MiR-4448 is involved in deltamethrin resistance by targeting CYP4H31 in Culex pipiens pallens

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Abstract

Background: Culex pipiens (Cx. pipiens) complex, which acts as a vector of viruses and is widespread and abundant worldwide, including West Nile virus, Japanese encephalitis virus, and Sindbis virus, can cause serious vector-borne diseases affecting human health. Unfortunately, mosquitoes have developed deltamethrin resistance because of its long-term overuse, representing a major challenge to mosquito control. Understanding the molecular regulatory mechanisms of resistance is vital to control mosquitoes. MicroRNAs (miRNAs) are short non-coding RNAs that have been demonstrated to be important regulators of gene expression across a wide variety of organisms, which might function in mosquito deltamethrin resistance. In the present study, we aimed to investigate the regulatory functions of miR-4448 and CYP4H31 in the formation of insecticidal resistance in mosquito Culex pipiens pallens.

Methods: We used quantitative real-time reverse transcription PCR to measure miR-4448 and CYP4H31 (encoding a cytochrome P450) expression levels. The regulatory functions of miR-4448 and CYP4H31 were assessed using dual-luciferase reporter assays. Then, oral feeding, RNA interference, and the American Centers for Disease Control and Prevention bottle bioassay were used to determine miR-4448's association with deltamethrin resistance by targeting CYP4H31 in vivo. Cell Counting Kit-8 (CCK-8) was also used to detect the viability of pIB/V5-His-CYP4H31-transfected C6/36 cells after deltamethrin treatment in vitro.

Results: MiR-4448 was downregulated in the deltamethrin-resistant strain (DR strain), whereas CYP4H31 was downregulated in deltamethrin-susceptible strain. CYP4H31 expression was downregulated by miR-4448 recognizing and binding to its 3′ untranslated region. Functional verification experiments showed that miR-4448 overexpression resulted in lower expression of CYP4H31. The mortality of miR-4448 mimic-injected DR strain mosquitoes was higher than that of the controls. CCK-8 assays showed that CYP4H31 decreased cellular resistance to deltamethrin in vitro and the mortality of the DR strain increased when CYP4H31 was knocked down in vivo.

Conclusions: In mosquitoes, miR-4448 participates in deltamethrin resistance by targeting CYP4H31. The results of the present study increase our understanding of deltamethrin resistance mechanisms.

Keywords: MicroRNA, Cytochrome P450, Insecticide resistance, CDC bottle, Mosquito

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representing a key threat for millions of people and animals worldwide [5, 6]. Among these vectors, *Culex pipiens* complex is widely distributed worldwide and plays important roles in the transmission of many human diseases [7]. Over the past 2 decades, insecticide-based approaches to control mosquito vectors have substantially reduced the prevalence of mosquito borne diseases, including malaria [8]. Deltamethrin is a pyrethroid insecticide that is commonly used and recommended for in-home insect control because of its high efficiency, broad spectrum, and relatively low toxicity to humans [9, 10]. Unfortunately, mosquito resistance to insecticides has developed because of their long-term heavy use [11].

Studies showed that the development of insecticide resistance in mosquitoes is a complex and heritable evolutionary phenomenon, involving multiple genes and mechanisms [12]. Improving our understanding of the molecular mechanisms of insecticide resistance would allow the formulation of novel strategies to minimize and prevent resistance development, thus controlling mosquitoes [13]. To date, research has largely focused on identifying insecticide resistance-related genes and has found that the evolution of insecticide resistance is mostly induced by changes in the expression of cytochrome P450 genes [14]. In resistant mosquitoes, several P450 genes are upregulated and overexpressed [15–17]. However, the regulatory mechanisms of P450s remain largely unknown.

MicroRNAs (miRNAs) are a class of evolutionarily highly conserved non-coding small RNAs. They are 21–22 nucleotides in size and are widely distributed in eukaryotic cells [18]. MiRNAs negatively regulate gene expression at the mRNA level by recognizing and binding to 3′ untranslated regions (UTRs) [19]. They could lead to degradation of the target mRNA or inhibition of its translation, resulting in decreased production of the protein [20]. Similar to those in other animals and insects, mosquito miRNAs (22–24 nt) degrade their target mRNAs to regulate the host-pathogen interactions, metabolism, development, and insecticide resistance; however, miRNAs’ precise role in deltamethrin resistance remains mostly unknown [21].

The present study aimed to use previously identified differentially expressed miRNAs between a deltamethrin-resistant (DR) strain and a deltamethrin-susceptible (DS) strain of *Culex pipiens pallens* [22] to investigate miRNAs related to mosquito deltamethrin resistance. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to verify the expression level of miR-4448 in DS and DR strain mosquitoes. Bioinformatic predictions and dual-luciferase assays were used to identify the potential target of miR-4448. Next, RNA interference (RNAi) was performed for miR-4448 and its target *CYP4H31* using oral feeding or microinjection, and then the mortality of mosquitoes was detected using CDC bottle bioassays in vivo. Cell Counting Kit-8 (CCK-8) was also applied to verify the viability of C6/36 cells transfected with *CYP4H31* in *in vitro*. These experiments helped us to determine whether miR-4448 is involved in deltamethrin resistance by targeting *CYP4H31* in *Culex pipiens pallens*.

**Materials and methods**

**Insects**

In this study, we used two strains of *Culex pipiens pallens* with different resistance levels to deltamethrin mosquitoes. The DS strain of *Culex pipiens pallens* used in the present study was obtained from Ji Nan University and maintained in the laboratory with a constant light/dark cycle (14:10 h) at 28 °C and 70–80% relative humidity. Adults were provided with 5% (w/v) sterilized sugar [glucose (5 g, GHTECH, Guangdong, China) was dissolved in 100 ml of deionized water and autoclaved] on a Scotch-Brite™ sponge wick (3 M, Shanghai, China) *ad libitum*. Mosquitoes were fed on mouse blood to reproduce the next generation. The DR strain was selected from the DS strain and was maintained via treatment with deltamethrin at the 50% lethal concentration (LC50) of each generation (G8). There were 4000 larvae for screening in each pool (three pools/G8). For the DS and DR strains, the LC50 values were 0.04 and 8.5 mg/l, respectively. Deltamethrin (technical, 99.0%) was a gift from Jiangsu Provincial Center for Disease Control and Prevention (Jiangsu, China). Procedures for blood-feeding with mice in our laboratory were monitored by The National Science and Technology Review Board (no. IACUC-1812047).

**Genomic DNA extraction, pre-miR-4448 amplification, and cloning**

Genomic DNA (gDNA) was extracted from 3-day post-eclosion (3 d PE) female adult mosquitoes (N = 1) using a MiniBEST Universal Genomic DNA Extraction Kit, version 5.0 (Takara, Dalian, China), following the manufacturer’s instructions. gDNA quantity and quality were checked using a Thermo Scientific™ NanoDrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA).

Using the gDNA as a template, PCR was performed using primers (Table 1) designed according the *Cx. pipiens pallens* pre-miRNA sequence with the following conditions: 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 10 s; and a
final extension step at 72 °C for 10 min. The PCR products were subjected to electrophoresis through a 2.0% agarose gel. A PCR fragment of around 80 bp was isolated from the gel and purified using a MiniBEST Agarose Gel DNA Extraction Kit version 4.0 (Takara) and then cloned into vector pMD 19-T (Takara). Ten microliters of the resultant plasmid was transferred into 100 μl of Escherichia coli One Shot® TOP10 Competent Cells (Invitrogen, Carlsbad, CA, USA) for amplification [23]. Colonies were selected and analyzed using PCR and sequencing.

Identifying the miR-4448 precursor and the potential target of miR-4448
First, the miR-4448 precursor (pre-miR-4448) sequence was identified to ensure that the miRNA actually existed in Cx. pipiens pallens rather than being a sequencing artifact. We obtained a 90-bp pre-miR-4448 nucleotide sequence (GCT CGC ACC ACA ACC CCG AAG CGA GAA TCA TAC CCC TAG ACC A) by sequencing. The miR-4448 sequence shown in bold.

To identify the putative gene targets of miR-4448, we used 3′ UTR sequences from the Cx. quinquefasciatus genome in the RNAhybrid target prediction program [24]. We focused on the CYP family genes that participate in the regulation insecticidal resistance of mosquitoes, and only CYP4H31 was identified as a potential target of miR-4448. To assess the conservation of the 3′ UTR, we amplified the 3′ UTR from Cx. pipiens pallens. The 3′ UTR sequence of CYP4H31 in Cx. pipiens pallens was 100% identical with that from Cx. quinquefasciatus. Then, qRT-PCR was used to detect the expression levels of miR-4448 and CYP4H31 in the DS and DR strains.

Quantitative real-time reverse transcription PCR (qRT-PCR) analyses
At 3 d PE, DS and DR strain female adult mosquitoes (N=10) were subjected to total RNA extraction using the RNAiso Plus reagent (Takara). The total RNA purity and concentration were checked using a NanoDrop spectrophotometer. The cDNA was synthesized from 1 μg of total RNA using a PrimeScript RT reagent Kit (Takara) and PrimeScript™ RT Master Mix (Takara) according to the manufacturer’s protocol. The cDNA was diluted 1:10, and 4 μl of the diluted cDNA solution was used as template for quantitative real-time PCR (qPCR) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR was performed in a 20-μl reaction mix containing 10 pmol of forward and reverse PCR primers [designed using Primer Premier 6.0 software (PREMIER Biosoft International, San Francisco, CA, USA)] for miR-4448 and CYP4H31 (Table 1). MiR-4448 expression was measured using the Stem-loop RT-PCR method [25] with the following reaction conditions: 50 °C for 2 min and 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by melting-curve analysis on an ABI Prism 7300 real-time PCR Instrument (Applied Biosystems). The relative expression level of miR-4448 was normalized to the internal control U6 small nuclear (U6), and the CYP4H31 expression level was normalized to that of β-actin from the DS and DR strains. The DS strain expression level was designated as 1. For each experiment, RNA from three biological replicates was used, and PCR amplification of each cDNA sample was performed in triplicate. The 2−ΔΔCt method was used to calculate the relative expression levels [26].

pMIR-REPORT vector construction, cell culture, and dual-luciferase reporter assay
We identified the region of the CYP4H31 3′ UTR that included the complementary sequences predicted to bind miR-4448. To mutate this region, the binding site

| Name                  | Forward (5′–3′)                           | Reverse (5′–3′)              |
|-----------------------|-------------------------------------------|-------------------------------|
| pre-miR-4448          | GCTCGACCCACAACCCCCG                       | AAGCGAGAATCATAACCCCCCTAGACCA |
| miR-4448 (RT-stem ring)| CTCAGCTTGTTGCGAGTGGCAATTCTGAGCATACCCCC   | TGGTGCTGAGATGCG               |
| miR-4448              | GCTCGACCCACAACCCCCG                       | AAGCGAGAATCATAACCCCCCTAGACCA |
| U6                    | ACACCTGGTGGGCTGAGTGTGTGGCAATTCTGAGCATACCC| TGGTGCTGAGATGCG               |
| CYP4H31               | ACTTTGTGAGCGCTGGATAGC                     | AATCCCCGAAAGGACTGAC           |
| β-actin               | AGCGTGAATGAGCGCTGCGTGTGTGGCAATTCTGAGCATACCC| AATCCCCGAAAGGACTGAC           |
| CYP4H31 3′ UTR-WT     | CGAGCTCGAAGACCTTGATATATATATATATATATATATATAGCA| AATCCCCGAAAGGACTGAC           |
| CYP4H31 3′ UTR-Δ       | CGAGCTCGAAGACCTTGATATATATATATATATATATATATAGCA| AATCCCCGAAAGGACTGAC           |
| pIB-VS-His-CYP4H31    | GGAAGCTGAGATGAGGGAATGCTGAGATATATATATATATATATATATATAGCA| AATCCCCGAAAGGACTGAC           |
complementary region (AUGCAGC) was replaced by UUGGGUGG (3′ UTR-Δ). Two pairs of primers were designed according to the transcript sequences from Cx. quinquefasciatus to amplify the wild-type (WT) 3′ UTR and 3′ UTR-Δ of CYP4H31 (Table 1). Luciferase constructs were made by amplifying and sequencing the Cx. quinquefasciatus putative target 3′ UTR-WT/3′ UTR-Δ sequence of the CYP4H31 mRNA (containing the putative seed region of the miR-4448 binding sites) and using the T/A cloning method to insert them into the HindIII and XbaI sites located downstream of the Renilla translational stop codon within the pMIR-REPORT mRNA Expression Reporter Vector (Promega, Madison, WI, USA) [17].

At 48 h after transfection of the pMIR-REPORT constructs, assays were performed using the dual-luciferase reporter assay system (Promega). 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco) in a 5% CO₂-humidified incubator at 37 °C [18]. Then, 6 × 10⁴ cells/well in 2.5 ml of complete growth medium was seeded and incubated in a 6-well plate for 24 h until they reached >80% confluency. Then, 6 ng of pMIR-REPORT-UTR-WT or pMIR-REPORT- UTR-Δ treated with 6 μl of miR-4448 mimic and miRNA negative control (NC1) (GenePharma, Shanghai, China) along with 6 ng of PGL4.7 (Promega) was cotransfected using the FuGENE HD transfection reagent (Promega). Vector PGL4.7, which constitutively expresses Renilla luciferase, was cotransfected as an internal control to correct for differences in the efficiency of transfection and harvest between the groups. In each sample, Renilla luciferase was normalized using Firefly luciferase expression [19]. An M200 microplate fluorescence reader (Tecan, Lyon, France) was used to detect the luciferase activity. Cells were treated in triplicate, and the transfections were repeated three times.

**Oral feeding**

For the oral feeding experiments, all the materials (e.g. water, glucose, and sponges) were treated with diethyl pyrocarbonate (DEPC; Sangon Biotech, Shanghai, China) to remove RNase. In each cage, DR strain pupae (N = 120) of Cx. pipiens pallens were collected in a plastic cup. The post-eclosion (PE) mosquitoes were starved for 12 h. The 12-h PE adults of the blank group (WT) were treated with 5% glucose water, while the negative control group (NC1) was given the miRNA mimic control dissolved in 5% glucose water, at a final dose of 100 nmol/l. The experimental group (miR-4448 mimic) was parallelly supplied with the miR-4448 mimic (100 nmol/l). At 48 h after treatment, RNA was extracted from female adult mosquitoes to validate the expression of miR-4448 and its target gene CYP4H31. The miR-4448 mimic and miRNA control mimic were obtained from GenePharma (Table 2).

**Microinjection of miR-4448 mimic and CYP4H31 siRNA (si-CYP4H31)**

Microinjections were conducted using a Nanoject III aspirator tube assembly (cat. no. 3-000-207, Drummond Scientific Co., Broomall, PA, USA) fitted with a needle puller (Sutter P-97, Sutter Instrument, Novato, CA, USA) and a glass capillary needle (3.5″, Drummond). GenePharma designed and synthesized a small interfering RNA targeting the open reading frame (ORF) of CYP4H31 (si-CYP4H31) (Table 2). For the microinjection of miRNA, DR strain female adult mosquitoes were collected within 12 h PE and frozen at −20 °C for 3–5 min. These mosquitoes were divided into three groups and injected in the thorax with different moieties. The negative control group (NC1) was injected with 0.5 μl of miRNA control mimic at a dose of 20 nmol/l, and the experimental group (miR-4448 mimic) was injected with 0.5 μl of the miR-4448 mimic under the same conditions at a final dose of 20 nmol/l. For the microinjection of siRNA, the negative control group (NC2) was injected with 69 nl of control at a dose of 5 μg/μl, and the experiment group (si-CYP4H31) was injected with 69 nl of si-CYP4H31 under the same conditions at a final dose of 5 μg/μl. Thereafter, the mosquitoes were transferred to holding tubes and maintained in our laboratory with a constant light/dark cycle (14:10 h) at 28 °C with 70–80% humidity. After 72 h, the expression levels of miR-4448 and its target gene CYP4H31 were validated using qRT-PCR. Three biological replicates with three technical replicates, each replicate containing 20 female mosquitoes, were performed.

**Eukaryotic expression vector pIB/V5-His construction, cell culture, and transfection**

Standard molecular biology procedures were used for plasmid construction [27]. Overlap PCR was performed to amplify the ORF of CYP4H31 using corresponding primer pairs (Table 1) from Cx. quinquefasciatus, which was inserted between unique restriction enzyme sites (Spel/Xhol) of the eukaryotic expression vector, pIB/V5-His (Invitrogen). The positive recombinant plasmid, named pIB/V5-His-CYP4H31, was confirmed using DNA sequencing.

*Aedes albopictus* C6/36 cells (CRL-1660; ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% (v/v) FCS. The C6/36 cells were grown in a 6-well plate at 28 °C in a 5% CO₂-humidified incubator at. The cells were then plated at 5 × 10⁵ cells/well and
incubated for 24 h in a 6-well plate in 2.5 ml of complete growth medium. The cells were transfected when they reached 60–80% confluence. The transfection protocol was as follows: the plasmid DNA (pIB/V5-His-CYP4H31) was diluted to 1.5 ng per 100 µl in complete growth medium followed by the addition of 5 µl of FuGENE HD transfection reagent. The DNA mixture was incubated at room temperature for 25 min and then added to the medium below the surface. The plate was rocked back and forth and from side to side to ensure distribution over the entire plate surface. Meanwhile, C6/36 cells transfected with pIB/V5-His were used as controls. Three biological replicates with three technical replicates were performed.

qRT-PCR and Western blotting analysis of CYP4H31 in the transfection cells

At 48 h after transfection, the transiently transfected C6/36 cells were subjected to western blotting and qRT-PCR. To evaluate the CYP4H31 transfection efficiency, total RNA was isolated from the transfected cells, and qRT-PCR was performed, as described above, to check the expression level of CYP4H31.

Transfected cells were washed with phosphate-buffered saline (PBS). Protein was extracted from the cells after digestion with trypsin solution and lysis using radiol-munoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Protein concentrations were tested using a bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA). Soluble protein (50 µg) was denatured and subjected to 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using Trans-Blot SD Cell and Systems for 60 min at 300 mA (Bio-Rad, Hercules, CA, USA). The membrane was washed twice in 1× Tris-buffered saline-Tween 20 (TBS-T), and then blocked for 60 min at 37 °C in 5% Difco™ Skim Milk (BD Biosciences, San Jose, CA, USA). The membrane was incubated with anti-His-Tag monoclonal primary antibodies (1:1000, NovaGen, Madison, WI, USA) and β-actin monoclonal primary antibodies (1:2000, ABGENT, Suzhou, China), with shaking overnight at 4 °C. The membranes were then washed with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies (1:2000, BioWorld, Shenzen, China) in blocking buffer at 37 °C for 2 h. The membranes were washed thoroughly with TBS-T before imaging using BIO-RAD UNIVERSAL HOOD II and Pierce™ ECL Western Blotting Substrate, according to the manufacturer's instructions.

**Table 2** List of the miR-4448 mimic, miRNA control mimic (NC1), si-CYP4H31, and control siRNA (NC2) sequences used for RNAi in Culex pipiens pallens

| Name                        | Sense (5′–3′)                              | Antisense (5′–3′)                  |
|-----------------------------|--------------------------------------------|-----------------------------------|
| miR-4448                    | GGCUCGAUGUCCUAGGUGGUAUG                   | UACCACCAGCACCGAGGCUUU            |
| miRNA control mimic (NC1)   | UUCUCGGAAACGCUCAGCGGTT                    | ACGUGACGCGUUCGGAGATT             |
| si-CYP4H31                  | GGGCAGAAGAUUGAGAGCAGCAA                   | AUUIUGUCGAUUCGAGCCTT             |
| Control siRNA (NC2)         | UUCUCCGAACGUGUCAGCAGTT                    | ACGUGACGCGUUCGGAGAATT            |

American CDC bottle bioassay

According to published guidelines, American Centers for Disease Control and Prevention (CDC) bottle bioassays were conducted to detect the sensitivity of mosquitoes injected with the miR-4448 mimic and s-CYP4H31 to deltamethrin [30]. Each 250-ml bottle and its cap were coated with 1 ml of deltamethrin solution using inversion and rolling of the bottles. Control bottles were coated using 1 ml of acetone. A sheet was used to cover all bottles, which were left to dry in the dark. Twenty
mosquitoes were placed in each bottle and exposed to deltamethrin or acetone for 120 min. Following exposure, mosquitoes subjected to CYP4H31 knockdown were monitored at 15-min intervals up to 2 h. Mosquitoes were considered dead if they could no longer stand [31]. The percent mortality (y-axis) was plotted against time (x-axis) using a linear scale.

**Statistical analyses**

Statistically significant qualitative variables were detected using the GraphPad Prism 8.0 software (GraphPad Inc., La Jolla, CA, USA), and statistical significance was accepted at *P* < 0.05. Qualitative variables were analyzed using the Chi-square test, while quantitative variables were assessed using analysis of variance [32, 33].

**Results**

**MiR-4448 targets CYP4H31**

Preliminary Solexa sequencing results showed that the expression of miR-4448 was significantly different between the DS and DR strains [22]. In this study, miR-4448 showed 6.49-fold higher expression in the DS strain compared with that in the DR strain (Fig. 1a, ***P* < 0.001), while the predicted target gene, CYP4H31, showed 2.77-fold lower expression in the DS strain than that in the DR strain (Fig. 1b, *P* < 0.05). The contrasting expression patterns suggested that CYP4H31 might be the target gene of miR-4448. Dual-luciferase report assays were then used to determine the interaction between miR-4448 and CYP4H31 in vitro. Plasmids inserted with the 3' UTR-WT or 3' UTR-Δ of CYP4H31 along with the control plasmid, pGL4.7, were cotransfected into HEK 293-T cells and then treated with the miRNA-4448 mimic or miRNA negative control (NC1). The results showed that miR-4448 treatment inhibited the luciferase activity from the WT 3' UTR construct markedly (by 24.67%), while no significant change occurred when the cells were treated with the negative control. Meanwhile, no increase in luciferase activity was observed in the 3' UTR-Δ group when treated with the miR-4448 mimic (Fig. 2b, **P* < 0.01). Therefore, CYP4H31 was verified as a target of miR-4448 in vitro.

**MiR-4448 modulates mosquito deltamethrin resistance**

To determine whether miR-4448 could regulate deltamethrin resistance in mosquitoes, the miR-4448 mimic or miRNA mimic control was supplied to DR strain mosquitoes via oral feeding. The relative miR-4448 expression was 1.97-fold higher in the mosquitoes fed with the miR-4448 mimic compared with those in the NC1 control group (Fig. 3a, ***P* < 0.001), which suggested that in the DR strain, miR-4448 was successfully overexpressed. In these cells, the transcription level of CYP4H31 was decreased about 47.85% (Fig. 3b, *P* < 0.05), which suggested that CYP4H31 is a direct in vivo target of miR-4448. In the CDC bottle bioassay, the group fed with the miR-4448 mimic had a higher mortality rate compared with those in the NC1 groups. At 90 and 105 min, the mortality rate of miR-4448 mimic-fed mosquitoes was 52.5% (21/40) and 65.0% (26/40), respectively, which was higher than those in the NC1 groups (42.4% [14/33] and 51.5% [17/33]) (Fig. 3c, *P* < 0.05). To further validate the results obtained by oral feeding, we injected the miR-4448 mimic or miRNA mimic control into each mosquito at 12 h PE. The microinjection results showed the efficient overexpression of miR-4448 (by 3.53-fold) and the significantly decreased expression level of CYP4H31 (65.82%) in the miR-4448 mimic injection group (Fig. 4a,
**CYP4H31 functions in mosquito deltamethrin resistance**

To determine the function of CYP4H31 in mosquito deltamethrin resistance, transient transfection assays were performed in C6/36 cells in vitro, and their sensitivity to deltamethrin was determined after transfection. The results showed the expression level of CYP4H31 was 851.0-fold higher in the experimental group than in the NC group (Fig. 5a, **P<0.01**). Western blotting demonstrated that the band could be detected using anti-His-tag antibodies in the pIB/V5-His-CYP4H31 group (Fig. 5b). Thus, transcript and protein level detection proved the transfection was successful. To investigate the sensitivity of the transiently transfected C6/36 cells to deltamethrin, a CCK-8 kit was employed to detect cell viability after deltamethrin treatment. The percentage of viable cells among those transfected with pIB/V5-His-CYP4H31 was significantly higher than those in the NC and WT groups (Fig. 5c, ***P<0.001, **P<0.01, *P<0.05). The data showed that CYP4H31 could increase mosquito cell resistance to deltamethrin.

To further evaluate whether CYP4H31 participates in mosquito resistance to deltamethrin in vivo, we conducted phenotypic experiments using CYP4H31 RNAi knockdown (si-CYP4H31) in DR strain mosquitoes. We expected that the RNAi-mediated ablation of the physiologically relevant target of miR-4448 would display the same phenotype as that caused by miR-4448 over-expression. CYP4H31 expression decreased by 41.90% in the si-CYP4H31 injection group compared with that in the negative control group (NC2) (Fig. 6a, **P<0.01). RNAi silencing of CYP4H31 in mosquitoes resulted in increased sensitivity to deltamethrin. At 120 min, the mortality rate was 74.3% (29/39) in si-CYP4H31-injected mosquitoes, while it was 46.7% (21/45) in the NC2 group and 48.7% (20/41) in the WT group (Fig. 6b, **P<0.01). These results suggested that CYP4H31 does indeed play a role in mosquito deltamethrin resistance.
Discussion

Our results showed downregulation of miR-4448 in the DR strain, which suggested that miR-4448 might be involved in the regulation of deltamethrin resistance in *Cx. pipiens pallens*. Multiple approaches to miRNA target prediction were used to identify the physiologically relevant miR-4448 target contributing to the miR-4448-mimic phenotypes. We identified *CYP4H31* as a direct target of miR-4448 in vitro and in vivo. The results from bioinformatic predictions showed that miR-4448 might directly regulate the expression of the P450 gene, *CYP4H31*. A dual-luciferase reporter assay using a luciferase reporter vector containing *Cx. pipiens pallens CYP4H31* 3′ UTR cotransfected with the miR-4448 mimic resulted in a decrease in *Renilla* luciferase activity in vitro. Overexpression of miR-4448 by oral feeding and
microinjection of an miR-4448 mimic reduced CYP4H31 expression and increased the mosquitoes’ sensitivity to deltamethrin in vivo. By contrast, CYP4H31 overexpression decreased mosquito cell sensitivity to deltamethrin, and intrathoracic microinjection of dsRNA of CYP4H31 (si-CYP4H31) increased the mosquitoes’ sensitivity to deltamethrin. Taken together, these results further confirmed CYP4H31 as an authentic miR-4448 target and indicated that miR-4448 might participate in deltamethrin-resistance by regulating CYP4H31 in mosquitoes.

MicroRNAs (~23 nt) are endogenous RNAs that play an important gene-regulatory role by pairing with the 3’ UTR of protein-coding gene mRNAs to direct their posttranscriptional repression [34]. Dysregulation of miRNAs has been reported in host-pathogen interactions, metabolism, development, and insecticide
resistance [21]. Previously, our group performed Solexa high-throughput sequencing and showed that miR-4448 was highly expressed in DS strain mosquitoes [22]. In this study, to further investigate the function of miR-4448 in deltamethrin-resistant mosquitoes, we first identified that the pre-miR-4448 sequence was present in Cx. pipiens pallens. The qRT-PCR results showed that the conserved miRNA, miR-4448, was enriched in the DS strain mosquitoes.

Many studies have shown that multiple, complex resistance mechanisms, particularly increased metabolic detoxification of insecticides, are likely to be responsible for insecticide resistance (reviewed in [16]). Commonly, metabolic detoxification, especially by CYPs, is the main molecular mechanism of insecticide resistance [35]. The overproduction of CYPs in resistant populations could, in principle, lead to a negative cross-resistance between different insecticides in insects, in which detoxification of one insecticide (for example pyrethroids) occurs at the same time as activation of another pro-insecticide (such as organophosphates, ketoenols, or clorfenapyr) [36]. Recently, researchers reported that miRNAs could mediate insecticide resistance through CYP genes [37–39]. In the present study, CYP4H31 was identified as a direct in vitro and in vivo target of miR-4448. A dual-luciferase reporter assay comprising a CYP4H31 3′ UTR-containing luciferase reporter vector, which was cotransfected together with the miR-4448 mimic, produced decreased in vitro Renilla luciferase activity. Meanwhile, microinjection of the miR-4448 mimic decreased the CYP4H31 transcript level in vivo, which further confirmed that in mosquitoes, CYP4H31 is a target gene of miR-4448. CYP4H31 belongs to the CYP4 family as a member of the monoxygenase cytochrome P450 (CYPs) superfamily [40]. CYP4 family genes were proposed as the most important P450 genes involved in pyrethroid resistance in Anopheles sinensis [41].

Next, using oral feeding and RNAi technology, in combination with the CDC bottle assay, the present study made significant progress toward determining the
regulatory role of miRNAs in insecticide resistance. In mosquitoes, miR-4448 function decreases deltamethrin resistance by inhibiting CYP4H31 expression. However, the mortality of miR-4448-mimic-supplied mosquitoes showed no significant change compared with the controls, possibly because we could not control the amount of microRNA mimic taken up by each mosquito. To date, novel strategies, including transgenic plants, engineered microorganisms, and nano-scale formulations, have been developed to improve the efficacy of miRNA; however, many hurdles must be overcome before this technology becomes a reliable method of pest management [42]. Notwithstanding, in our study, the miR-4448-mimic-injected mosquitoes displayed drastically higher
sensitivity to deltamethrin and resulted in significantly increased mortality in the DR strain. Furthermore, our study showed that high expression of CYP4H31 could increase resistance to deltamethrin and consequently improve cell viability. In contrast, low expression of CYP4H31 RNAi, resulted in increased sensitivity to deltamethrin and higher mortality. Taken together, our findings demonstrated that CYP4H31 is related to mosquito deltamethrin resistance.

Conclusion
Our study has established a fundamental role for miR-4448 in the regulation of mosquito deltamethrin resistance through its target, CYP4H31, in mosquitoes (Fig. 7). Further investigation of CYP4H31, e.g. using gene editing, is warranted to determine its exact function in deltamethrin resistance. Our findings revealed a mechanism of insecticide resistance, which could lead to new methods to control mosquito populations.

Abbreviations
miRNA: MicroRNA; DR strain: Deltamethrin-resistant strain; DS strain: Deltamethrin-susceptible strain; qRT-PCR: Quantitative real-time reverse transcription PCR; gDNA: Genomic DNA; 3′ UTR: 3′ Untranslated region; 3 d PE: 3 days post-eclosion; CCK-8: Cell Counting Kit-8.

Acknowledgements
We are grateful to Prof. Maocing Gong of Ji Nan University for their kind help and donation of Culex pipiens pallens mosquitoes. A team of workers from Ji Nan University (E117°, N36°38’) sampled the Culex pipiens pallens mosquitoes from Tangkou town, Ji Nan city, Shandong province.

Authors’ contributions
XXL, SLH, HTY, and HB2 performed the experiments. XXL and SLH wrote the manuscript and prepared the figures. YS, DZ, LM, BS, and CLZ conceived the idea and coordinated the project. All authors read and approved the final manuscript.

Funding
This work was funded by the National Natural Science Foundation of China (grant nos. 81672058, 81672056, 81772227, and 81971970) and the National S and T Major Program (grant no. 2017ZX10303404-002-006). The funding bodies had no role in the design of the study and the collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
Data supporting the conclusions of this article are included within the article. All data are fully available without restriction upon request.

Declarations
Ethics approval and consent to participate
All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University for the use of laboratory animals (no. IACUC-1812047).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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