MiR-4448 Is Involved in Deltamethrin Resistance by Targeting *CYP4H31* in Culex Pipiens Pallens

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Abstract

Background: Mosquitoes are vectors of serious 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 as important regulators of gene expression across a wide variety of organisms, which might function in mosquito deltamethrin resistance.

Methods: In the present study, 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*. A 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. negatively regulated *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 *CYP4H31 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.

Background

Many serious diseases, including Zika, neglected tropical diseases (NTDs), yellow fever, dengue fever, West Nile fever, and malaria, are transmitted by mosquitoes [1-4]. Globalization and international travel have contribute to transnational pathogen and vector dispersion, representing a key threat for millions of people and animals worldwide [5, 6]. Over the past two decades, insecticide-based approaches to control mosquito vectors have substantially reduced the prevalence of malaria and contained outbreaks of the virus [7]. Pyrethroids, represented by deltamethrin, are commonly used as insecticides and are recommended for in-home insect control because of their properties of high efficiency, broad spectrum, and relatively low toxicity to humans [8, 9]. Unfortunately, mosquito resistance to insecticides has developed because of their long-term heavy use [10]. The emergence of mosquito resistance to insecticides has resulted in the resurgence of some diseases that had been controlled before. For example, the resurgence of neurological disease caused by West Nile virus (WNV) resulted in 243 deaths in the United States in 2012 [11].

Studies showed that the development of insecticide resistance in mosquitoes is a complex and heritable biological evolutionary phenomenon, involving multiple genes and mechanisms [12]. Improving our understanding of the molecular mechanisms of insecticide resistance would allow the formation of novel strategies to minimize and prevent resistance development, thus controlling mosquitoes [13]. To date, research has largely focused on seeking insecticide resistance-related genes, which found that the development of insecticide resistance is mostly induced by changes in the expression of the identified insecticide resistance-associated genes. Genes encoding cytochrome P450s are closely involved in resistance development [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 of 21-22 nucleotides in size, which are widely distributed in eukaryotic cells. MiRNAs negatively regulate gene expression at the mRNA level by recognizing and binding to 3' untranslated regions (UTRs), which leads to degradation of the target mRNA or inhibition of its translation, resulting in decreased production of the protein [18-20]. 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) verified the downregulation of miR-4448 in the DR-strain, which suggested that miR-4448 might be involved in the regulation of deltamethrin resistance. Bioinformatic predictions and functional assays suggested that miR-4448 might directly regulate the expression of the P450 gene, *CYP4H31*. Overexpression of miR-4448 by oral feeding and microinjection of an miR-4448 mimic reduced *CYP4H31* expression and increased sensitivity to deltamethrin in mosquitoes. *CYP4H31* overexpression decreased mosquito cell sensitivity to deltamethrin and intrathoracic microinjection of dsRNA of *CYP4H31* (si-*CYP4H31*) increased the sensitivity to deltamethrin in mosquitoes. Taken together, these results indicated that miR-4448 might participate in deltamethrin-resistance by regulating *CYP4H31* in mosquitoes.

Methods

Insects

DS- and DR-strains of mosquito (*Cullex pipiens pallens*) used in the present study were obtained from Tangkou, Shandong province, and maintained in the laboratory with a constant light/dark cycle (14 h:10 h) at 28 °C and 70–80% humidity. Adults were provided with 5% sterilized sugar on sponge wick ad libitum. The DR-strain was selected from the DS-strain and was maintained via treatment with deltamethrin at the 50% lethal concentration (LC_{50}) of each generation. For the DS- and DR-strains, the LC_{50} values were 0.04 mg/L and 8.5 mg/L, respectively.

Genomic DNA Extraction, pre-miR-4448 Amplification and cloning

Genomic DNA (gDNA) was extracted from 3-d post-eclosion (3 d PE) female adult mosquitoes using a MiniBEST Universal Genomic DNA Extraction Kit Ver. 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 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 Ver. 4.0 (Takara), and then cloned into vector pMD 18-T (Takara). The resultant plasmid was transferred into *Escherichia coli* Top10 cells for amplification and sequencing.

Quantitative real-time reverse transcription PCR (qRT-PCR) analyses

At 3 d PE, DS-strain and DR-strain female adult mosquitoes 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 [23] using 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 $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels [24].

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 complementary region (AUCGAGC) was replaced by UUGGUGG (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 miRNA 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) supplemented with 10% (v/v) fetal calf serum (FCS) in a 5% CO_2-humidified incubator at 37 °C [18]. Then, 6×10^4 cells/well in 2.5 ml of complete growth medium were 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) were 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 in triplicate, and the transfections were repeated three times.

Oral feeding

For the oral feeding experiments, all of the materials (e.g., water, glucose, and sponges) were treated with diethyl pyrocarbonate (DEPC) to remove RNase. DR-strain pupae of *Cx. pipiens pallens* were collected and placed in three cages until eclosion, and then starved for 12 h. The 12 h eclosion (12 h PE) mosquitoes of blank group (WT) were treated with 5% glucose water, while the negative control group (NC1) were 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 supplied with the miR-4448 mimic dissolved in 5% glucose water at final a dose 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*)

GenePharma deigned 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 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 h: 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, each containing 20 female mosquitoes, with three technical replicates, were performed.

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

Standard molecular biology procedures were used for plasmid construction. 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 (*SpeI*/*XhoI*) of the eukaryotic expression vector, pIB/V5-His. The positive recombinant plasmid, named pIB/V5-His-CYP4H31 was confirmed using DNA sequencing.

Aedes albopictus C6/36 cells (CRL-1660; ATCC) 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% CO_2-humidified incubator at. The cells were then plated at 5×10^5 cells/well and incubated for 24 h in a 6-well 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 Radioimmunoprecipitation 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™ Skin 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.

Cell viability assay using a Cell Counting Kit-8

CYP4H31 overexpression in relation to deltamethrin resistance was assessed by measuring cell viability using a Cell Counting Kit-8 (CCK-8; Dojindo, Japan) [25]. Cells (100 μl) were added to each well of a 96-well plate 5×10^3 cells/well and incubated in a 5% CO_2-humidified incubator at 28 °C for 24 h. Then, the cells were treated with various concentrations of deltamethrin in 100 μl (0, $10^{0.5}$, 10^1, $10^{1.5}$, 10^2, and $10^{2.5}$ mg/L) [26]. Twenty-four hours later, CCK-8 solution (10 μl) was added to the wells and incubated for 28 °C for 3 h. The absorbance was then detected using dual wavelength spectrophotometry at 450 nm and 630 nm in a microplate reader. Dimethyl sulfoxide (DMSO, Sigma, St, Louis, MO, USA) was used to dissolve deltamethrin and the final concentration of DMSO was 0.5% (v/v) for the different concentrations of deltamethrin [22]. Three biological replicates with three technical replicates were performed.

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 si-*CYP4H31* to deltamethrin [27]. 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, knockdown mosquitoes were monitored at 15 min intervals up to 2 h and the percent mortality (Y axis) was plotted against time (X axis) using a linear scale.

Statistical Analysis

Statistical significance was accepted at $P < 0.05$. Qualitative variables were detected using the chi-square test, while quantitative variables were detected using analysis of variance [28, 29].

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. First, the miR-4448 precursor (pre- miR-4448) sequence was identified to ensure that the miRNA actually exists in *Cx.*

pipiens pallens rather than being a sequencing artefact. We obtained a 90 bp pre-miR-4448 nucleotide base sequence (GCTCGCACCACAACCCCGAATCACCGCGAGCGTACCGCCACTCCAGCACTCATGGCAC**GGCTCGATGGTCTAGGGGTATG**ATTCTCGCTT) by sequencing. The miR-4448 sequences are shown in bold. To identify the putative gene targets of miR-4448, we used 3' UTR sequences from the *Cx. quinquefasciatus* genome for use in RNAhybrid target prediction program [30]. *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. MiR-4448 showed 6.56-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 2.90-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 assay were then used to determine the interaction between miR-4448 and *CYP4H31in vitro*. Plasmids inserted with the 3' UTR-WT or 3' UTR-Δ of *CYP4H31* (pMIR-REPORT-UTR-WT and pMIR-REPORT-UTR-Δ) 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, while no significant change occurred when the cells were treated with the negative control. No increase in luciferase activity was observed in the pMIR-REPORT- 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 mosquitoes via oral feeding. The relative miR-4448 expression was 1.88-fold higher in the insects 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 49.20% (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% and 65.0%, respectively, which was higher than NC1 groups (45% and 52%) (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 3.59-fold) and the significantly decreased expression level of *CYP4H31* (68.0%) in the miR-4448 mimic injection group (Fig. 4A; Fig. 4B, ***$P < 0.001$). The expression trend was consistent with the results of the oral feeding experiment. Additionally, the results of CDC Bottle Bioassay showed significantly higher mortality rates after injecting miR-4448 mimic compared with the control. At 105 min, in the mimic group, the mortality rate was 45.5%; in the NC1 group, the mortality rate was 31.3%; and in the WT group, the mortality rate was 27.5%. Furthermore, at 120 min, in the mimic group, the mortality rate was 78.9%, while it was 57.2% in the NC1 group and 45.7% in the WT group (Fig. 4C, **$P < 0.01$, *$P < 0.05$). Taken together, oral feeding and microinjection both demonstrated that miR-4448 could modulate deltamethrin resistance of mosquitoes by downregulating the expression of *CYP4H31*.

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 *CYP4H31* ORF (GenBank: KM056314.1) of *Cx. pipiens pallens* was amplified, inserted into vector pIB/V5-His vector, and transfected into C6/36 cells. Total RNA and protein were extracted from C6/36 cells of blank group (WT), the pIB/V5-His control group (NC), and the experimental group (pIB/V5-His-*CYP4H31*). The results showed the expression level of *CYP4H31* was 445.1-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 investigative 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 overexpression. *CYP4H31* expression was lower in the si-*CYP4H31* injection group than in the blank group (WT) and 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% in si-CYP4H31-injected mosquitoes, while it was 48.9% in the NC2 group and 46.7% in the WT group (Fig. 6B, $^{**}P < 0.01$). These results suggested that *CYP4H31* does indeed play a role in mosquito deltamethrin resistance.

Discussion

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 [31]. 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]. To further investigate the function of miR-4448 in deltamethrin resistant mosquitoes, we 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.

A large number of 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 [32]. The overproduction of CYPs in resistant populations could, in principle, lead to a negative cross-resistance between different insecticides in insects, in which detoxification one insecticide occurs at the same time as activation of another pro-insecticide [33]. Recently, researchers reported that miRNAs could mediate insecticide resistance through *CYP* genes [34-36]. 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 vitroRenilla* 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 CYP4 family as a member of monooxygenase cytochrome P450 (CYPs) superfamily [37]. CYP4 family genes were proposed as the most important *P450* genes involved in pyrethroid resistance in *Anopheles Sinensis* [38].

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 miRNA in insecticide resistance. In mosquitoes, miR-4448 function decreases deltamethrin resistance by inhibiting *CYP4H31* expression. However, miR-4448 mimic administration through feeding resulted in no significant change in miR-4448-mimic-supplied mosquitoes, 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 [39]. Notwithstanding, in our study, the miR-4448-mimic-injected mosquitoes displayed drastically lower 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* after miR-4448 mimic oral feeding or microinjection, and *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 mosquitoes. We identified *CYP4H31* as a target of miR-4448, which functions in the regulation of mosquito deltamethrin resistance. 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.

List Of Abbreviations

miRNAs	MicroRNAs
DR-strain	Deltamethrin-resistant strain
DS-strain	Deltamethrin-susceptible strain
qRT-PCR	Quantitative real time reverse transcription PCR
gDNA	Genomic DNA
3' -UTRs	3' untranslated regions
3 d PE	3-d post-eclosion
CCK-8	Cell Counting Kit-8

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.

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.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XXL, SLH, HTY and HBZ 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.

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Tables

Table 1: Oligonucleotide sequences used in the present study

Name	Forward (5'-3')	Reverse (5'-3')
pre-miR-4448	GCTCGCACCACAACCCCG	AAGCGAGAATCATACCCCTAGACCA
miR-279-3p (RT-stem ring)	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCATACCCC	
miR-4448	ACACTCCAGCTGGGGGCTCGATGGTCTAGG	TGGTGTCGTGGAGTCG
U6	GCTTCGGCTGGACATATACTAAAAT	GAACGCTTCACGATTTTGCG
CYP4H31	ACTTTGATGGCGTTGGATAGC	AATCCCGCAAGAGGACTGAC
β-actin	AGCGTGAACTGACGGCTCTTG	ACTCGTCGTACTCCTGCTTGG
CYP4H31 3' UTR-WT	CGAGCTCAAACCTGTTGATATTTTACTGGCA	CCAAGCTTTTTTTGCGCTCGATGGTTT
CYP4H31 3' UTR-Δ	CGAGCTCAAACCTGTTGATATTTTACTGGCA	CCAAGCTTTTTTTGCCCACCAAGGTTT
pIB/V5-His-*CYP4H31*	GGACTAGTGAGATGGAAATGCTGATTGAGATCGTACTGG	CCGCTCGAGCGGTTTCGCGCCATAATCTTCA

Table 2: List of the miR-4448 mimic, miRNA control mimic (NC1), *siCYP4H31, and* control siRNA (NC2) sequences

Name	Sense (5'-3')	Antisense (5'-3')
miR-4448	GGCUCGAUGGUCUAGGGGUAUG	UACCCCUAGACCAUCGAGCCUU
miRNA control mimic (NC1)	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
siCYP4H31	GGGCAAAGAUUCGACAAAUTT	AUUUGUCGAAUCUUUGCCCTT
control siRNA (NC2)	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Figures

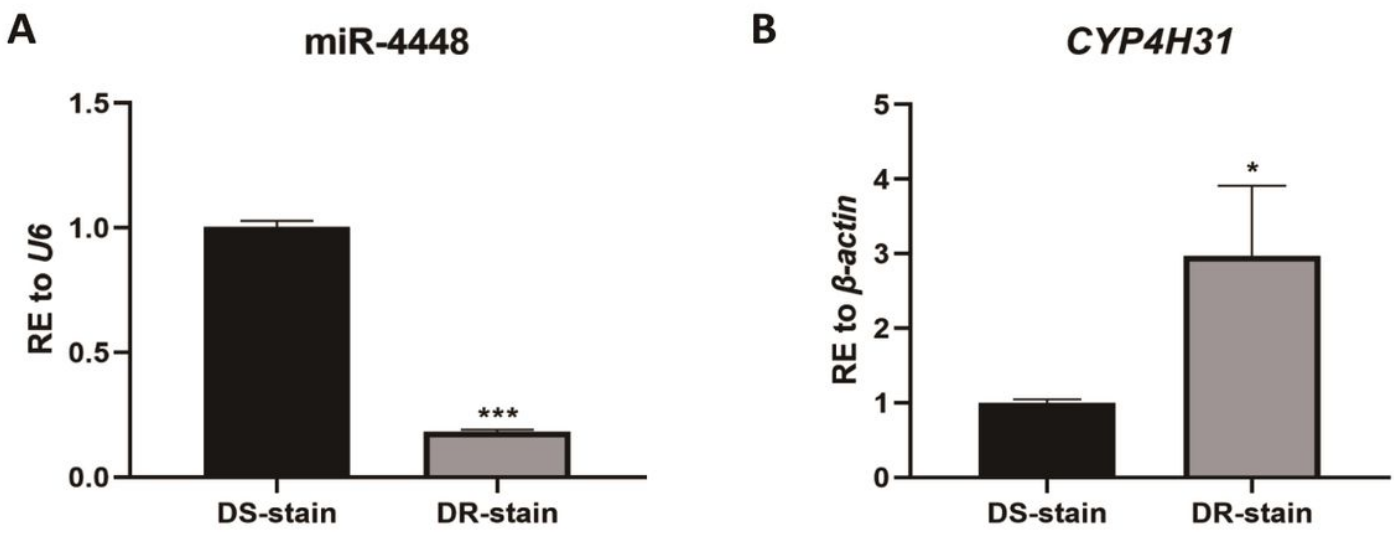
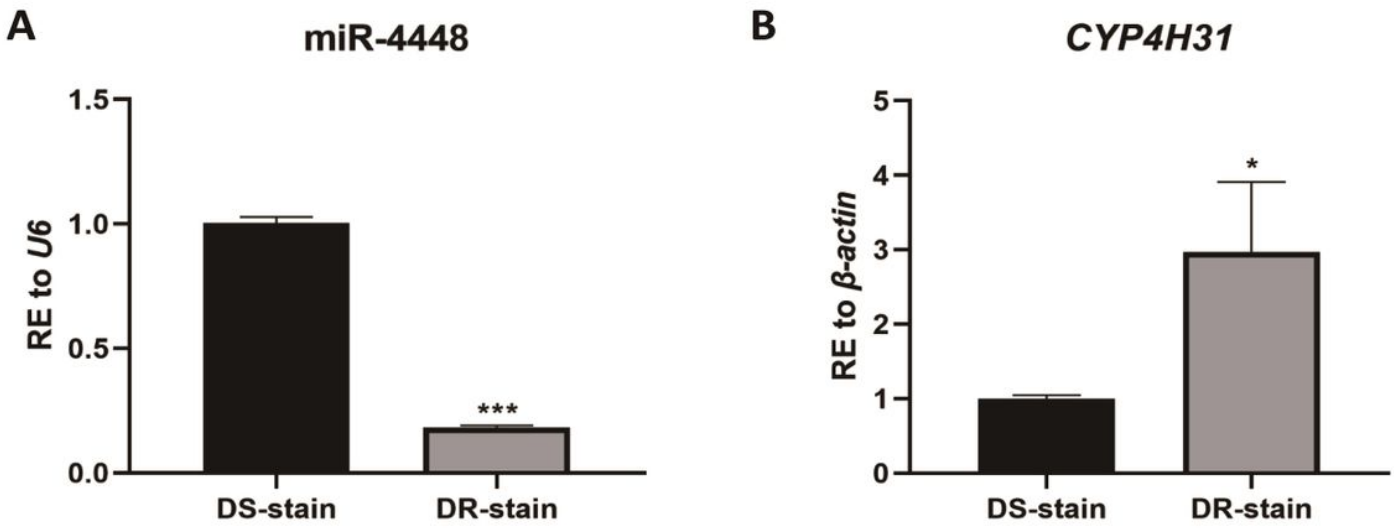


Figure 1

The expression of miR-4448 was lower and that of CYP4H31 was higher in the DR-strain than that in the DS-strain. (A) The expression of miR-4448 was 6.56-fold higher in the DS-strain than in the DR-strain. (B) The expression of CYP4H31 was 2.90-fold higher in the DR-strain than in the DS-strain. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; *P < 0.05, *** P < 0.001.



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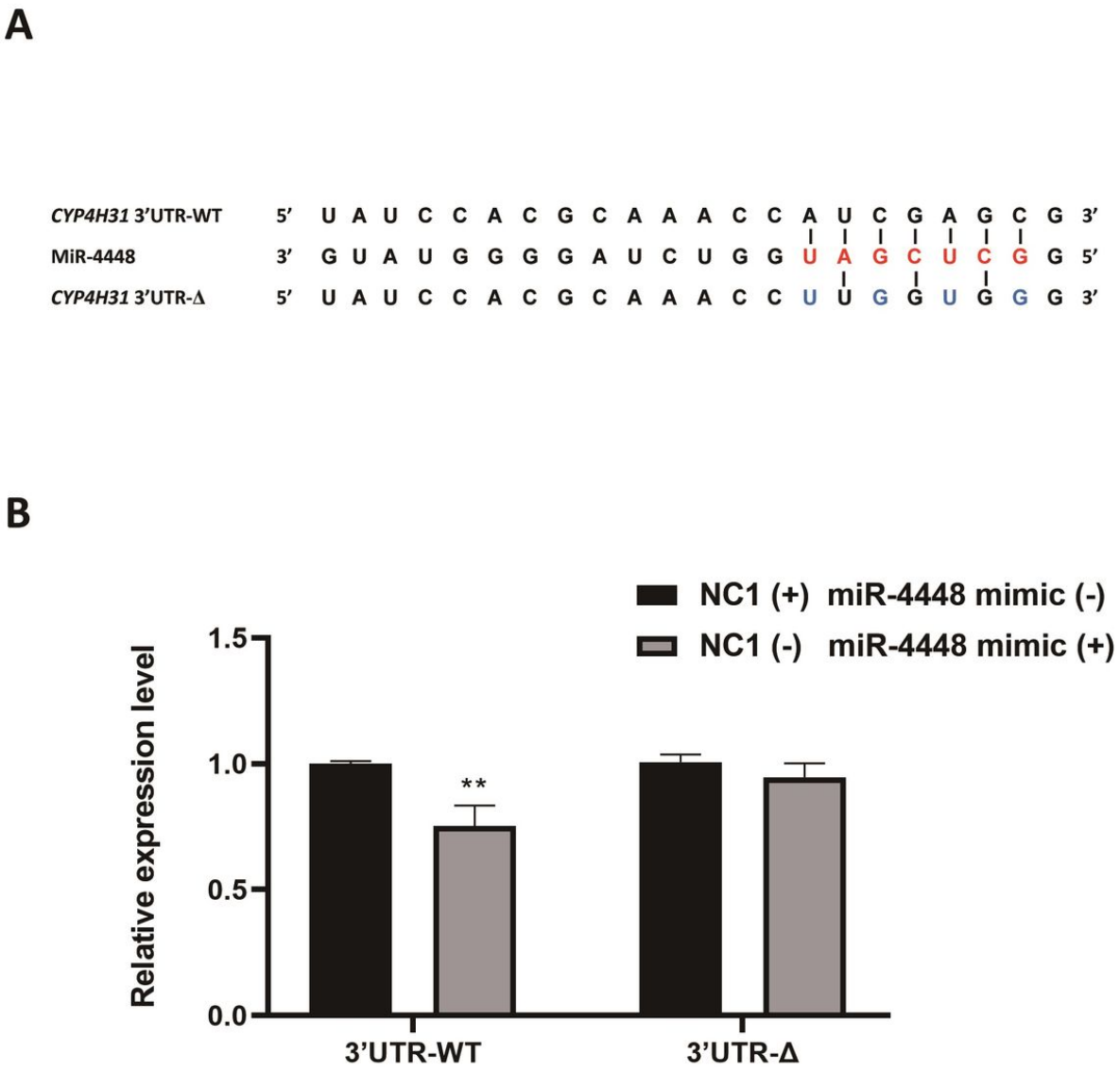
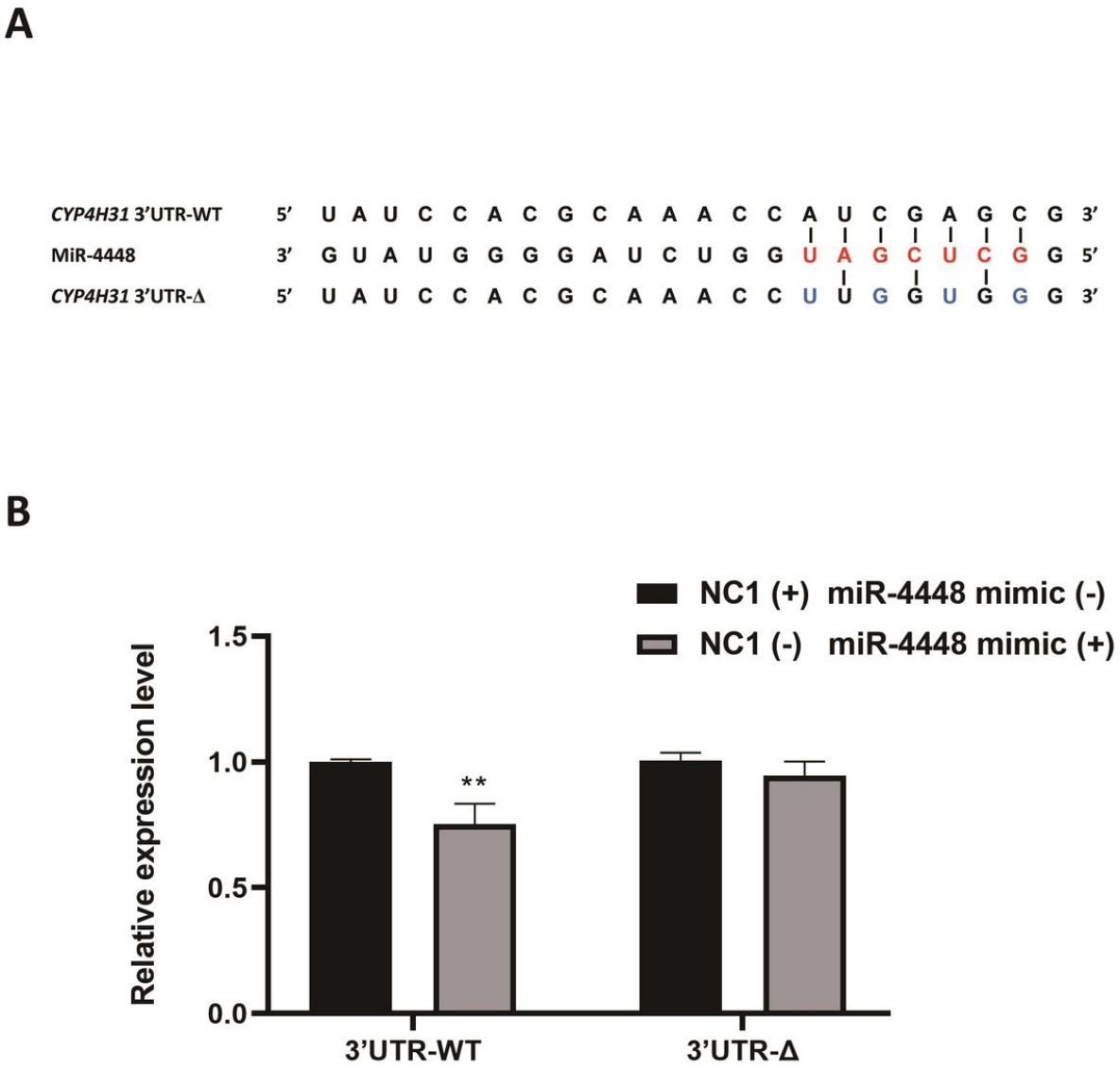


Figure 2

MiR-4448 directly regulated CYP4H31 expression through 3' UTR sites. (A) The predicted CYP4H31 3' UTR miR-4448 binding site. (B) CYP4H31 assessed using a Dual-luciferase reporter assay. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; **P < 0.01.



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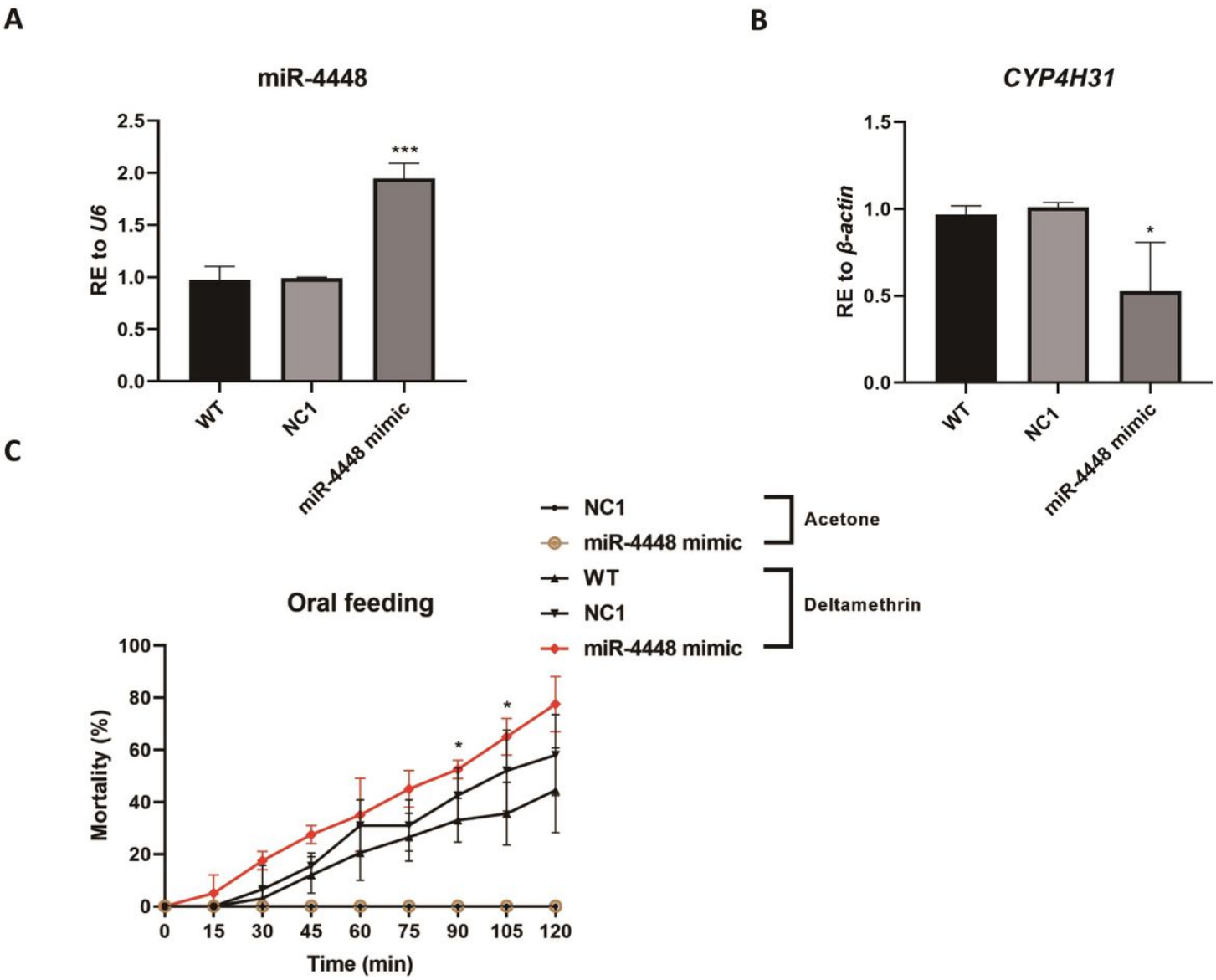
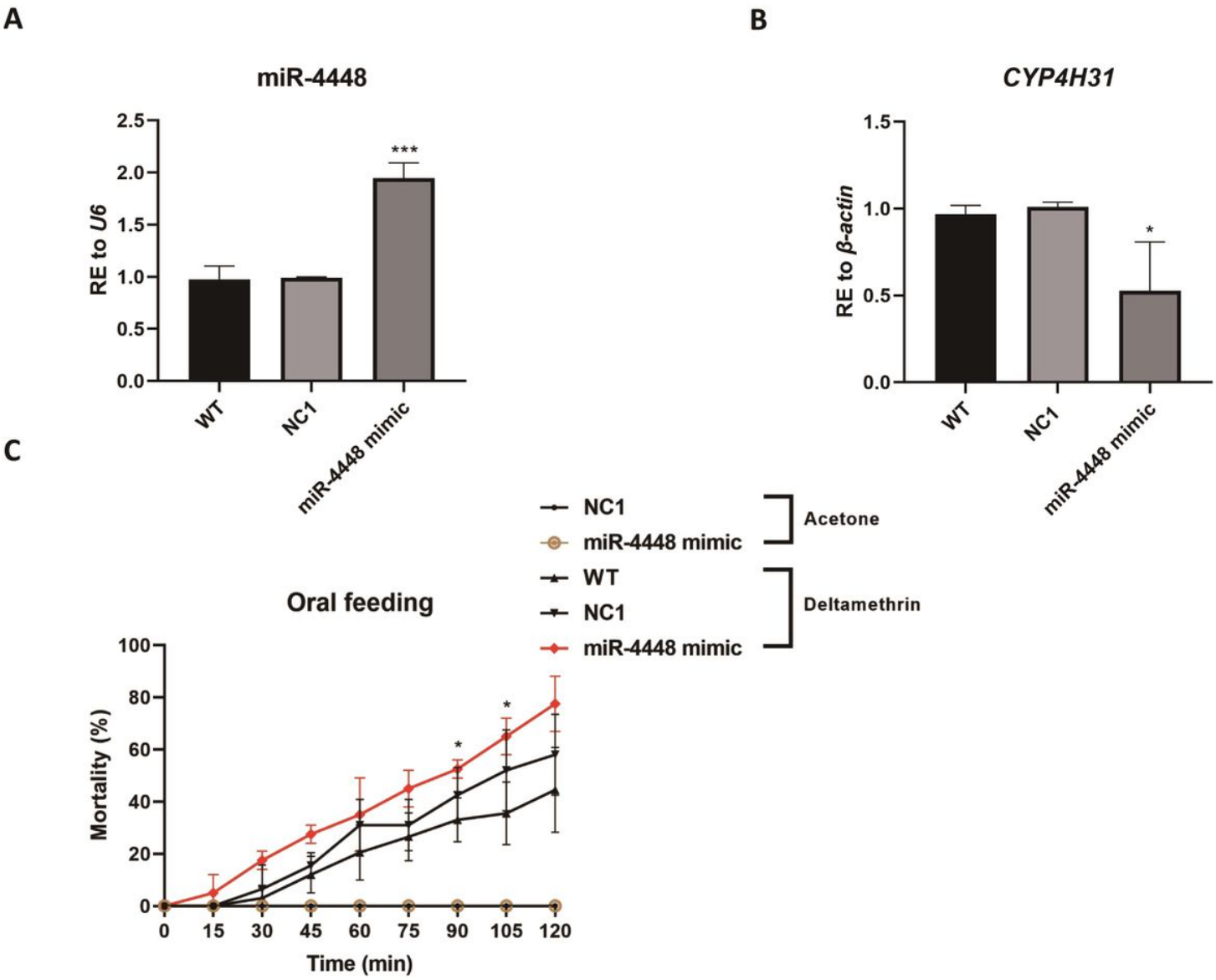


Figure 3

Oral feeding with the miR-4448 mimic could reduce mosquito resistance to deltamethrin. (A-B) Oral feeding of the miR-4448 mimic upregulated the expression of miR-4448 and downregulated the expression of CYP4H31 in DR-stain. (C) Mosquito mortality was assessed after incubation for a 2 h in a CDC bottle containing 7 mg/L deltamethrin. The mortality of the miR-4448 mimic group was higher than that of the acetone control, WT, and miRNA control mimic (NC1) groups. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; *** $P < 0.001$, *$P < 0.05$.



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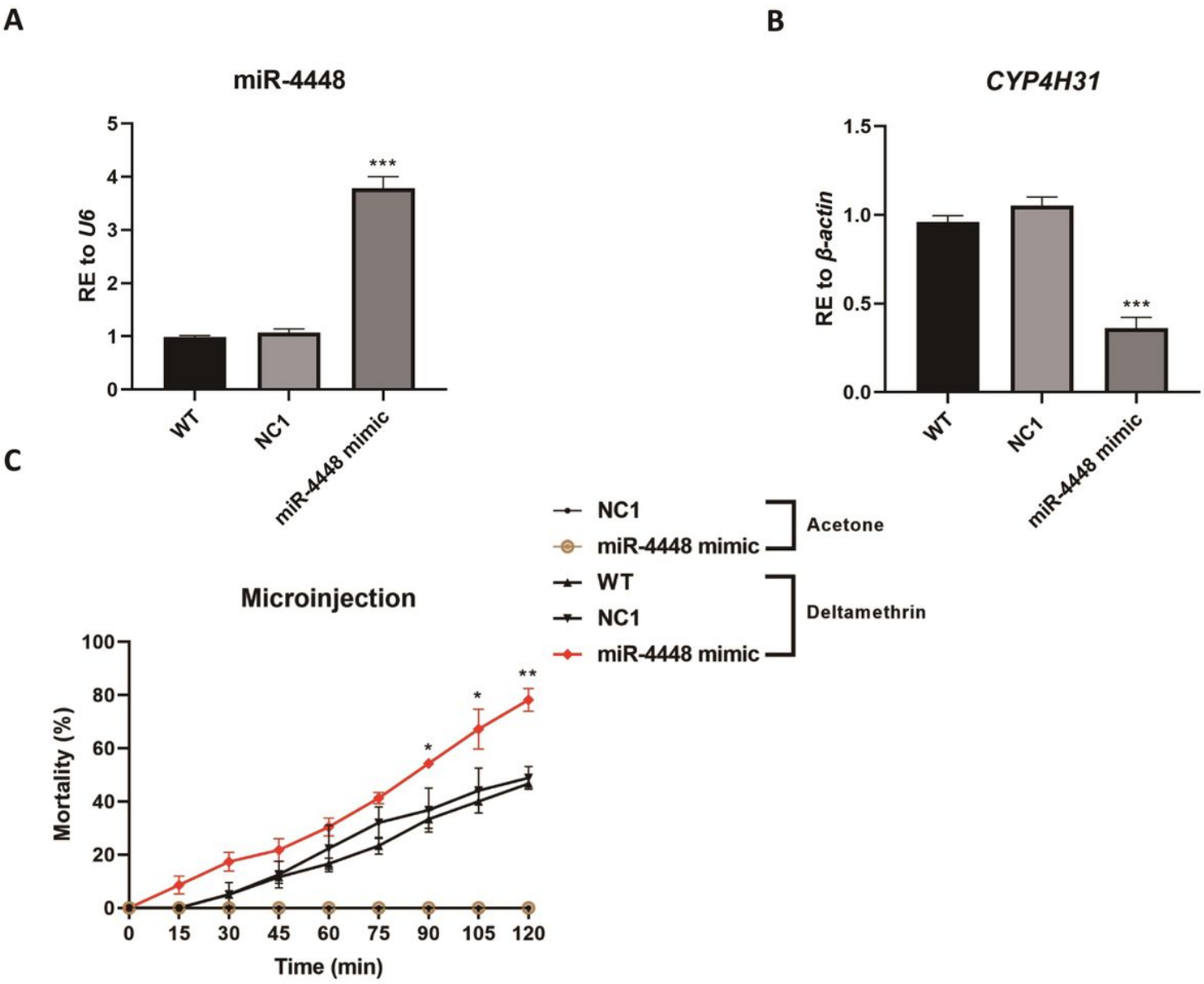
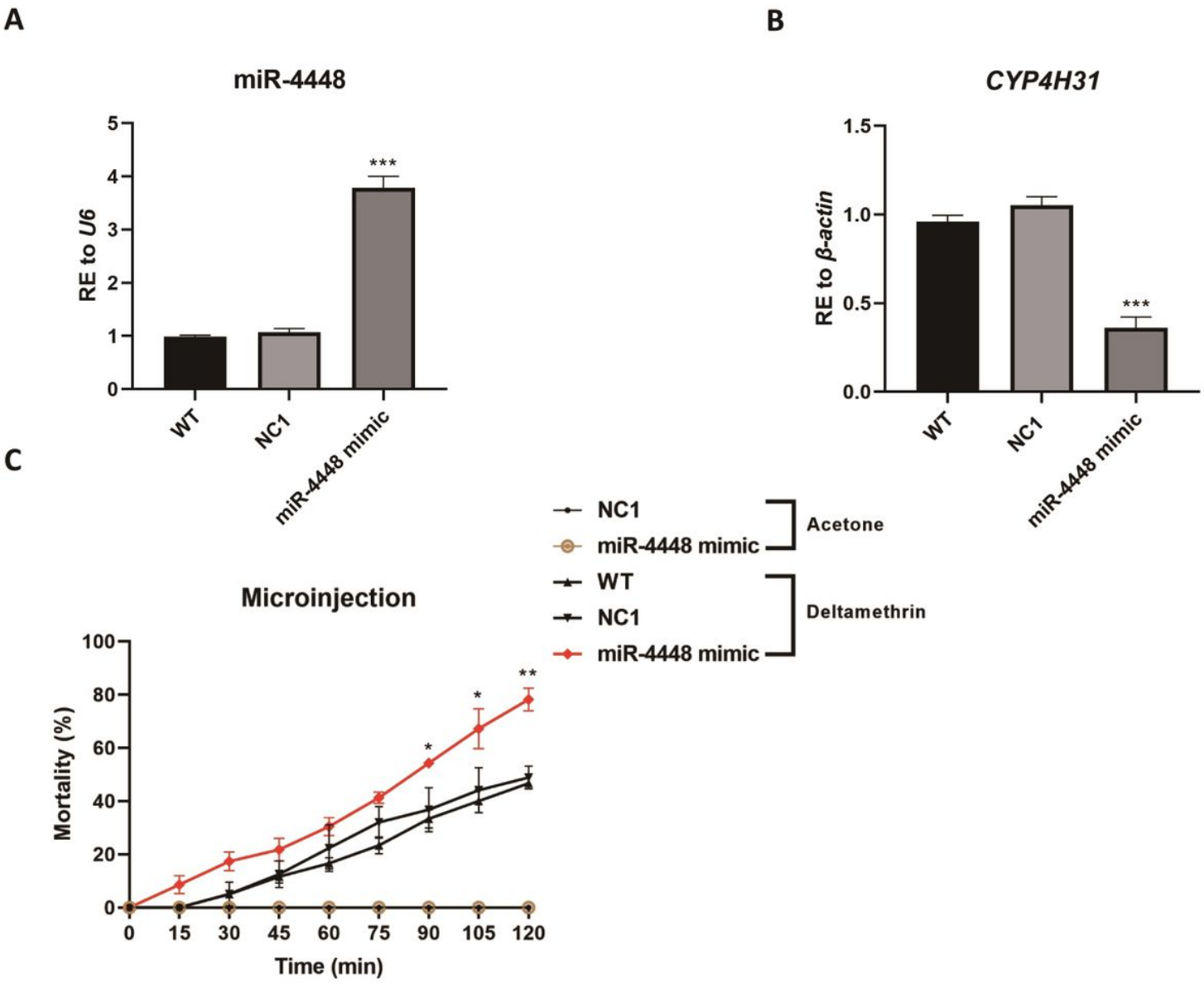


Figure 4

Microinjection of miR-4448 mimic could reduce mosquito resistance to deltamethrin. (A-B) Microinjection of the miR-4448 mimic upregulated the expression of miR-4448 and downregulated the expression of CYP4H31 in the DR-stain. (C) Mosquito mortality was assessed after incubation for a 2 h in a CDC bottle containing 7 mg/L deltamethrin. The mortality of the miR-4448 mimic group was higher than that of the acetone control, WT, and miRNA control mimic (NC1) groups. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; *** $P < 0.001$, ** $P < 0.01$, *$P < 0.05$.



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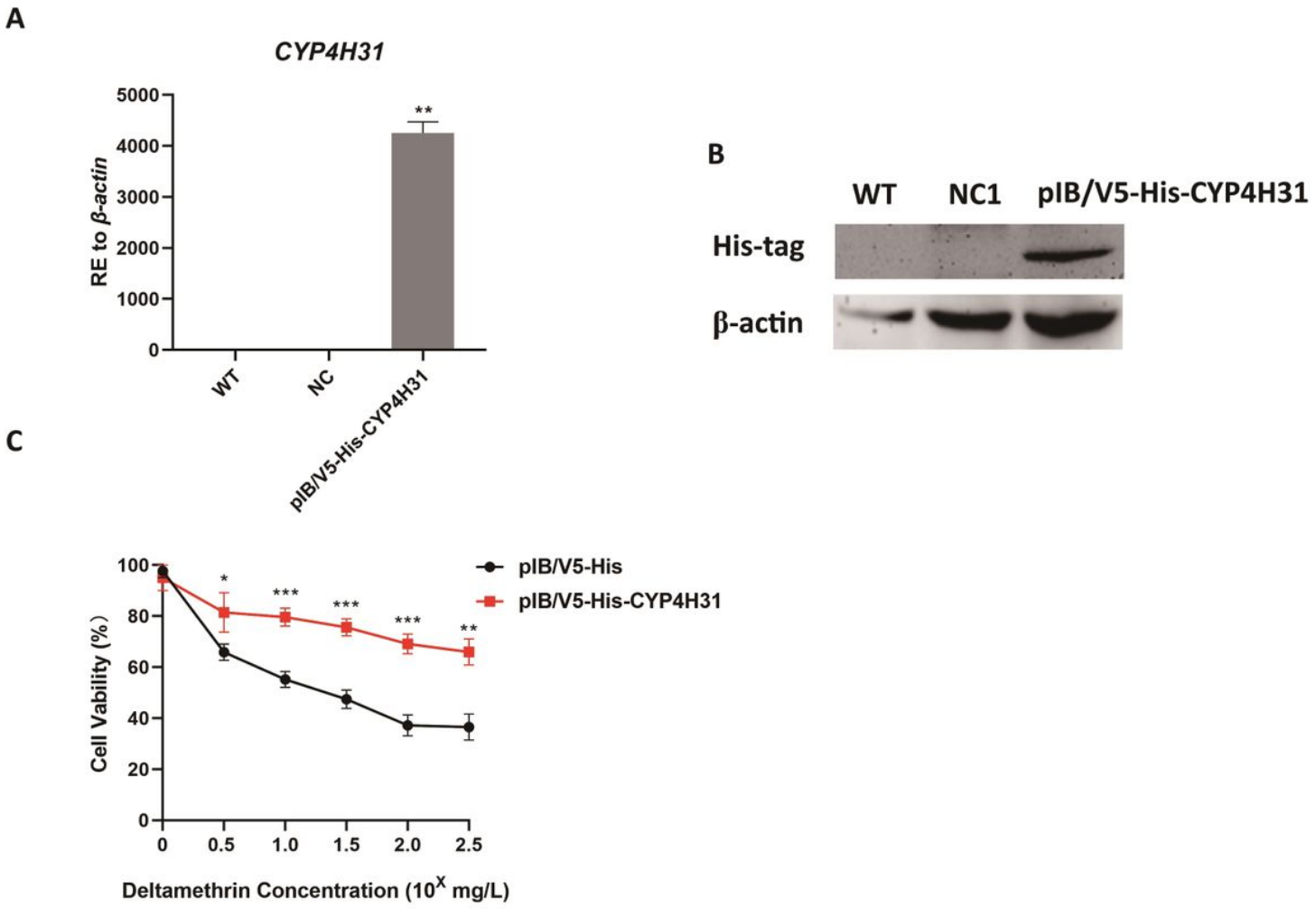
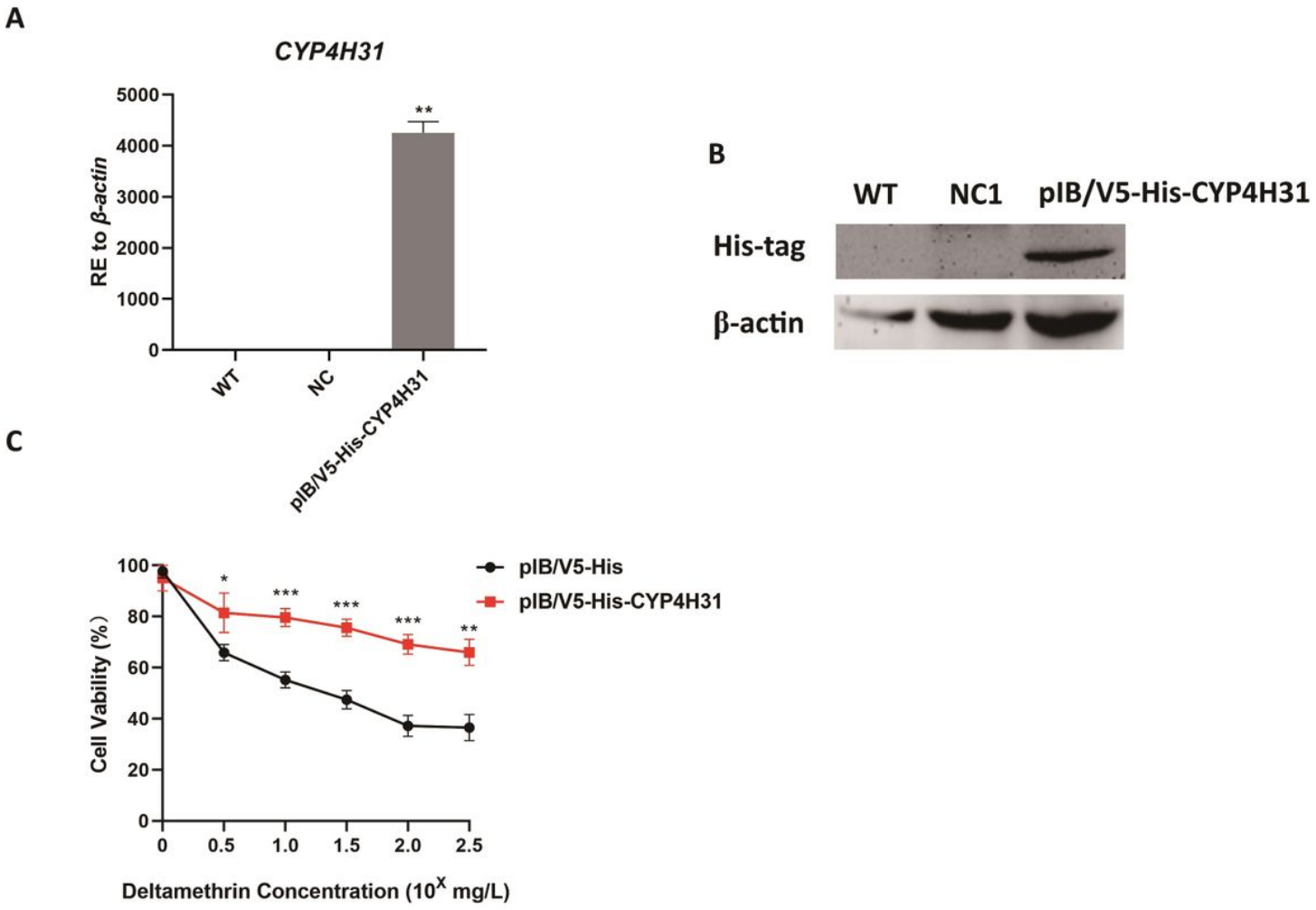


Figure 5

Overexpression of CYP4H31 upregulated the cell viability of C6/36 cells under deltamethrin stress. (A) qRT-PCR analysis of CYP4H31 in transfected C6/36 cells. The cells transfected with pIB/V5-His-CYP4H31 showed a significantly higher transcription level (445.1-fold). (B) Western blotting experiments using His-tag antibodies to detect the CYP4H31 protein. (C) The pIB/V5 His CYP4H31 construct-transfected C6/36 cells were treated with deltamethrin and their viability was calculated using a CCK-8 kit. The viability of pIB/V5-His-CYP4H31-transfected cells was higher than that the pIB/V5-His group. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; ***P < 0.001, ** P < 0.01, *P < 0.05.



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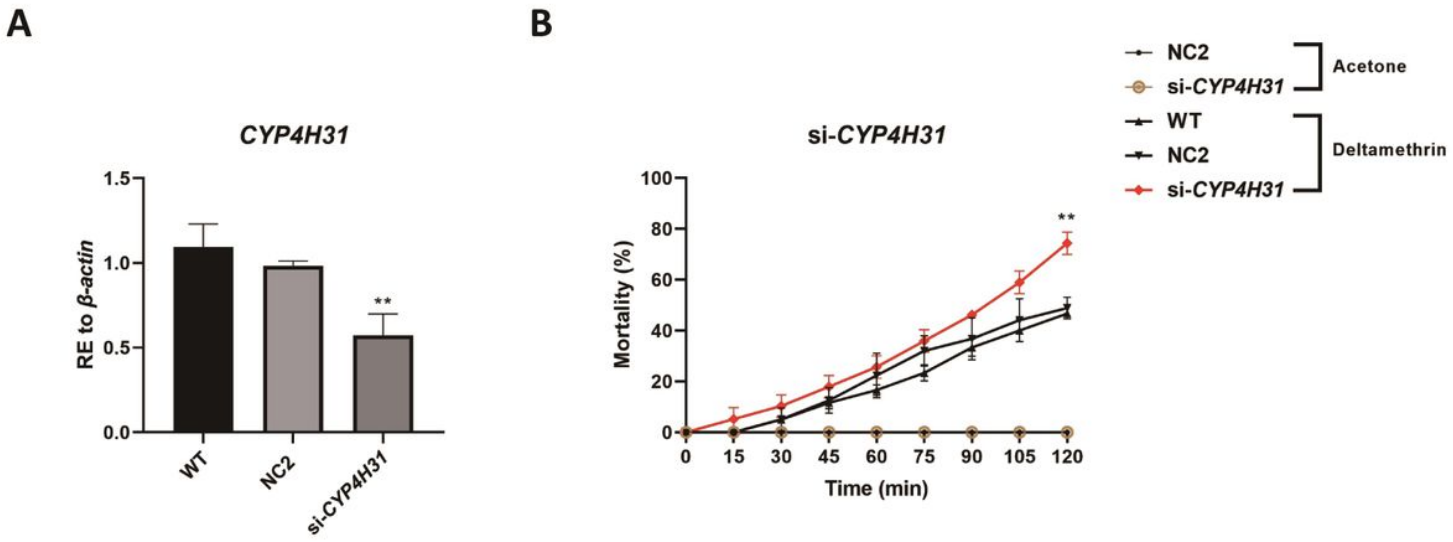
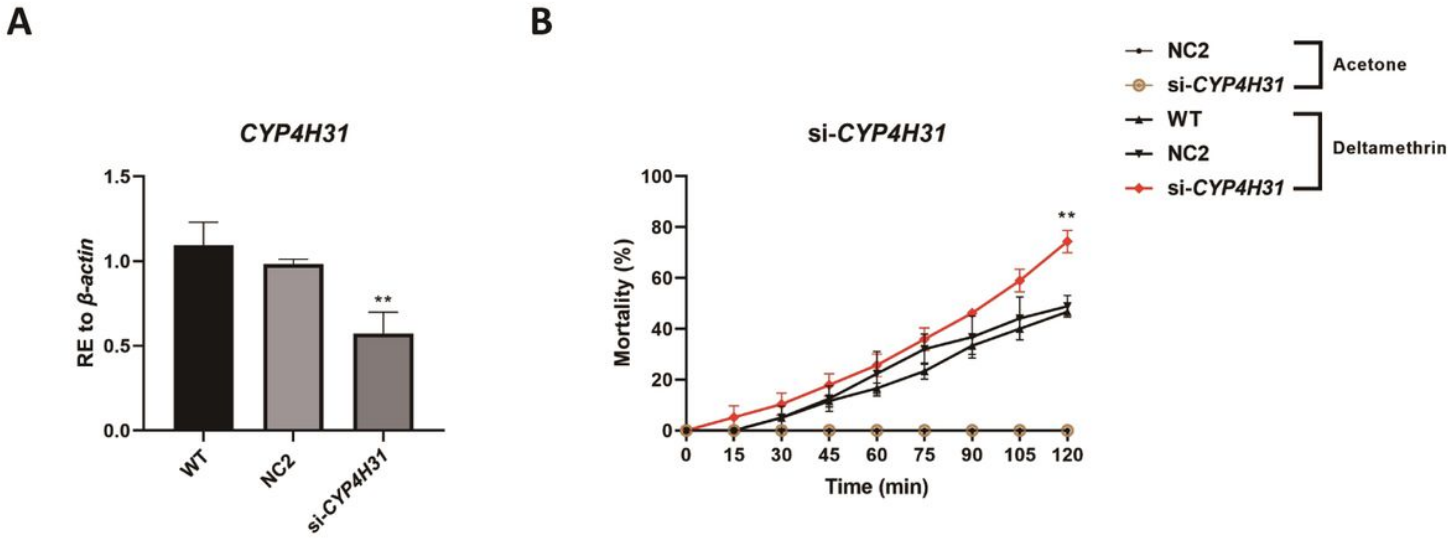


Figure 6

Microinjection of an siRNA targeting CYP4H31 (si-CYP4H31) in female adult mosquitoes reduced their resistance to deltamethrin. (A) Microinjection of si-CYP4H31 downregulated the expression of CYP4H31 in the DR-stain. (C) Mosquito mortality was assessed after incubation for a 2 h in a CDC bottle containing 7 mg/L deltamethrin. The mortality of the si-CYP4H31 group was higher than that in the acetone control, WT, and control siRNA (NC2) groups. Data are representative of three technical replicates of three biological replicates and are indicated as the mean ± SE; ** P < 0.01.



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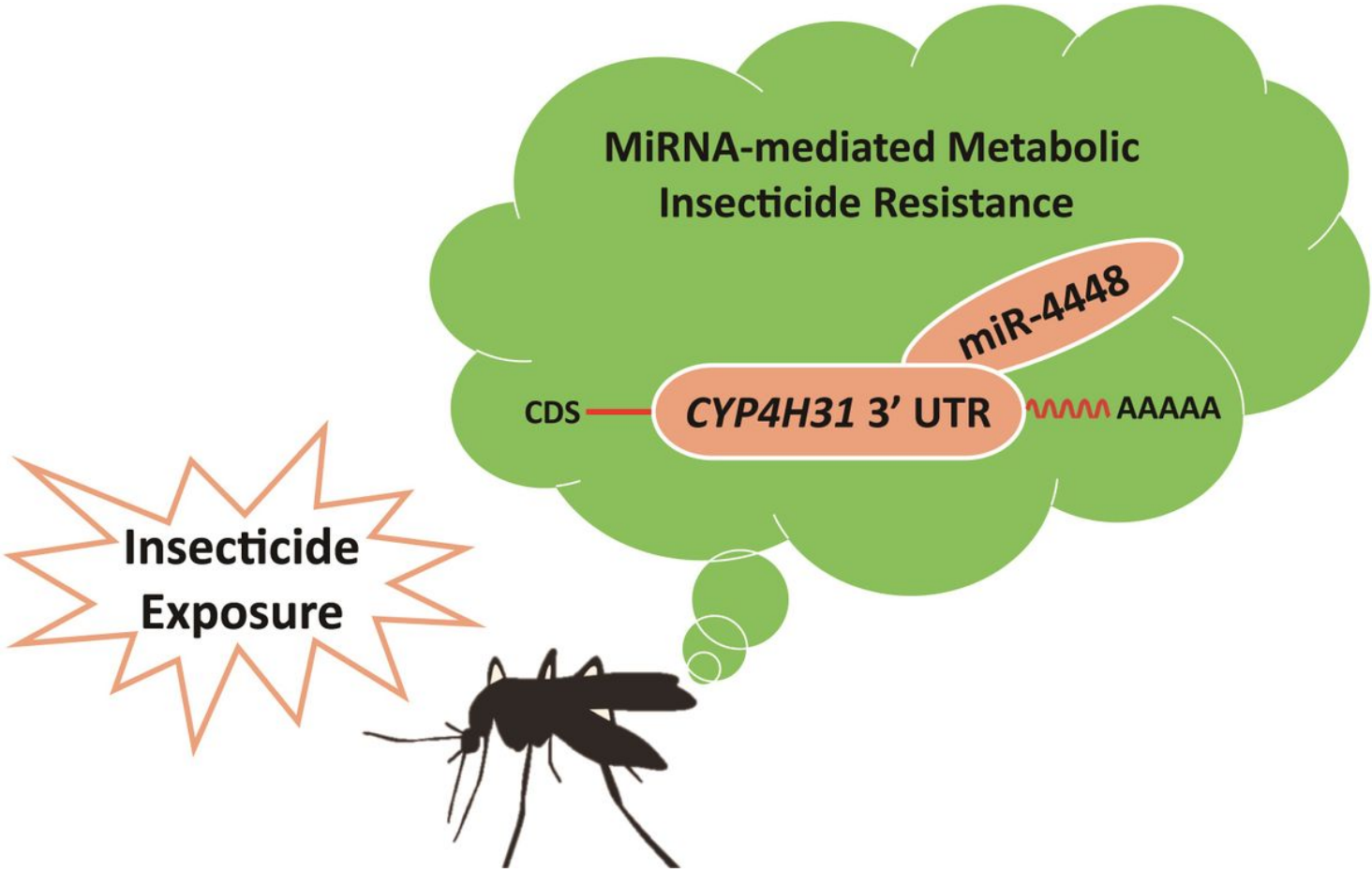
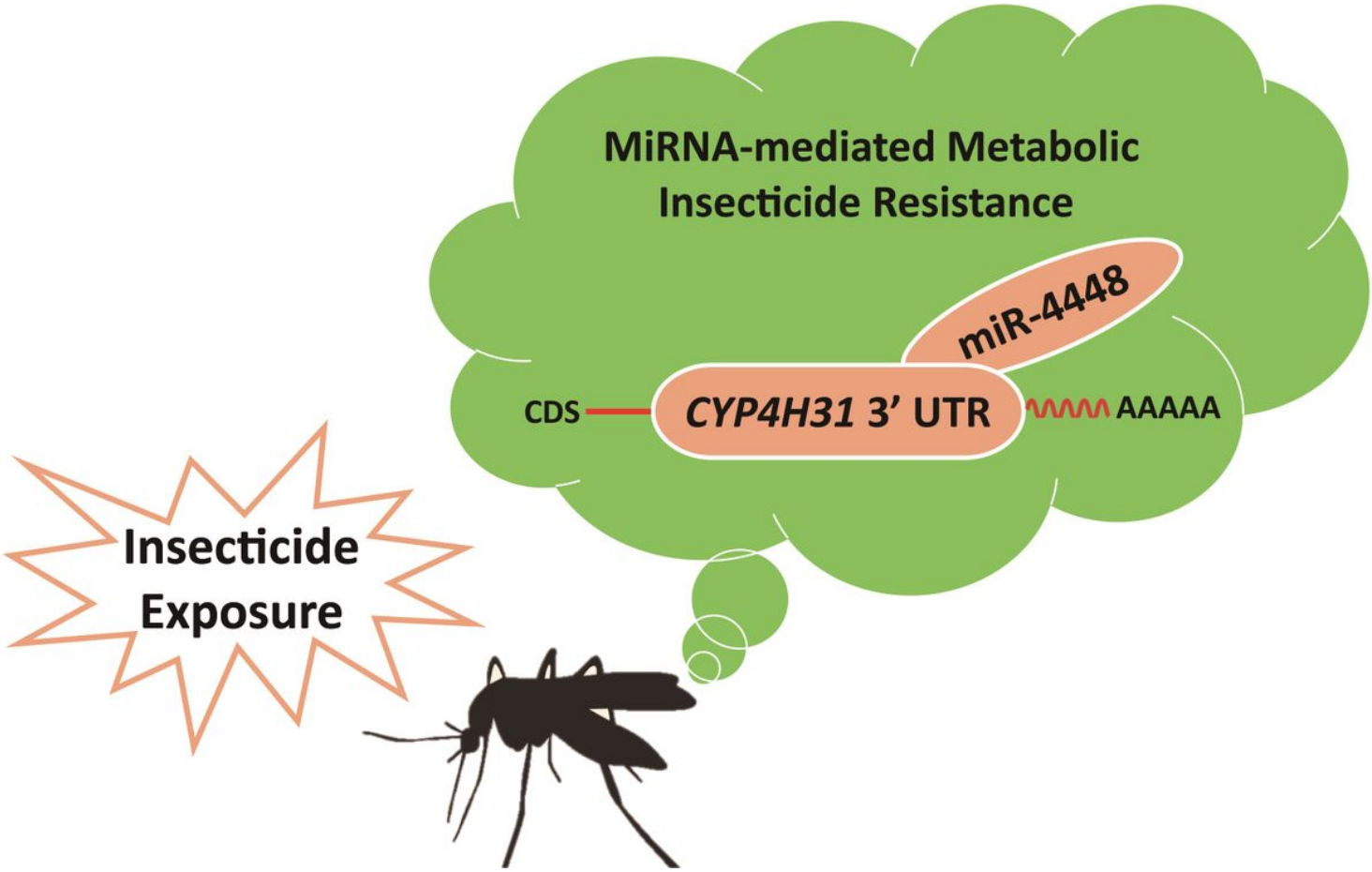


Figure 7

The regulatory map of miR-4448's involvement in deltamethrin resistance by targeting CYP4H31 in Culex pipiens pallens.



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