Tang Y, He QY, Qiao L, Yan ZT, Fu WB, et al. De novo transcriptome sequencing and sequence analysis of the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Parasite Vector*. 2014;7:314.
9. Qiu X, Zhou D, Liu X, Sun Y, Ma L, Shen B, Zhu C. Genomic Analysis of Detoxification Supergene Families in the Mosquito *Anopheles sinensis*. *PLoS ONE*. 2015;10(11):e0143387.
10. Wu XM, Xu BY, Si FL, Li JY, Yan ZT, Yan ZW, He X, Chen B. Identification of carboxylesterase genes associated with pyrethroid resistance in the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Pest Manag Sci*. 2018;74(1):159–69.
11. Liu BQ, Qiao L, He QY, Zhou Y, Ren S, Chen B. Genome-wide identification, characterization and evolution of cuticular protein genes in the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Insect Sci*. 2018;25(5):739–50.
12. Wang T-T, Si F-L, He Z-B, Chen B. Genome-wide identification, characterization and classification of ionotropic glutamate receptor genes (iGluRs) in the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Parasite Vector*. 2018;11(1).
13. He X, He ZB, Zhang YJ, Zhou Y, Xian PJ, Qiao L, Chen B. Genome-wide identification and characterization of odorant-binding protein (OBP) genes in the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Insect Sci*. 2016;23(3):366–76.
14. Chang ZX, Akinyemi IA, Guo DY, Wu Q. Characterization and comparative analysis of microRNAs in the rice pest *Sogatella furcifera*. *PLoS ONE*. 2018;13(9):e0204517.
15. Chang ZX, Tang N, Wang L, Zhang LQ, Akinyemi IA, Wu QF. Identification and characterization of microRNAs in the white-backed planthopper, *Sogatella furcifera*. *Insect Sci*. 2016;23(3):452–68.
16. Asgari S. MicroRNA functions in insects. *Insect Biochem Molec*. 2013;43(4):388–97.
17. Menzel P, McCorkindale AL, Stefanov SR, Zinzen RP, Meyer IM. Transcriptional dynamics of microRNAs and their targets during *Drosophila* neurogenesis. *RNA Biol*. 2019;16(1):69–81.
18. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(D1):D68–73.
19. Li Y, Li SJ, Li RM, Xu J, Jin P, Chen LM, Ma F. Genome-wide miRNA screening reveals miR-310 family members negatively regulate the immune response in *Drosophila melanogaster* via co-targeting *Drosomycin*. *Dev Comp Immunol*. 2017;68:34–45.
20. Gendron CM, Pletcher SD. MicroRNAs mir-184 and let-7 alter *Drosophila* metabolism and longevity. *Aging Cell*. 2017;16(6):1434–8.
21. Fu X, Dimopoulos G, Zhu J. Association of microRNAs with Argonaute proteins in the malaria mosquito *Anopheles gambiae* after blood ingestion. *Sci Rep-UK*. 2017;7(1).
22. Dennison NJ, BenMarzouk-Hidalgo OJ, Dimopoulos G. MicroRNA-regulation of *Anopheles gambiae* immunity to *Plasmodium falciparum* infection and midgut microbiota. *Dev Comp Immunol*. 2015;49(1):170–8.
23. Zhang GM, Hussain M, O'Neill SL, Asgari S. *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *P Natl Acad Sci USA*. 2013;110(25):10276–81.
24. Hussain M, Walker T, O'Neill SL, Asgari S. Blood meal induced microRNA regulates development and immune associated genes in the Dengue mosquito vector, *Aedes aegypti*. *Insect Biochem Molec*. 2013;43(2):146–52.
25. Hong S, Guo Q, Wang W, Hu S, Fang F, Lv Y, Yu J, Zou F, Lei Z, Ma K, et al. Identification of differentially expressed microRNAs in *Culex pipiens* and their potential roles in pyrethroid resistance. *Insect Biochem Molec*. 2014;55:39–50.
26. Guo Q, Huang Y, Zou F, Liu B, Tian M, Ye W, Guo J, Sun X, Zhou D, Sun Y, et al. The role of miR-2 approximately 13 approximately 71 cluster in resistance to deltamethrin in *Culex pipiens pallens*. *Insect Biochem Molec*. 2017;84:15–22.
27. Ma K, Li X, Hu H, Zhou D, Sun Y, Ma L, Zhu C, Shen B. Pyrethroid-resistance is modulated by miR-92a by targeting *CpCPR4* in *Culex pipiens pallens*. *Comp Biochem Phys B*. 2017;203:20–4.
28. Feng X, Wu J, Zhou S, Wang J, Hu W. Characterization and potential role of microRNA in the Chinese dominant malaria mosquito *Anopheles sinensis* (Diptera: Culicidae) throughout four different life stages. *Cell Biosci*. 2018;8:29.
29. Chen B, Zhang Y-J, He Z, Li W, Si F, Tang Y, He Q, Qiao L, Yan Z, Fu W, et al. De novo transcriptome sequencing and sequence analysis of the malaria vector *Anopheles sinensis* (Diptera: Culicidae). *Parasite Vector*. 2014;7(1):314.

30. Li S, Mead EA, Liang S, Tu Z. Direct sequencing and expression analysis of a large number of miRNAs in *Aedes aegypti* and a multi-species survey of novel mosquito miRNAs. *BMC Genomics*. 2009;10:581.
31. Zhong X, Heinicke F, Rayner S. miRBaseMiner, a tool for investigating miRBase content. *RNA Biol*. 2019;16(11):1534–46.
32. Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*. 2012;40(1):37–52.
33. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in *Drosophila*. *Genome Biol*. 2004;5(1).
34. Kruger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res*. 2006;34:W451–4.
35. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al. Gene Ontology: tool for the unification of biology. *Nat Genet*. 2000;25(1):25–9.
36. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*. 2000;28(1):27–30.
37. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44–57.
38. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat Protoc*. 2006;1(3):1559–82.
39. Riveron JM, Irving H, Ndula M, Barnes KG, Ibrahim SS, Paine MJI, Wondji CS. Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*. *P Natl Acad Sci USA*. 2013;110(1):252–7.
40. Tian M, Liu B, Hu H, Li X, Guo Q, Zou F, Liu X, Hu M, Guo J, Ma L, et al. MiR-285 targets P450 (CYP6N23) to regulate pyrethroid resistance in *Culex pipiens pallens*. *Parasitol Res*. 2016;115(12):4511–7.
41. Lei Z, Lv Y, Wang W, Guo Q, Zou F, Hu S, Fang F, Tian M, Liu B, Liu X, et al. MiR-278-3p regulates pyrethroid resistance in *Culex pipiens pallens*. *Parasitol Res*. 2015;114(2):699–706.
42. Liu B, Tian M, Guo Q, Ma L, Zhou D, Shen B, Sun Y, Zhu C. MiR-932 Regulates Pyrethroid Resistance in *Culex pipiens pallens* (Diptera: Culicidae). *J Med Entomol*. 2016;53(5):1205–10.
43. Guo Q, Huang Y, Zou F, Liu B, Tian M, Ye W, Guo J, Sun X, Zhou D, Sun Y, et al. The role of miR-2~13~71 cluster in resistance to deltamethrin in *Culex pipiens pallens*. *Insect Biochem Molec*. 2017;84:15–22.
44. Li S, Xu X, Zheng Z, Zheng J, Shakeel M, Jin F. MicroRNA expression profiling of *Plutella xylostella* after challenge with *B. thuringiensis*. *Dev Comp Immunol*. 2019;93:115–24.
45. Zhang YL, Huang QX, Yin GH, Lee S, Jia RZ, Liu ZX, Yu NT, Pennerman KK, Chen X, Guo AP. Identification of microRNAs by small RNA deep sequencing for synthetic microRNA mimics to control *Spodoptera exigua*. *Gene*. 2015;557(2):215–21.
46. Peng T, Pan Y, Gao X, Xi J, Zhang L, Ma K, Wu Y, Zhang J, Shang Q. Reduced abundance of the CYP6CY3-targeting let-7 and miR-100 miRNAs accounts for host adaptation of *Myzus persicae nicotianae*. *Insect Biochem Molec*. 2016;75:89–97.
47. Perrier S, Moreau E, Deshayes C, El-Adouzi M, Goven D, Chandre F, Lapied B. Compensatory mechanisms in resistant *Anopheles gambiae* *AcerKis* and *KdrKis* neurons modulate insecticide-based mosquito control. *Commun Biol*. 2021;4(1):665.
48. Li T, Cao C, Yang T, Zhang L, He L, Xi Z, Bian G, Liu N. A G-protein-coupled receptor regulation pathway in cytochrome P450-mediated permethrin-resistance in mosquitoes, *Culex quinquefasciatus*. *Sci Rep-UK*. 2015;5:17772.
49. Campbell CL, Saavedra-Rodriguez K, Kubik TD, Lenhart A, Lozano-Fuentes S. Black WCT: Vgsc-interacting proteins are genetically associated with pyrethroid resistance in *Aedes aegypti*. *PLoS ONE*. 2019;14(1):e0211497.
50. Terhzaz S, Cabrero P, Brinzer RA, Halberg KA, Dow JA, Davies SA. A novel role of *Drosophila* cytochrome P450-4e3 in permethrin insecticide tolerance. *Insect Biochem Molec*. 2015;67:38–46.
51. He Q, Yan Z, Si F, Zhou Y, Fu W, Chen B. ATP-Binding Cassette (ABC) Transporter Genes Involved in Pyrethroid Resistance in the Malaria Vector *Anopheles sinensis*: Genome-Wide Identification, Characteristics, Phylogenetics, and Expression Profile. *Int J Mol Sci*. 2019;20(6).
52. Pignatelli P, Ingham VA, Balabanidou V, Vontas J, Lycett G, Ranson H. The *Anopheles gambiae* ATP-binding cassette transporter family: phylogenetic analysis and tissue localization provide clues on function and role in insecticide resistance.

53. Green AJ, Rivers SL, Cheesman M, Reid GA, Quaroni LG, Macdonald IDG, Chapman SK, Munro AW. Expression, purification and characterization of cytochrome P450 Biol: a novel P450 involved in biotin synthesis in *Bacillus subtilis*. *J Biol Inorg Chem*. 2001;6(5-6):523–33.

Figures

Figure 1

Annotation of total reads from the seven samples of libraries in *An. sinensis*. AH (a), CQ(b) and YN(c) indicate Anhui, Chongqing and Yunnan province, respectively; FR and FS indicate the pyrethroid-resistant and -susceptible field populations, respectively; WX-LS(d) presents the pyrethroid-susceptible laboratory strain from Wuxi, Jiangsu province. The total reads can be divided into eleven categories: exon-antisense, exon-sense, intron-antisense, intron-sense, miRNA, rRNA, repeat, snRNA, snoRNA, tRNA and unmapped.

Figure 2

Identification of miRNAs from 7 *Anopheles sinensis* libraries (AH-FR, AH-FS, CQ-FR, CQ-FS, YN-FR, YN-FS, WX-LS). **a**: the number of miRNAs in the seven libraries of *Anopheles sinensis*; **b**: the number of miRNAs between the four sensitive and three resistant strains of *Anopheles sinensis*; **c**: the expression of the first 35 miRNAs in the seven libraries of *Anopheles sinensis*.

Figure 3

Identification of miRNAs and their functions related to pyrethroid resistance in *Anopheles sinensis*. **a**: the numbers of commonly and significantly-down-regulated miRNAs among three field pyrethroid-resistant populations in comparison of laboratory pyrethroid-susceptible strain in *Anopheles sinensis*; **b**: expression profile of significantly-down-regulated 39 miRNAs in three field pyrethroid-resistant populations comparative with laboratory pyrethroid-susceptible strain of *Anopheles sinensis*. The color code (on the right panel) indicates the multiples of expression quantity between pyrethroid-resistant and -susceptible strains; gene ontology annotation (**c**) and KEGG pathway (**d**) of the target genes relative with pyrethroids, which are significantly negatively regulated by miRNAs.

Figure 4

Interaction between pyrethroid resistance-related miRNAs and target genes. **a**: miRNA-mRNA interaction network. The squares and ovals represent the significantly-down-regulated miRNAs and their target genes associated with pyrethroid resistance, respectively. The connecting lines demonstrate the regulatory relationship between miRNAs and their target genes. The color code (on the right panel) denotes the multiple of expression quantity; **b**: the relative expression of miRNAs and target genes associated with pyrethroid resistance, identified through three field populations. 13 commonly significantly-down-regulated miRNAs match 33 commonly up-regulated mRNAs and 17 commonly down-regulated mRNAs.

Figure 5

Experimental verification of differentially expressed miRNAs and their target genes. **a**: eleven differentially expressed miRNAs transcription levels in RNA-seq; **b**: Validation of the significantly-down-regulated miRNAs by quantitative real-time PCR. The transcript levels of eleven miRNAs of the three resistant strains were calculated relative to the amount of the susceptible strains after normalization. **c**: Target identification of asi-miR-87 in cells. Sinensis_GLEAN_10013226(TF) 3'UTR luciferase reporter gene detection. NC represents an unrelated small RNA sequence, WT represents recombinant reporter plasmid with TF 3'UTR and MUT represents a recombinant reporter plasmid with mutant TF 3'UTR, which were used as controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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