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Comparative study of transferrin gene expression level from different strains of anopheles sinensis

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Abstract :Quantitative RT-PCR analysis indicated that the Anopheles sinensis transferrin transcription level was 11 .5-fold higher in deltamethrin resistant strain and 8 .3 fold higher in local collected strain than that of sensitive strain .The maximal level of transferrin mRNA was detected in adult female mosquito ,followed by the 3rd instar larvae and the egg .Anopheles sinensis deltamethrin resistant strain and local collected strain have 3-to 4-fold increase of transferrin transcription compared to sensitive strain .These results indicated the transferrin express level was enhanced synchronously with the deltamethrin resistance level of Anopheles sinensis ,and transferrin may confer some deltamethrin resistance in Anopheles sinensis .

Key words :Anopheles sinensis ;Transferrin ;Deltamethrin ;Resistance
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中华按蚊不同株转铁蛋白基因表达水平的比较研究

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摘 要 定量 RT-PCR 分析表明,中华按蚊溴氰菊酯抗性品系的转铁蛋白转录水平比敏感品系高 11 .5 倍,现场采集品系比敏感品系高 8 .3 倍。不同发育阶段的基因表达差异分析表明,成年雌蚊转铁蛋白 mRNA 的表达水平最高,其次是三龄幼虫和卵。中华按蚊溴氰菊酯抗性品系和本地收集的菌株有 3 到 4 倍的增加相比,和敏感品系相比,中华按蚊溴氰菊酯抗性品系和现场采集品系的转铁蛋白表达水平均有 3 到 4 倍的增加。这些结果表明,转铁蛋白的表达水平与中华按蚊溴氰菊酯的抗性水平的提高有同步性,提示转铁蛋白对中华按蚊溴氰菊酯对杀虫剂的抗性形成具有一定的作用。

关键词 中华按蚊;转铁蛋白;溴氰菊酯;抗药性

1 Introduction

Mosquitoes are not only the cause of nuisance by their bites but also transmit deadly diseases like malaria ,filariasis ,yellow fever ,dengue ,and Japanese encephalitis[1] . Fighting these diseases takes many efforts including pesticide research ,vaccine development and mosquito control .Insecticides play a central role in controlling mosquitoes .Unfortunately ,resistance to insecticides has appeared in mosquitoes more and more seriously ,and against every chemical class of insecticides . Insecticide resistance jeopardizes mosquito-borne disease control ,and creates a major public health concern[2] .

Pyrethroids such as deltamethrin are a group of chemicals interact with insect ion channels causing a disruption to transmembrane potentials ,and then interfere with the normal functions of the insect nerve system .Deltamethrin is commonly used for the treatment of bed nets and as a residual spray to help control malaria transmission . Unfortunately the wide spread use and incorrect application of deltamethrin and other synthetic pyrethroids have accelerated the emergence of resistance in both targeted Anopheles species and off-target species such as Anopheles sinensis[3] . The elucidation of the resistance mechanism be-

comes important to guide the use of deltamethrin and the development of its substitutes .

To study the mechanism of deltamethrin resistance ,a resistant strain of Anopheles sinensis , An-D ,has been previously established in laboratory of Shandong Institute of Parasitic Diseases by selecting stadium larvae with increasing concentrations of deltamethrin over 42 generations ,and the deltamethrin-resistant level is 160 .43 times higher than that of sensitive strain .Two-dimensional electrophoresis was used to screen differences in protein expression between An-D strain and sensitive strain (An-S) of Anopheles sinensis .One of the identified protein segment has high sequence homology with part of Anopheles Sinensis transferrin protein sequence .Transferrin delivers iron to many cells by way of a membrane transferrin receptor ,which has a high affinity for diferric transferrin ,less affinity for the monoferric form ,and low affinity for apotransferrin at physiological pH .Transferrin has remained highly conserved during evolution .Iron is delivered to tissues by circulating transferrin ,a transporter that captures iron released into the plasma mainly from intestinal enterocytes or reticuloendothelial macrophages .The binding of iron-laden transferrin to the cell-surface transferrin receptor 1 results in endocytosis and uptake of the metal cargo[4] .When diferric transferrin bound to its membrane receptor is taken into an endocytotic vesicle that becomes an acidic endosome ,iron dissociates from transferrin and is taken into the cell cytoplasm ,whereas the transferrin receptor and apotransferrin are recycled to the plasma membrane , where the apotransferrin is released into the blood .But the correlation between the function of transferrin and insecticide resistance of Anopheles sinensis has not been reported to date .

In the present study , Real-time quantitative RT-PCR indicated that this gene is transcribed to a greater extent in the An-D strain than in the An-S strain and local strain (An-L) of Anopheles sinensis .We also established the expression profile of the gene in the mosquito life cycle . The transferring expression level increased combined with the deltamethrin resistance level in different strains of Anopheles sinensis indicated that the transferrin gene involved in the deltamethrin resistance mechanism of Anopheles sinensis .

2 Materials and methods

2 .1 Mosquitoes

An-D , An-S and An-L strain used in this study were reared at 28℃ ,with 70% ～80% humidity and a constant light :dark cycle (14 :10) . The mosquitoes were fed with mouse blood .The An-D colony has been selected from a susceptible strain ,and the resistance has been maintained by treatment with deltamethrin at LC50 of each generation .The LC_{50} of An-D strain is 6 .71mg/L , 163-fold greater than that in the susceptible strain (0 .041mg/L) . An-L strain was collected from Tangkou town of Jining ,Shandong province .

2 .2 Real-time quantitative RT-PCR analysis

Total RNA was extracted from approximately 20 mg of 1st ,2nd ,3rd and 4th instar larvae and female adult Anopheles sinensis mosquitoes ,using Trizol reagent (Gibco BRL , Grand Island , NY , USA) according to the manufacturer's protocol .

Real-time quantitative RT-PCR was done using a LightCycler-RNA amplification Kit syBR Green I (Roche ,Germany) .The reaction was repeated using three independently purified RNA samples ,and threshold cycle number was determined using LightCycler software version . Two pairs of primer were designed for this experiment : transferrin (forward : 5′-TACAAATTGAAGCCCATCC-3′,reverse :5′-AAATAAGCCGCACCACC-3′) and β-actin (forward :5′-AGCGTGAACTGACGGCTCTTG-3′, reverse : 5′-ACTCGTCGTACTCCTGCTTGG-3′) .To confirm the accuracy and reproducibility of real-time quantitative RT-PCR ,the experiment was determined in three repeats within one LightCycler run .The results of transferrin were normalized to housekeeping β-actin gene .Overexpression fold was calculated according to the $2^{(Rt-Et)}=2^{(Rn-En)}$ formula ,where Rt is the threshold cycle number for

the reference gene in the An-D strain , Et is the threshold cycle number for the experimental gene in the An-D strain , Rn is the threshold cycle number for the reference gene in the susceptible strain and En is the threshold cycle number for the experimental gene in the susceptible strain . Sample that had expression lever five-fold was considered overexpressed .

2 .3 Semi-quantitative RT-PCR analysis of different life cycle

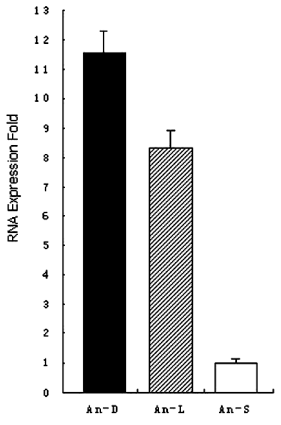
RT-PCR was also done with samples isolated from An-D , An-L and An-S strains of egg , 1st , 2nd , 3rd and 4th instar larvae , pupa and female adult mosquitoes to confirm the expression levels at each developmental stage using the primers described above . Mosquito β-actin gene cDNA was amplified by PCR using the forward primer 5′-TACGGTGGTGCGGCTTAT-3′, and the reverse primer 5′-CAGGGTGAAATCTGATGGTT-3′.

The PCR conditions were : 95 ℃ for 5min followed by 25 cycles of 95℃ for 40 s , 53℃ for 40s , 72 ℃ for 1min with a final 10-min extension at 72 ℃ . The transferrin and β-actin gene PCR products were resolved by electrophoresis on 1% agarose gels . Gels were photographed using Molecular Analyst 1 .4 .1 (Bio-Rad , Hercules , USA) and the images were analyzed by using BandScan 5 .1 software . The magnitude of transferrin expression in An-D strain compared to the deltamethrin sensitive strain was calculated by the following formula : (RL/Rb)/ (SL/Sb) . RL is the band intensity of An-D strain transferrin ; Rb is the band intensity of An-D strain β-actin ; SL is the band intensity of susceptible strain transferrin ; Sb is the band intensity of susceptible strain β-actin .

3 Results

3 .1 Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was used to analysis the amplification fold of transferrin among An-D , An-L and An-S strains of Anopheles sinensis . The cycle number of transferrin at which the amplification reached the threshold was normalized against β-actin cycle number to determine the relative copy numbers among An-D , An-L and An-S strains of Anopheles sinensis . The transferrin expression level exhibited 11 .5-fold higher in An-D strain and 8 .3 fold higher in An-L strain than that of An-S strain . The results suggested the transferrin expression level had the correlation with the deltamethrin resistant level in Anopheles sinensis . (Fig .1) .

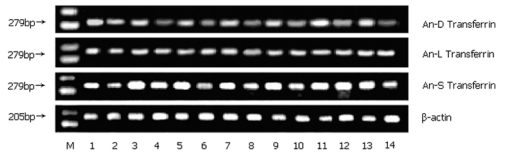


An-D : Anopheles sinensis .resistant strain ;
An-L : Anopheles sinensis .Local strain ;
An-S : Anopheles sinensis .sensitive strain ;

Fig .1 Quantitative RT-PCR assay of Transferrin mRNA in Cr-C and susceptible strains of Anopheles sinensis

3 .2 Expression of transferrin gene at various developmental stages of An-D , An-L and An-S strains of Anopheles sinensis

Transferrin stage-specific expression was determined by semi-quantitative RT-PCR with different tissue RNAs , including those from egg , 1st , 2nd , 3rd and 4th instar larvae , pupa and adult females from above strains of Anopheles sinensis . A 279-bp cDNA fragment was selectively amplified using the specific primers . The amount of amplified cDNA product was normalized by comparison with the amplification product of the β-actin gene from Anopheles sinensis (205 bp) . The maximal level of transferrin mRNA was detected in adult female mosquito , followed by the 3rd instar larvae and the egg . In all of the developmental stages , An-D and An-L strain had 3-to 4-fold increase of transferrin transcription compared to An-S strain (Fig .2) .



M ,marker ;1 ,Cr-C strain egg ;2 ,susceptible strain egg ;
3 ,Cr-C strain 1st larvae ;4 ,susceptible strain 1st larvae ;
5 ,Cr-C strain 2nd larvae ;6 ,susceptible strain 2nd larvae ;
7 ,Cr-C strain 3rd larvae ;8 ,susceptible strain 3rd larvae ;
9 ,Cr-C strain 4th larvae ;10 ,susceptible strain 4th larvae ;
11 ,Cr-C strain pupa ;12 ,susceptible strain pupa ;
13 ,Cr-C strain female mosquito ;
14 ,susceptible strain female mosquito .

Fig .2 Semi-quantitative RT-PCR of each life stage showed the relative amount of amplified transcripts of transferrin (panel 1 ,2 ,3) in comparison with amplified mosquito b-actin gene transcripts (panel 4)

4 Discussion

Insecticide resistance has become a serious public health problem ,which limited the effectiveness of pest control and presented an obstacle to the controlling of vector borne diseases[5] . Here we reported the characterization of one deltamethrin resistance associated gene of Anopheles sinensis . The writer reported insecticide-resistance ribosomal protein L39 gene of Cx . pipiens pallens[6] ,while other researchers in the same laboratory identified several genes differentially expressed in permethrin-resistant strain of the mosquito previously[7-9] .Some of the candidate genes encode for enzymes such as chymotrypsin[10] , trypsin[11] ,CYP450[12] which are likely involved in more direct functions , e . g . oxidation of deltamethrin ,others are likely more upstream factors , such as ribosomal protein L22[8] and ribosomal protein S4 . The predicted Anopheles sinensis transferrin protein has many characteristics common to the transferrin family , such as a typical transferrin domain and the first 19 amino acid signal peptide sequence . The Anopheles sinensis transferrin protein sequence shared 95 .74% identity with that of Cx . anthropophagus . These results strongly suggest that this protein is the transferrin of Anopheles sinensis

Iron is an essential metal not only in oxygen delivery , but also in cell proliferation and drug metabolism ,while it is a very toxic metal producing reactive oxygen species (ROS) .In order to avoid the toxicity and shortage of iron ,the level of iron is strictly regulated in the body and cells .The amount of cellular iron is regulated by the IRE (iron responsive element) and IRP (iron regulatory protein) system .The most common inherited sideroblastic anemia is X-linked sideroblastic anemia (XLSA) caused by mutations of the erythroid-specific delta-aminolevulinate synthase gene (ALAS2), which is the first enzyme involved in heme biosynthesis in erythroid cells . However , there are still significant numbers of cases with genetically undefined , inherited sideroblastic anemia . Molecular analysis of these cases will contribute to the understanding of mitochondrial iron metabolism .

The transferrin family spans both vertebrates and invertebrates . It includes serum transferrin , ovotransferrin , lactoferrin , melanotransferrin , inhibitor of carbonic anhydrase , saxiphilin , the major yolk protein in sea urchins , the crayfish protein , pacifastin , and a protein from green algae . Most (but not all) contain two domains of around 340 residues , which are thought to have evolved from an ancient duplication event . For serum transferrin , ovotransferrin and lactoferrin each of the duplicated lobes binds one atom of Fe (III) and one carbonate anion . With a few notable exceptions each iron atom is coordinated to four conserved amino acid residues ; an aspartic acid , two tyrosines , and a histidine , while anion binding is associated with an arginine and a threonine in close proximity . These six residues in each lobe were examined for their evolutionary conservation in the homologous N-and C-lobes of 82 complete transferrin sequences from 61 different species . Of the ligands in the N-lobe , the histidine ligand shows the most variability in sequence . Also , of note , four of the twelve insect transferrins have glutamic acid substituted for aspartic acid in the N-lobe (as seen in the bacterial ferric binding proteins) . In addition , there is a wide spread substitu-

tion of lysine for the anion binding arginine in the N-lobe in many organisms including all of the fish ,the sea squirt and many of the unusual family members .No association between transferrin and pyrethroid resistance was established before . In this study ,we found higher transcriptional level of transferrin in the An-D strain than in the susceptible strain .We determined the expression profile of transferrin mRNA during the mosquito life cycle ,and the results demonstrated that the levels of transferrin expression are developmentally regulated ,and the expression was the highest in adult mosquito .Ectopic expression of transferrin in C3/36 cells also increases resistance against deltamethrin .The up-regulation of transferrin in adult mosquito may be indicative of an adaptive ability of the mosquito to regulate the synthesis of other insecticide resistance proteins and consequently to have enhanced resistance to insecticides .Although we do not have enough information to pinpoint the exact role of transferrin in deltamethrin resistance ,our results suggest that transferrin is a good candidate for future studies of pyrethroid resistance .

In this study ,real-time quantitative RT-PCR proved that the transferring gene has strong relationship with the deltamethrin resistance of Anopheles sinensis .The expression profile study of transferring gene in the Anopheles sinensis life cycle proved the increased expression level of this gene were combined with the high deltamethrin resistance level in different strains of Anopheles sinensis .The data in the present study suggests that transferrin may confer some deltamethrin resistance in Anopheles sinensis . Research carried out to date has provided a basis for further studies on the gene function associated with insecticide resistance ,which will improve our understanding of the molecular basis of transferrin mediated resistance in Anopheles sinensis .

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