Glucose transporter Glut1 influences Plasmodium berghei infection in Anopheles stephensi

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Research

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Abstract

Background Sugar feeding provides energy for mosquito. Facilitated glucose transporters (GLUT) are responsible for cellular uptake of glucose. However, the knowledge of GLUT function in *Anopheles* mosquito is limited. **Methods** Phylogenetic analysis of GLUTs in *Anopheles stephensi* (AsteGlut) was performed by the maximum likelihood and Bayesian method. The spatial and temporal expression patterns of three AsteGluts were analyzed by qPCR. The function of AsteGlut1 was examined using a dsRNA-mediated RNA interference method. Transcriptome analysis was used to understand the influence of AsteGlut1 on mosquito vector competence. **Results** We identified 3 glut genes, AsteGlut1, AsteGlut2 and AsteGlut3 in *An. stephensi*. AsteGlut1 and AsteGlut3 were mainly localized in the midgut. The expression of all three AsteGluts were strongly induced after blood meal. All three genes were knocked down successfully, but only abrogation of AsteGlut1 significantly increased the susceptibility of *An. stephensi* to *Plasmodium berghei* infection. Our transcriptome analysis revealed that knockdown of AsteGlut1 differentially regulated expression of genes associated with the functional clusters including detoxification, serine proteases, and immunity. The dysregulation of multiple pathways might contribute to the increased *P. berghei* infection. **Conclusions** Our study shows that AsteGlut1 plays a role in defense against *P. berghei* in *An. stephensi*. The regulation of AsteGlut1 on vector competence might through modulating multiple biological processes, including detoxification and immunity.

Background

Anopheles mosquitoes transmit *Plasmodium* parasites that kill over 450,000 people annually [1]. To transmit to the next mammalian host, malaria parasite has to complete multiple development stages in mosquito including gametogenesis, fertilization, zygote-to-ookinete conversion and oocyst formation [2]. During this process, complicated interactions between *Anopheles* mosquito and *Plasmodium* parasite occur [3]. The nutrient availability is one of the key factors that determine the infection outcome [4, 5]. Sugar affects the survival and fecundity of mosquitoes [6, 7, 8, 9]. Trehalose, the main circulating sugar, is a nonreducing disaccharide composed of two glucose molecules linked by an α - α -1,1-glycosidic bond. It enters cell metabolism after catabolized into glucose [10, 11]. Trehalose transporter AgTreT1 is responsible for the transportation of trehalose from fat body to hemolymph [12]. Knocking down of TreT1 leads to the reduction of hemolymph trehalose and inhibition of *Plasmodium falciparum* infection [12].

Glucose is the primary source of energy for both mosquito and *Plasmodium* [13, 14]. During the blood stage and liver stage of malaria infection, the *Plasmodium* parasites drive host cells to absorb more glucose by enhancing the translocation of GLUT1 to the cell membrane of infected cells [15, 16]. Subsequently, glucose is transported intracellularly by *Plasmodium* facilitative hexose transporter (PfHT) [17]. In mosquito, the function of only one glucose transporter, *AGAP007752*, is characterized. Blocking *AGAP007752* in mosquito salivary glands inhibits *Plasmodium* sporozoite infection [18, 19, 20]. However, the function of other GLUTs remains unknown.

In this study, we identified three *Asteglut* genes in *An. stephensi*. RNAi-mediated silencing of *Asteglut1* specifically increased *P. berghei* infection and promoted the midgut intake of glucose. The regulation of *Asteglut1* on vector competence might through modulating multiple biological processes, including detoxification, and immunity.

Methods

Mosquito rearing and maintenance

Anopheles stephensi (strain Hor) was reared at 28°C, 80% relative humidity. Adults were maintained on 2% sucrose solution. Adult female mosquitoes were fed on BALB/c mice for a blood meal.

Plasmodium berghei infection

Plasmodium berghei ANKA parasites were maintained by passing through BALB/c mice by mosquito biting [21]. When parasitemia of *P. berghei* infected mice rises to 3%~6%, mosquitoes starved overnight were allowed to feed on it. Engorged mosquitoes were maintained at 20 °C and unengorged ones were removed 24 h post blood meal. Midguts were dissected and oocyst number were counted under fluorescence microscope 8 days post infection.

Phylogenetic analysis

Sequences were aligned using the default settings in MEGA X software [22]. Phylogenetic tree was constructed using the Maximum Likelihood method based on a bootstrapping method with 1000 replicate. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Twenty five protein sequences were included in Phylogenetic analysis. The sequences were obtained from database under the accession numbers: 3 glucose transporters of *Anopheles stephensi*, ASTE005839, ASTE003001 and ASTE008063; 3 homologous sugar transporters of *Anopheles gambiae*, AGAP007340, AGAP005238 and AGAP003020; 3 homologous sugar transporters of *Aedes aegypti*, AAEL020018, AAEL006264 and AAEL010868; 3 homologous sugar transporters of *Drosophila melanogaster*, GLUT1 (FBpp0305693), SUT-1 (FBpp0087855) and MFST (FBpp0075675). And 13 glucose transporters of *Homo sapiens*, GLUT1 (NP_006507.2), GLUT2 (XP_011511389.1), GLUT3 (NP_008862.1), GLUT4 (AAI13593.1), GLUT5 (NP_001315548.1), GLUT6 (XP_016869725.1), GLUT7 (XP_011539126.1), GLUT8 (XP_011516904.1), GLUT9 (XP_011512158.1), GLUT10 (XP_011527362.1), GLUT11 (NP_110434.3), GLUT12 (XP_006715412.1) and GLUT14 (XP_024304616.1).

RNA interference

The *Asteglut* genes were amplified by the corresponding primers. Asteglut1, F:5'-ACA GTA CAA CAG GTG AAG GAA GAG-3' and R: 5'-GTA ATC CTA CGG TCA CAG CCA AT-3', Asteglutx, F:5'-GCT GTC AGG AAT CAA TGC CGT CTT-3' and R:5'-CGC CAC CTC CGT TAC CTC TTG-3'; Asteglut3, F:5'- GCA TTG TTG AGC CAG CCC AAA-3' and R:5'-CTG CCT CGC CTA GTC CAT TCC-3'. Primers with T7 promoter sequence (TAATACGACTCACTATAGGG) were used to generate templates for double-stranded RNA (dsRNA) synthesis using the MEGAscript RNA kit (Ambio, Invitrogen, Shanghai, China). The plasmid eGFP (BD Biosciences, Shanghai, China) was used as controls. Four days-old mosquitoes were injected with 69 nl dsRNA (4 µg/µl) using a nanoject II microinjector (Drummond, City, USA, America). The dsRNA-treated mosquitoes were collected two days post-treatment and knocking down efficiency was verified by qPCR as previously described [23].

RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

Total RNA was extracted from mosquitoes using TRIzol reagent (Sigma-Aldrich, Shanghai, China) according to the manufacturer's protocol. One µg of total RNA was used to synthesize cDNA using 5' All-in-One MasterMix (AccuRT Genomic DNA Removal Kit, ABM, Shanghai, China). The qPCR was performed by Roche LightCycler 96 Real Time PCR Detection System using SYBR Green qPCR Master Mix (Biomake, Shanghai, China) according to a previously described protocol [23]. The data were processed and analyzed with LightCycler 96 software. Ribosomal gene *s7* was used as the internal reference gene.

Sugar measurement

The glucose and trehalose level in mosquito hemolymph and midgut were examined as described [12, 24]. Briefly, 30 µl hemolymph was collected from 10 mosquitoes. Ten midguts were pooled together and homogenized in 30 µl PBS buffer. 30 µl of midgut homogenates were collected for glucose and trehalose measurement. One third volume of each sample was taken to measure the glucose level with the Glucose Kit (Megazyme; K-GLUC, Bray, Ireland). The second one third volume of the sample was first treated with trehalase enzyme (Megazyme; K-TREH, Bray, Ireland), and then examined for glucose concentration. Trehalose concentration was calculated as described [12]. The remaining one third volume of the sample was used to extract and quantify the amount of genomic DNA [25]. The measured concentration of glucose and trehalose were normalized to the amount of genomic DNA, respectively.

RNA Sequencing

Mosquitoes treated with *dsAsteglut1* and *dsGFP* 24 hours post infectious blood meal were collected for RNA sequencing. Four mosquitoes were pooled for one sample and three biological replicates were prepared from each treatment. Total RNA was extracted using TRIzol® Reagent according the

manufacturer's instructions (Sigma-Aldrich, Shanghai, China) and sent to Majorbio (Shanghai, China) for library construction and sequencing using Illumina HiSeq 10. Clean data were aligned to the reference genome AsteS1.6 (<https://www.vectorbase.org/organisms/anopheles-stephensi>). To identify DEGs (differential expression genes) between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method [26]. R statistical package software EdgeR (Empirical analysis of Digital Gene Expression in R, <http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>) was used for differential expression analysis[27]. GO functional enrichment were carried out by Goatools (<https://github.com/tanghaibao/Goatools>). Heat map and Venn map are performed by TBtools software (v0.66831, <https://github.com/CJ-Chen/TBtools-Manual>)[28]. Pie chart, scatter chart and histogram were performed by GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). Raw RNA-Seq sequencing data has been uploaded to the National Center for Biotechnology Information's Sequence Read Archive (Accession no. PRJNA597441).

Statistical Analysis

All statistical analyses were performed by GraphPad Prism version 6. Statistics of gene expression and sugar levels were determined using the Student's *t*-test. The Mann-Whitney test was used to determine the significance of oocyst intensities of dsRNA- treated mosquitoes.

Results

The phylogenetic analysis of glucose transporters in *Anopheles stephensi*

There are three genes annotated as glucose transporter, ASTE005839, ASTE003001 and ASTE008063 in the database of *An. stephensi* gene (AsteS1.6). To investigate the phylogenetic relationships of these proteins between *An. stephensi* and other organisms, a phylogenetic tree was constructed based on the amino acid sequence of known glucose transporters from *Anopheles gambiae*, *Anopheles stephensi*, *Aedes aegypti*, *Drosophila melanogaster* and *Homo sapiens* by the maximum likelihood and Bayesian phylogenetic analyses (Fig. 1). The family of GLUT transporters known in humans can be divided into three classes, class 1 (GLUT1, GLUT2, GLUT3, GLUT4 and GLUT14), class 2 (GLUT5, GLUT7, GLUT9, and GLUT11) and class 3 (GLUT6, GLUT8, GLUT10 and GLUT12)[29, 30, 31, 32]. Amino acid sequence of ASTE005839 has the greatest similarity to the GLUT1 of *Drosophila melanogaster* (FBpp0305693) and *Homo sapiens* (NP 006507.2) that belong to class1. We herein named it *Asteglut1*. While ASTE008063 was categorized into GLUT- class3, and ASTE003001 was not phylogenetically close to GLUT- class1 or GLUT- class 2, so we named it *Asteglut3* and *Asteglutx*, respectively (Fig 1).

Expression pattern of *Astegluts* in *An. stephensi*

To determine the expression pattern of *Astegluts* in *An. stephensi*. We analyzed the expression level of these genes in the midgut and carcass 24 h before and after infectious blood meal by qPCR, respectively. *Asteglut1* and *Asteglut3* were mainly localized in the midgut tissue of *An. stephensi* (Fig. 2A and 2C). *Asteglutx* was distributed in both midgut and carcass (Fig. 2B). Infectious blood meal stimulated the expression of all three genes (Fig. 2A to C).

Knockdown of *Asteglut1* facilitates *Plasmodium berghei* infection in *An. stephensi*

To investigate the role of *Asteglut1*, *Asteglutx* and *Asteglut3* in the susceptibility of *A. stephensi* to *P. berghei*, double-stranded RNA (dsRNA)-mediated silencing was employed. The expression level of *dsAsteglut1*, *dsAsteglutx* and *dsAsteglut3* was examined two days post dsRNA treatment. The expression level of these genes were significantly decreased by 57.8% ($P=0.02$), 40% ($P<0.0001$) and 65% ($P=0.0002$) comparing to *dsGFP* control, respectively (Fig 3A to C). Silencing *Asteglut1* led to a significant increase in the number of oocysts of *P. berghei*, while *dsAsteglutx* and *dsAsteglut3* treatments had no apparent effect on malaria infection (Fig 3D to F). Such effect was due to the specific knockdown of *Asteglut1* instead of the compensatory expression of other *Astegluts* because silencing *Asteglut1* didn't change the expression level of either *Asteglutx* or *AteGlut3* (Fig 3G & H).

Role of *Asteglut1* in maintaining mosquito hemolymph glucose homeostasis

We next analyzed the influence of *Asteglut1* on glucose level in *An. stephensi*. The glucose and trehalose levels in the midgut and hemolymph of dsRNA treated mosquitoes were examined. The glucose level of *Asteglut1*-knockdown group was significantly higher than that in dsGFP controls before blood-feeding (Fig. 4A). However, its level in hemolymph is comparable to that in dsGFP (Fig. 4C). Knocking down of *Asteglut1* didn't change the level trehalose either in midgut or in hemolymph (Fig. 4B&D). Thus, *Asteglut1* might play a role in maintenance of glucose homeostasis in mosquito midgut.

Transcriptional analysis of *Asteglut1*-knockdown mosquitoes

To explore the mechanism that could be responsible for the influence on *P. berghei* infection, we performed a transcriptome analysis of mosquito's midgut microinjected with *dsAsteglut1* and *dsGFP* 24 h post blood-meal, respectively. The Venn diagram shows that the expression of 10240 genes were overlapped in the two groups (Fig. 5A). A total 46 genes were differentially expressed (Fig. 5B, Table S1) with 26 up-regulated and 20 down-regulated. These differentially expressed products were belong to the functional clusters including cytoskeletal and structural, immunity, metabolism, proteolysis, redox, transport and unknown function (Fig. 5C).

Among the 'redox' functional cluster, five genes encoding cytochrome P450 (CYP450) were upregulated, indicating that the detoxification mechanism was activated in mosquito [33]. Gene encoding peroxiredoxin was significantly up-regulated, peroxiredoxin is known to control cytokine-induced peroxide levels in cells [34]. However, DUOX, which has been widely reported to be associated with *Plasmodium* elimination, showed significant down-regulated expression [35].

A cluster containing numerous differentially expressed genes is belong to 'proteolysis' in *dsAsteglut1* treated mosquitoes. The class of serine proteases, also known as CLIP family, was considered to be involved in immune response, among them two genes were found to be up-regulated while two were down-regulated. The down-regulated transcript genes under this GO term was the CLIPB2 and CLIPB19, while the upregulated gene was CLIPB3 [36, 37]. Two genes encoding allantoinase (ASTE005468 and ASTE014323) were significantly up-regulated, and these genes are involved in the process of nitrogenous waste in mosquitoes [38].

In the cluster associated with the immune response, *caudal*, the negative regulator of Imd pathway was significantly up-regulated [39]. There were 4 genes related to innate immune responses in mosquitoes, the peptidoglycan recognition proteins, *pgrp-la*, *-lc*, *-ld*, and the antimicrobial peptides, *defensin* were significantly down-regulated [23, 39, 40, 41, 42].

Discussion

The glucose transporter family, functionally conserved from insects to mammals, plays a role in transporting glucose across the plasma membrane in all cells [43]. In mammals, GLUT1 is one of the earliest cloned membrane transporters and has been widely studied in the past half century [44]. It is a ubiquitous glucose transporter in skeletal, muscle, heart, and other tissues, but predominantly functions in erythrocytes and blood–brain barrier [31, 44, 45]. In *An. stephensi*, we identify 3 *glut* genes, *Asteglut1*, *Asteglut3*, and *AsteglutX*. They are differentially regulated in response to blood feeding in *An. stephensi*, suggesting its potential role in regulating glucose transportation. We also notice that knockdown of *Asteglut1* caused the increase of glucose level in midgut, further confirming that its role in maintaining the homeostasis of intestinal blood glucose. However, we did not observe significant changes of glucose and trehalose level in the hemolymph. It is possible that sugar level in hemolymph is controlled by multiple factors and functional redundancy exists between members of the AsteGlut family.

In addition, we also find that knocking down *Asteglut1* influences mosquito's susceptibility to *P. berghei* infection. In agreement with our findings, GLUT1 is also involved in the regulation of pathogen infection. For example, GLUT1 is a receptor of human *T lymphovirus*, which plays an important role in the activation of CD4 T cell [46, 47]. In plant, sugar transporter can also affect the growth of pathogens by manipulating glucose metabolism [48]. In *An. stephensi*, invasion of *P. berghei* into salivary glands induces the expression of solute carrier proteins (SLCs) *prestinA* and *NDAE1* that transport solutes, such as ions and metabolites maintain ionic homeostasis using ion or electrochemical gradients [49]. Knockdown of both genes reduces the burden of *P. berghei* sporozoites in the salivary glands. [18, 19, 20].

The influence of AsteGlut1 on parasite infection might through the modulation of intestinal glucose homeostasis, metabolism and immune response. Our transcriptome analysis reveals that a considerable number of up-regulated genes are cytochrome P450, which is involved in detoxification. It is highly possible that mosquitoes of which *Asteglut1* is knocked down suffered more severe parasite mediated toxicity than that in controls [50, 51]. The role of CLIP family functions as activators or suppressors of the melanization process responsible for elimination of *P. berghei* in *An. gambiae* [37]. CLIPB3 is one of the suppressors of melanization pathway that inhibits *P. berghei* infection in *An. gambiae* [37]. Our transcriptome data show that dsAsteGlut1 treatment upregulates expression of CLIPB3, suggesting that the regulation of AsteGlut1 on CLIPB3 might contribute to the elevated *P. berghei* infection levels.

Notably, genes related to immune deficiency (Imd) signal pathway are differentially regulated. The upregulation of the negative regulator, *caudal*, and the downregulation of receptors, peptidoglycan recognition proteins, *pgrp-la*, *-lc*, *-ld*, and the antimicrobial peptides, *defensin* suggest that Glut1 might influence mosquito's susceptibility to *P. berghei* infection through regulating immune responses [23, 39, 52]. However, how Glut1 regulates Imd pathway needs to be investigated in the future.

Conclusion

In summary, we identify 3 Glut members in *An. stephensi*. Asteglut1 influences the susceptibility of *An. stephensi* to *P. berghei* infection. The regulation of Asteglut1 on vector competence might through modulating multiple biological processes, including detoxification and immunity (Fig 6). Our findings pave the way for further understanding of how the sugar transporter regulate vector-parasite interaction and will explore new targets for developing control measures.

Abbreviations

AsteGlut: glucose transporter in *Anopheles stephensi*; CYP450: cytochrome P450; DUOX: dual oxidase; CLIP: class of serine proteases.

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

All animals were handled strictly in accordance with the guidelines of the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare, China. The research protocol was approved by the Institutional animal care and use committee, Department of Laboratory Animal Science, Fudan University, China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MF performed the experiments, drafted the manuscript and data analysis. MF and JW revised the manuscript. All authors read and approved the final manuscript.

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Additional File Legend

Additional file 1: Table S1. List of significantly differentially expressed genes.

Figures

Figure 1

Phylogenetic analysis of glucose transporters (GLUTs) in *An. stephensi* and other organisms. The genes involved in this study were marked with red stars. The family of GLUT transporters known in humans are divided into three classes, class 1 (blue box), class 2 (green box) and class 3 (red box).

Figure 2

Expression patterns of *Asteglut1*, *Asteglutx*, *Asteglut3* in *An. stephensi* before and after blood meal. Relative gene expression of *Asteglut1*, *Asteglutx*, *Asteglut3* in the midgut and carcass of mosquitoes before (-24 h) and after (+24 h) feeding with infectious blood meal respectively. Error bars indicate standard error of the mean (n = 8). Results from one of two independent experiments are shown.

Figure 3

Knockdown of Asteglut1, Asteglutx, Asteglut3 in *An. stephensi* and *P. berghei* oocysts in dsRNA treated mosquitoes. a, b, c Asteglut1, Asteglutx, Asteglut3 silencing efficiency. Relative expression level of Asteglut1, Asteglutx, Asteglut3 were normalized to that in dsGFP controls. Ribosomal gene s7 used as an internal control. Error bars indicate standard error of the mean (n=10). Results from one of three independent experiments are shown. Significance was determined by Student's -t- test. d, e, f Median number of oocyst in dsRNA treated mosquitoes. Each dot represents an individual mosquito and horizontal lines represent the medians. Results from one of three independent experiments are shown. Significance was determined by Mann-Whitney test. g, h Specificity of dsAsteglut1 treatment. Relative expression level of Asteglutx, Asteglut3 in dsAsteglut1 mosquitoes were normalized to that in dsGFPs. Error bars indicate standard error of the mean (n=10). Results from one of three independent experiments are shown. Significance was shown by Student's -t- test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 4

Role of Asteglut1 in maintaining mosquito glucose homeostasis. a The relative concentration of glucose (a and c) and trehalose (b and d) level in midgut and hemolymph of GFP or Asteglut1 dsRNA- injected *An. stephensi* 4 d after injection. Sugar concentrations were normalized to genomic DNA extracted from

Figure 6

Model of influence of dsAsteglut1 in *An. stephensi*. Abrogation of Asteglut1 significantly increased the susceptibility of *An. stephensi* to *Plasmodium berghei* infection. The regulation of Asteglut1 on vector competence might through modulating multiple biological processes, including detoxification and immunity.

Supplementary Files

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- [TableS1.xlsx](#)