MiR-932 Regulates Pyrethroid Resistance in *Culex pipiens pallens* (Diptera: Culicidae)

Bingqian Liu, Mengmeng Tian, Qin Guo, Lei Ma, Dan Zhou, Bo Shen, Yan Sun, and Changliang Zhu¹

Department of Pathogen Biology, Nanjing Medical University, 140 Hanzhong Rd., Nanjing, Jiangsu 210029, China (fuandniu@126.com; tmm0201@126.com; guoqin2782@163.com; malei@njmu.edu.cn; zhoudan@njmu.edu.cn; shenbo@njmu.edu.cn; sunyan@njmu.edu.cn; clzhu@njmu.edu.cn), and ¹Corresponding author, e-mail: clzhu@njmu.edu.cn

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Abstract

MicroRNAs (miRNAs) play notable role in regulation of gene expression at the posttranscription level, and have been involved in many biological processes, including insecticide resistance. In this study, we investigated the role of miR-932 in the molecular mechanisms of pyrethroid resistance in *Culex pipiens pallens* (L.). Overexpression of miR-932 in the DS-strain made the mosquitoes more resistant to deltamethrin, while inhibiting the expression of miR-932 in the DR-strain made the mosquitoes more sensitive to deltamethrin. Further, we also identified *CpCPR5* as a target gene of miR-932. Sustained overexpression of miR-932 resulted in repression of *CpCPR5*, and that knockdown of miR-932 increased *CpCPR5* expression. In addition, knockdown of *CpCPR5* decreased the sensitivity of mosquitoes to deltamethrin in the DS-strain. In conclusion, our study finds a molecular link between miR-932 and *CpCPR5* and provides a novel insight into the mechanism of insecticide resistance.

Key words: *Culex pipiens pallens*, pyrethroid, microRNA, *CpCPR5*

Mosquitoes transmit a lot of pathogens, causing severe diseases such as malaria, dengue fever, West Nile fever, encephalitis, and filariasis, which threat public health worldwide (Andriessen et al. 2015, Fortuna et al. 2015, Paredes-Esquivel et al. 2015). Recent estimates show that there are about 400 million infections of dengue fever annually around the world (Sood et al. 2015). According to the WHO world malaria report, there are still 214 million new infections worldwide in 2015. Therefore, it is imperative for controlling and preventing mosquito-borne diseases. However, due to the lack of effective vaccines and drugs, vector control mostly relies on insecticides to reduce the number of mosquitoes (Glunt et al. 2015). But continuous and lasting application of insecticides has led to a constant increasing resistance, which makes antivector strategies more challenging (Rasic et al. 2015). Insecticide resistance arises from complex polygenic inheritance and many genes involved (Yu et al. 2014, Bonizzoni et al. 2015). These genes can be regulated at transcriptional and posttranscriptional levels and microRNA (miRNA) is known to be a posttranscriptional gene regulator (Wang et al. 2016).

MiRNA is a class of small noncoding RNA, with about 22 nucleotides in length. It has been shown to repress translation via imperfectly pairing with complementary sites within the 3'UTR of target mRNA (Liu et al. 2014, Munoz et al. 2015). In diverse cellular programs, miRNA plays a key role and involves in cell growth, differentiation, apoptosis, and many other physiological processes (Liu et al. 2015, Park et al. 2015, Zheng et al. 2015). In insects, the studies on

miRNAs are mostly focused on the development, behavior, and host-pathogen interactions (Lucas et al. 2015). Recent evidence has shown that a few miRNAs participate in the regulation of insecticide resistance in mosquitoes, but the exact role of miRNA in pyrethroid resistance is still under little attention yet (Zhang et al. 2015).

Here, according to our previous study, we identified many different expressed miRNAs in deltamethrin-resistant strain (DR) and deltamethrin-susceptible strain (DS) of *Culex pipiens pallens* (L.) (Hong et al. 2014). Among these miRNAs, miR-932 is down-regulated in the DS-strain, suggesting that this miRNA could involve in the regulation of pyrethroid resistance. Besides, a few cuticle proteins have been reported to be associated with deltamethrin resistance (Fang et al. 2015). Based on the genomes of all cuticle proteins from *Culex quinquefasciatus* and the mature miR-932 sequence, we found that the 3'UTR sequence of *CpCPR5* contained the seed region of miR-932. In this study, we examined the role of miR-932 and identified its target, *CpCPR5*, and investigated their regulatory mechanism in pyrethroid resistance.

Materials and Methods

Mosquito Strains

Two laboratory strains of *Cx. pipiens pallens* were used in this study. The DS-strain (the 50% lethal concentration $LC_{50} = 0.04$ mg/

Table 1. List of primers for qRT-PCR and vector constructs

Primers	Forward primer (5' to 3')	Reverse primer (5' to 3')
miR-932	TGCAAGCAATGTGGAAGTGAAG	TGGTGTCTGCTGGAGTCCG
U6	GCTTCGGCTGGACATATACTAAAT	GAACGCTTCACGATTTTTCG
CpCRP5	GAGACCAGAAGGAACAGCAGC	TGGGATCGGCGGGTGAAT
β -actin	AGCGTGAAGTACGGCTCTG	ACTCGTCTGACTCCTGCTTGG
CpCRP5-3'UTR	CGAGCTCACTGTTATCGAATGTTTGTGACTG	CCAAGCTTAAGCAAGCATGATTTGAGCA
CpCRP5-MUT	CGAGCTCACTGTTATCGAATGTTTGTGACTG	CCAAGCTTAAGGATGGATGATTTGAGCAC

liter) was picked up from Tang Kou county of Shan Dong province in 2009 and later on reared in our lab without exposure to any insecticides. The DR-strain was selected from its early fourth-instar larvae with deltamethrin for >65 generations and its LC_{50} was 7.3 mg/liter. Both the lab strains were maintained at 28°C, 70–80% humidity, and a photoperiod of 16:8 (L:D) h.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from five female mosquitoes for each group using TRIzol Reagent (Invitrogen, Carlsbad, CA). The integrity of total RNA was assessed by denaturing agarose gel electrophoresis and the purity and concentration were authenticated by the NanoDrop spectrophotometer (Nano Drop, Wilmington, DE). cDNA was synthesized using PrimeScriptRT Reagent Kit (TAKARA, Tokyo, Japan) from 500 ng total RNA according to the manufacturer's protocol.

Quantitative RT-PCR Analysis

Quantitative real-time PCR was performed on the ABI Prism 7300 HT Sequence Detection system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems) and the expression of miRNA was measured through Stem-loop RT-PCR method (Dedeoglu 2014). The relative expression level of *CpCRP5* was normalized to the internal control β -actin, and *U6 small nuclear (U6)* was used to normalize the expression level of miR-932. The expression level in DS-strain was considered as 1. The RT-PCR condition was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of forward and reverse primers specific for *CpCRP5* and miR-932 are listed in the Table 1.

Cell Culture and Luciferase Assay

Two pairs of specific primers in Table 1 (*CpCRP5* 3'UTR-F, *CpCRP5* 3'UTR-R; *CpCRP5*-MUT-F, *CpCRP5*-MUT-R) were designed based on the similar cDNA sequences from *Cx. quinquefasciatus* to amplify 3'UTR/MUT of *CpCRP5*. The region of *CpCRP5* 3'UTR included complementary sequence of seed region of miR-932, and the other one included the same sequence but with four bases change in the seed region. PCR was performed using the following conditions: denaturing at 95°C for 30 s followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. PCR products were purified using the MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TAKARA) after agarose gel electrophoresis and had been sequenced in Invitrogen, and then the purified DNA was ligated into the pMD19-T vector (TAKARA). Reporter constructs for the expression in 293T cells were generated by cloning the PCR product into the pMIR-REPORT miRNA Expression Reporter Vector (Applied Biosystems) using Sac I and Hind III restriction sites (TAKARA).

The 293T cells were seeded at 1×10^5 cells per well in 12-well plates and were cultured for 24 h to 60–70% confluence, and then were transfected with recombinant plasmids (60 ng), pGL4.74 plasmids (control reporter, 20 ng), and miR-932 mimic containing the final concentration varying from 25 nM to 200 nM or negative control (NC) (GenePharma, Shanghai, China) per well using the Fugene HD Transfection Reagent (Promega, WI). Luciferase activity was assayed with the Dual-Luciferase Assay System (Promega, WI) after 24 h according to the manufacturer's protocols. Normalized firefly luciferase activity (firefly luciferase activity/*Renilla* luciferase activity) was compared to that of the control. Experiments were repeated at least three times.

Microinjection and American CDC Bottle Bioassay

The miR-932 mimic/inhibitor and double-stranded RNA (dsRNA) targeting *CpCRP5* were designed and synthesized from GenePharma (GenePharma) at a concentration of 20 μ M. The details of the sequences are included in Table 2 (miR-932 inhibitor is single-stranded RNA molecules). Injection experiment was performed in the 1-d postemergence female mosquitoes. About 0.35 μ g miR-932 mimic/miR-932 inhibitor/dsRNA was microinjected into the thorax of the DS-strain, and equivalent volume of DEPC water (DEPC) and negative control (NC) served as control. After 3 d, we examined the transcript abundance through qRT-PCR. The mosquito's resistance to insecticides was detected by the means of American CDC Bottle Bioassay. According to the guideline of CDC bottle bioassay, we did a calibration assay to determine the diagnostic dose (http://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html). We used 6 mg deltamethrin per bottle (250 ml) for DR-strain, and 1 μ g for DS-strain. In each bottle, 15–20 mosquitoes were exposed to deltamethrin, and a bottle coating with acetone was used as a control. Every 15 min, the numbers of dead mosquitoes were recorded, until to 2 h. At last the mortality rate was calculated at the diagnostic time.

Statistical Analysis

Data were analyzed using Student's *t*-test and chi-square test. Probability values of <0.05 are considered significant. Data are presented as mean and the SEM of three independent experiments.

Results

Identification of miR-932 That Targets *CpCRP5*

Quantitative real-time PCR was used to detect the expression of miR-932 and its predicted target gene *CpCRP5* in DR-strain and DS-strain of *Cx. pipiens pallens*, and β -actin served as a normalization control. The expression level of miRNA-932 was 1.8-fold higher in the DR-strain compared to the DS-strain (Fig. 1A), and on the contrary, the expression level of *CpCRP5* was 2.8-fold lower in the DR-strain (Fig. 1B). The opposite phenomenon suggested that

Table 2. List of the miR-932 mimic/miR-932 inhibitor/dsRNA sequences

Name	Sense (5' to 3')	Antisense (5' to 3')
miR-932 mimic	UGCAAGCAAUGUGGAAGUGAAG	UCACUCCACAUUGCUUGCAU
miR-932 inhibitor	CUUCACUCCACAUUGCUUGCA	
dsRNA	GGUAAGAAUUUGAUGCCACTT	GUGGCAUCAAAUUCUUACCTT
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGAGAATT

Fig. 1. Expression levels of *CpCPR5* and miR-932 in DS-strain and DR-strain of *Cx. pipiens pallens*. (A) The expression of miR-932 is 1.8-fold higher than in DR-strain. (B) The expression of *CpCPR5* is 2.8-fold higher than in DS-strain. Figures show the mean $\pm$ SD of three independent experiments. * $P < 0.05$.

CpCPR5 could be the target gene of miR-932. To verify this, the plasmid inserted with 3'UTR/MUT of *CpCPR5* (named as pMIR-REPORT 3'UTR and pMIR-REPORT MUT) along with pGL4.7-Control plasmid were cotransfected into HEK 293T cells. The luciferase assays indicated that miR-932 greatly inhibited the activity of 3'UTR construct in a dose-dependent manner, while the activity of NC remained with no significant change (Fig. 2A). Besides, in the pMIR-MUT group, when transfected miR-932mimic, the luciferase activity didn't decrease (Fig. 2B). All the data above suggest that *CpCPR5* is a direct target of miR-932.

Regulation of miR-932 in the Deltamethrin Resistance

To determine the function of miR-932 in the mosquito, we overexpressed miR-932 by injecting mimic in the DS-strain. The relative expression of miR-932 was 88-fold higher compared to the control

Fig. 2. *CpCPR5* is a direct target of miR-932. (A) Luciferase assay of HKE 293T cells cotransfected by pMIR-REPORT-3'UTR together with increasing amounts (50, 100, and 200 nM) of miR-932. (B) The luciferase activity is assayed in 293T cells cotransfected by pMIR-REPORT-3'UTR or the mutant pMIR-REPORT-MUT construct together with miR-932. ** $P < 0.01$.

group (Fig. 3A), and the level of *CpCPR5* decreased about 24% (Fig. 3B), which suggested that the overexpression of miR-932 in DS-strain was constructed successfully. And the CDC Bottle Bioassay showed that the miR-932 mimic-injected group had a lower mortality rate than NC and DEPC groups. At 90 min, the mortality rate of mimic group was 30.4%, whereas that for the NC group was 61.2% and for the DEPC group was 59.4%. Furthermore, at 120 min, there was a huge gap between mimic group and NC/DEPC group; the mortality rate of mimic group was 45.7%, while that for the NC group was 79% and for the DEPC group was 78.2% (Fig. 3C). On the other hand, we inhibited miR-932 in DR-strains by the means of microinjecting inhibitor. Similarly, qRT-PCR and CDC Bottle Bioassay analysis were conducted. The qRT-PCR results showed that miR-932 was knock-down about 30% compared with the other groups (Fig. 4A), and the expression level of *CpCPR5* increased 67% correspondingly (Fig. 4B). The CDC Bottle Bioassay exhibited that the mosquitoes injected with inhibitor were more susceptible than the other groups when exposed to deltamethrin in 2 h (Fig. 4C). Thus, miR-932 plays a role in regulation of resistance to deltamethrin in mosquitoes.

CpCPR5 Plays a Role in the Deltamethrin Resistance of Mosquitoes

To test the function of *CpCPR5* in mosquitoes, we injected specific dsRNA into the DS-strain. Also, qRT-PCR and CDC Bottle

Fig. 3. Microinjection of the miR-932 mimic in female adult mosquitoes. (A) The expression of miR-932 in DEPC, NC, and mimic groups postinjection. (B) The expression of CpCPR5 after microinjection. (C) Morality of microinjected mosquitoes observed after 2-h exposure to deltamethrin (6 mg/ml) in CDC bottles. Two controls were performed in an equivalent volume of DEPC or NC. All mosquitoes in the acetone bottles were alive. * $P < 0.05$.

Fig. 4. Microinjection of the miR-932 inhibitor in female adult mosquitoes. (A) The expression of miR-932 in DEPC, NC, and mimic groups postinjection. (B) The expression of CpCPR5 after microinjection. (C) Morality of microinjected mosquitoes observed after 2-h exposure to deltamethrin (1 μ g/ml) in CDC bottles. The results were presented as mean $\pm$ SE of three independent experiments. * $P < 0.05$.

Fig. 5. Microinjection of the dsRNA of *CpCPR5* in female adult mosquitoes. (A) The expression of *CpCPR5* in DEPC, NC, and dsRNA groups postinjection. (B) Morality of microinjected mosquitoes observed after 2-h exposure to deltamethrin (1 μ g/ml) in CDC bottles. The results were presented as mean $\pm$ SE of three independent experiments. * $P < 0.05$.

Bioassay were performed. The group injected with dsRNA showed a reduction in *CpCPR5* expression of 44% compared to the control group (Fig. 5A). Based on the CDC Bottle Bioassay, the group injected with dsRNA had a lower mortality rate than the other two groups. At 90 min, the mortality rate of dsRNA group was 45%, whereas that for the NC group was 72.1% and for the DEPC group was 73.6%, and at 120 min, the mortality rate of dsRNA group was 53.1%, while that for the NC group was 79.8% and for the DEPC group was 80.8% (Fig. 5B). The results suggested that mosquitoes were more resistant to deltamethrin after *CpCPR5* knockdown and *CpCPR5* could affect deltamethrin resistance in mosquitoes.

Discussion

MicroRNAs are known to be the crucial regulators in posttranscriptional gene expression (Tuna et al. 2015). Previous works on miR-932 show that it can play different roles among insects. In *Drosophila*, miR-932 can specifically target Hh coreceptor Boi to modulate hedgehog signaling (Gao et al. 2013). And in the honeybee *Apis mellifera*, miR-932 can affect the memory by controlling actin-related plasticity mechanisms (Cristino et al. 2014). In this study, we find that the miR-932-mimic-injected mosquitoes become more resistant to deltamethrin, while the miR-932-inhibitor-injected mosquitoes become more susceptible to deltamethrin, suggesting that miR-932 participates in the regulation of pyrethroid resistance.

The role of miR-932 in pyrethroid resistance also offers new ideas about exploring the mechanisms of insecticide resistance, which would help to make more pertinent measures in controlling mosquito populations (Dusfour et al. 2015). All the way through, there are two main molecular mechanisms that can elucidate insecticide resistance, one is target site modification represented by knock-down effect, another is metabolic detoxification, such as the cytochrome P450 monooxygenases (Itokawa et al. 2015, Li et al. 2015, Ochomo et al. 2015, Vera-Maloof et al. 2015). Furthermore, cuticular proteins are also

responsible for insecticide resistance, by reducing the permeation of insecticides (Noh et al. 2015).

Cuticle proteins are important component of protective exoskeleton in insects, which help to prevent environmental and pathogenic challenges (Vannini et al. 2014). Recently, as the technology and methods in transcriptome have been getting more and more mature, some cuticle proteins that expressed differently between susceptible and resistant strains have been reported (Lv et al. 2015, Pan et al. 2015). However, the molecular characterization of cuticle proteins regulating insecticide resistance remains limited (Nkya et al. 2014). In this study, the target gene of miR-932, *CpCPR5*, is up-regulated in the DS-strains. The potential cause that *CpCPR5* gets involved in the pyrethroid resistance may be a change in the structure of cuticle morphology, and alerting cuticle structure could let insecticides get into the inside of mosquitoes more easily; however, it needs further study (Jones et al. 2013, Noh et al. 2015).

In conclusion, our study demonstrates that miR-932 participates in the regulation of pyrethroid resistance of mosquitoes by targeting *CpCPR5*. And the role of miR-932 in pyrethroid resistance provides novel insights into the mechanism of insecticide resistance.

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