Dynamics of Transferrin 2 (Tsf2) Expression in Various Developmental Stages of Culex quinquefasciatus

Vignesh Mariappan¹, Azhagu Rani Manoharan², Agieshkumar Balakrishna Pillai³

Abstract

Aim: Transferrin is an acute-phase protein known to be involved in insect immune responses. In the present study, we have assessed the transcript levels of transferrin 2 (Tsf2) in various developmental stages of vector mosquito Culex quinquefasciatus.

Materials and methods: Culex quinquefasciatus mosquitoes were reared in laboratory conditions. Total RNA was extracted from larvae, pupae, and adult mosquitoes using the trizol method. Complementary DNA (cDNA) was synthesized and utilized for gene expression analysis using quantitative real-time PCR (qRT-PCR) and the result was expressed in the percentage fold change.

Results: In this study, we observed an elevation in the fold change expression of Tsf2 in the larval to pupal stages followed by a decline in the fold of the adult stage. We speculate that the upregulation may be because of the microbial challenge or the aquatic stages are programmed to synthesis more Tsf2 to encounter the upcoming microbes present in the aquatic environment. However, the same should be ascertained by screening for the presence of microorganisms if any.

Conclusion: The study presents a dynamic change in the expression of Tsf2 during the developmental process of mosquito. The role of the protein in vector competence deserves further studies.

Keywords: Culex quinquefasciatus, Transferrin, Transferrin 2, Vector immunity.

SBV Journal of Basic, Clinical and Applied Health Science (2020): 10.5005/jp-journals-10082-02273

Introduction

Transferrin (Tsf) is an iron-binding glycoprotein with two iron binding sites and each located within a separate and similar lobe of protein.¹ In vertebrates, transferrin is predominantly employed in iron transport in the blood and finally utilized in heme production.¹,² It delivers iron to several cells via membrane transferrin receptor, which has a very low affinity toward apotransferrin, less affinity for the monoferric form, and a very high affinity for diferric transferrin at physiological pH. After binding to the membrane receptor, transferrin is taken inside the endocytotic vesicle, and iron dissociates from transferrin and is taken into the cytoplasm, while the transferrin receptor and apotransferrin are recycled in the plasma membrane, where the apotransferrin is released into the bloodstream.³ In vertebrates, serum transferrin is categorized as an acute-phase protein because, during the infection or stress condition, the level of transferrin will either rise or fall depending upon the animal host.⁴ For example, the human lactoferrin level is increased in plasma during infection and indicates that it might have an antibiotic role by interfering with the utilization of iron needed for pathogen proliferation.⁴ Similarly, ovotransferrin found in white avian eggs have protective action against the pathogen.⁵

Transferrin proteins, as well as complementary DNAs (cDNAs) encoding insect Tsfs, have been identified for several insect species.⁷ The role of transferrin and iron metabolism in insects and other organisms are studied very little, but enough information is known to indicate that there is a distinct difference between an insect and other organisms (specifically mammals). For example, insect transferrin has been demonstrated to play a vital role in iron transport and act as antibiotic agents, antioxidants, and vitellogenic protein.⁶ Similarly, transferrin can also contribute to the adaptation of insects to various stresses.⁸–¹¹ Transferrin 1 of Aedes aegypti and Drosophila melanogaster was found to be expressed in larval, pupal, and adult stage but not in embryo, with expression in Ae. aegypti were highly in the fat body where it is secreted in the hemolymph.¹²,¹³ About this line, in most organisms, iron-containing protein ferritin is stored in the cytoplasm while insect ferritin is deficient in the cytoplasm but found in the export pathway of cell and hemolymph.¹⁴ Later, transferrin 2 (Tsf2) was found in Ae. aegypti with low iron binding capacity due to the mutation in key amino acids in the binding site. Expression of these two genes in the adult female mosquitoes differs with the AatF1 expression elevated during 24-hour post blood meal and AatF2 expression elevated during 72-hour post blood meal when compared to sugar-fed mosquitoes.¹⁵ Other than Tsf1, two additional putative transferrins (Tsf2 and Tsf3) were identified in D. melanogaster. Similar, in Ae. aegypti and Anopheles gambiae, three additional putative transferrin have been discovered.¹⁶ Authors have earlier reported

© The Author(s). 2020 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted use, distribution, and non-commercial reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
the role of transferrin 1 (Tsf1) in the innate immune response of Culex quinquefasciatus to the filarial parasite Wuchereria bancrofti.\textsuperscript{17,18} This upregulation due to pathogen suggests that insect transferrin has a role in the vector immune system.\textsuperscript{4,13} However, to the best of our knowledge, no studies are available on Tsf2 in C. quinquefasciatus. Since the transferrin molecule could be a potential target for vector immunity the present study is designed to understand the expression dynamics of Tsf2 in filarial vector C. quinquefasciatus.

**Materials and Methods**

**Rearing of C. quinquefasciatus**

Culex quinquefasciatus egg rafts (field-collected) were placed in a tray containing tap water and incubated at room temperature, under covering conditions. The temperature of about 27–30°C provides a suitable condition for the growth of mosquito eggs. Mosquito eggs were fed with organic material for their essential growth for a certain period as prescribed by Kauffman et al.\textsuperscript{19} After 2 days, mosquito eggs transformed into the larval stage. The larvae pass through four larval instars, and toward the end of the fourth instar, they stop eating and undergo molting to give rise to pupae. After 36 hours (at 27°C), adults emerge from the pupal stage. It took 6–8 days for their complete development. Finally, the different developmental stages of C. quinquefasciatus, such as, larvae (L), pupae (P), and adults (A) were pooled stage-wise and stored in 1 mL of trizol –80°C until further use. All the samples were collected from one generation. The difference in expression patterns between developmental stages was observed in the present study.

**Vector Base and Primer Designing**

Culex quinquefasciatus mosquito’s Tsf2 gene sequence was searched in the vector base site (www.vectorbase.org). A particular sequence of Tsf2 gene sequences (XM001863377.1) was obtained and these sequences were used to design primers for quantitative real-time PCR (qRT-PCR). Primer 3 online software (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design the specific primer for both the gene sequence was searched in the database (http://www.ncbi.nlm.nih.gov/GenBank/). Tsf2 of different species (Table 2) was obtained and sequence similarity was analyzed using online CLUSTAL W software (https://www.genome.jp/tools-bin/clustalw).

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted using Trizol Method. Nanodrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) was used for assessing purity and concentration of RNA. One microgram of RNA was used to synthesize cDNA using PrimeScript RT Reagent Kit (Takara, Japan). The cDNA thus obtained was used to study the gene expression of specific endogenous genes (\(\beta\)-actin) and selected target gene (Tsf2) in a 40 cycle qRT-PCR.

**qRT-PCR Analysis**

The primers of the endogenous gene and target gene are listed in Table 1. All reactions were performed in duplicates with a standard run protocol of initial denaturation at 95°C for 30 seconds followed by 40 cycles of denaturation (95°C for 10 seconds) and combined annealing and extension (60°C for 30 seconds). Fold change between the groups was determined using 2\(^{-\Delta\Delta CT}\) values.

**Multiple Sequence Alignment of Tsf2 between Different Species**

A specific protein sequence of Tsf2 of different species (Table 2) was obtained and sequence similarity was analyzed using online CLUSTAL W software (https://www.genome.jp/tools-bin/clustalw).

**Results**

The different developmental stages of C. quinquefasciatus, such as, larvae, pupae, and adults were collected and stored in a separate container. It took about 6–8 days for the complete development of adult C. quinquefasciatus from the larval stage. Early (larvae group I—1st instar) and late (larvae group II—4th instar) larvae were analyzed. Similarly, early- (pupae group I) and late-stage (pupae group II) of pupae were analyzed. Late pupae were transferred into closed test tubes. Adults upon eclosion were immediately used. (Adult insects were not maintained.)

**Expression Level of Tsf2**

The gene expression level of Tsf2 in different developmental stages of C. quinquefasciatus is represented in Table 3 and Figure 1. Number of animals pooled in each stage: Larvae group I (L1–10 larvae), Larvae group II (L2–10 larvae), Pupae group I (P1–10 pupae), Pupae group II (P2–10 pupae), and Adult group I (A1–10 adult). In the larval stage, the expression level of Tsf2 was less when compared to the pupal stage.
and adult stages. On the one hand, the Tsf2 expression in the pupal stage is highly elevated when compared to the adult stage. On the other hand, the expression of Tsf2 in the adult stage is decreased when compared to the pupal stage.

**Homology of Tsf2—CLUSTAL W**

We found that the Tsf2 of *C. quinquefasciatus* shares 82.75, 80.73, 70.23, and 58.45% of sequence similarity with *Aedes albopictus*, *Ae. aegypti*, *An. gambiae*, and *D. melanogaster*, respectively. The multiple sequence alignment output represents in Figure 2.

**Discussion**

We have earlier demonstrated the role of iron-binding Tsf2, lipid-binding proteins lipophorin, other effector molecules like defensins in serpins in regulating the development of filarial parasite *Wuchereria bancrofti*.\(^{17,20,21}\) The importance of these molecules in functional genomics studies in vector competence was recently reviewed.\(^{22}\) In this context, the present study was designed to assess the levels of Tsf2 in *C. quinquefasciatus*. Molecular studies

![Fig. 1: Expression level of Tsf2 in larval, pupa and adult stages of C. quinquefasciatus](image)
Dynamics of Transferrin 2 (Tsf2) Expression in Various Developmental Stages of Culex quinquefasciatus

SBV Journal of Basic, Clinical and Applied Health Science, Volume 3 Issue 4 (October–December 2020)

The present study exhibited the expression of Tsf2 in the different developmental stages of C. quinquefasciatus, such as, larvae, pupae, and adults (Fig. 3). The results reveal that there was an elevation in the fold change expression of Tsf2 in the larval to pupal stages followed by a decline in the fold of the adult stage as shown in Figure 1. Based on our previous report, we speculate that this may be because of the microbial challenge or the aquatic stages are programmed to synthesize more Tsf2 to encounter the upcoming microbes present in the aquatic environment. This shows that there is a distinct role of the Tsf2 gene and iron metabolism in the different development stages of C. quinquefasciatus. Several studies reported the elevation of mosquito Tsf when treated with heat-killed bacteria and filarial worms. Later studies were done with honeybees, moths, termites, and Drosophila exhibit that Tsf is upregulated upon wounding and bacterial/fungal infection. Together indicates that transferrin plays a major role in vector immunity. In our result,

Fig. 2: Homology sequence alignment output of Tsf2 in five different species of Diptera

Fig. 3: Developmental stages of C. quinquefasciatus

on Tsf protein from several infects has shown that, like their vertebrates homolog, they are multifunctional.
the expression level of Tsf2 is very high in the pupal stage of C. quinquefasciatus indicates that the pupae stage may be highly resistant to bacterial/fungal infection when compared to larvae and adult stage. Similarly, the upregulation of Tsf in mosquito in blood-fed female mosquito follows closely the decrease in juvenile hormones (JH). This suggests that JH suppresses the mosquito Tsf expression. The present study showed that the pupae stage may be highly resistant to bacterial/fungal infection when compared to larvae and adult stage. Similarly, the upregulation of Tsf in mosquito in blood-fed female mosquito follows closely the decrease in juvenile hormones (JH). This suggests that JH suppresses the mosquito Tsf expression. The present study showed that the pupae stage may be highly resistant to bacterial/fungal infection when compared to larvae and adult stage. Similarly, the upregulation of Tsf in mosquito in blood-fed female mosquito follows closely the decrease in juvenile hormones (JH). This suggests that JH suppresses the mosquito Tsf expression. The present study showed that the pupae stage may be highly resistant to bacterial/fungal infection when compared to larvae and adult stage. Similarly, the upregulation of Tsf in mosquito in blood-fed female mosquito follows closely the decrease in juvenile hormones (JH). This suggests that JH suppresses the mosquito Tsf expression.

**Conclusion**

The present study showed the dynamics of Tsf2 expression in the different developmental stages of C. quinquefasciatus. The elevation in the fold change expression of Tsf2 in the larval to pupal stages followed by a decline in the fold of the adult stage may be because of the pre-existing challenge or the aquatic stages are programed to synthesis more Tsf2 to encounter the upcoming microbes present in the aquatic environment via JH. However, the same should be ascertained by screening for the presence of microorganisms if any. Together based on the dynamic changes in the gene expression if Tsf2 during various developmental stages shows that the protein may be involved in innate immune responses of the vector mosquito. In addition, this difference in the expression of Tsf2 in C. quinquefasciatus may be also due to a distinct role of the transferrin gene in iron metabolism. However, the precise role of this gene remains to be determined.

**Acknowledgments**

The authors would like to thank Prof Adithan C, for providing “Dr Rajitha” to carry out the research work. We would like to thank Sri Balaji Vidyapeeth (Deemed to be University) for providing the basic facility and instrumentation.

**References**

1. Zhang A-S, Enns CA. Molecular mechanisms of normal iron homeostasis. Hematology Am Soc Hematol Educ Program 2009(1):207–214. DOI: 10.1182/ash.a2009-207.207.
2. Smith BN. Iron metabolism in health and disease, ed., Brock JH, Halliday JW, Pippard MJ, Powell LW London: Saunders; 1994. p. 495. 579. Hematology. 1995;21(3):889–90.
3. Thorstensen K, Romso I. The role of transferrin in the mechanism of cellular iron uptake. Biochem J 1990;271(1):1–9. DOI: 10.1042/bj2710001.
4. Yoshiga T, Hernandez VP, Fallon AM, Law JH. Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proc Natl Acad Sci U S A 1997;94(23):12337–12342. DOI: 10.1073/pnas.94.23.12337.
5. Kontogiorghes GJ, Weinberg ED. Iron: Mammalian defense systems, mechanisms of disease, and chelation therapy approaches. Blood Rev 1995;9(1):33–45. DOI: 10.1016/0268-963X(95)90038-1.
6. Farnaud S, Evans RW. Lactoferrin—a multifunctional protein with antimicrobial properties. Mol Immunol 2003;40(7):395–405. DOI: 10.1016/S0161-5809(03)00152-4.
7. Nichol H, Law JH, Winzlerling JF. Iron metabolism in insects. Annu Rev Entomol 2002;47(1):535–559. DOI: 10.1146/annurev.ento.47.091201.145237.
8. Geiser DL, Winzlerling JF. Insect transferrins: multifunctional proteins. Biochim Biophys Acta 2012;1820(3):437–451. DOI: 10.1016/j.bbabio.2011.07.011.
9. Kim BY, Lee KS, Choo YM, Kim I, Hwang JS, Sohn HD, et al. Molecular cloning and characterization of a transferrin cDNA from the white-spotted flower chafer, protaetia brevitarsis. DNA Seq 2008;19(2):146–150. DOI: 10.1080/10425270701461854.
10. Kim BY, Lee KS, Choo YM, Kim I, Je YH, Woo SD, et al. Insect transferrin functions as an antioxidant protein in a beetle larva. Comp Biochem Physiol B, Biochem Mol Biol 2008;150(2):161–169. DOI: 10.1016/j.cpb.2008.02.009.
11. Lee KS, Kim BY, Kim HJ, Seo SJ, Yoon HJ, Choi YS, et al. Transferrin inhibits stress-induced apoptosis in a beetle. Free Radic Biol Med 2006;41(11):1151–1161. DOI: 10.1016/j.freeradbiomed.2006.07.001.
12. Yoshiga T, Georgieva T, Dunkov BC, Harizanova N, Ralchev K, Law JH. Drosophila melanogaster transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. Eur J Biochem 1999;260(2):414–420. DOI: 10.1046/j.1432-1327.1999.00173.x.
13. Harizanova N, Georgieva T, Dunkov BC, Yoshiga T, Law JH. Aedes aegypti transferrin. Gene structure, expression pattern, and regulation. Insect Mol Biol 2005;14(1):79–88. DOI: 10.1111/j.1365-2583.2004.00533.x.
14. Locke M, Nichol H. Iron economy in insects: Transport, metabolism, and storage. Annu Rev Entomol 1992;37(1):195–215. DOI: 10.1146/annurev.en.37.010192.001211.
15. Zhou G, Velasquez LS, Geiser DL, Mayo JJ, Winzlerling JF. Differential regulation of transferrin 1 and 2 in Aedes aegypti. Insect Biochem Mol Biol 2009;39(3):234–244. DOI: 10.1016/j.ibmbi.2008.12.004.
16. Dunkov B, Georgieva T. Insect iron binding proteins: Insights from the genomes. Insect Biochem Mol Biol 2006;36(4):300–309. DOI: 10.1016/j.ibmbi.2006.01.007.
17. Paily KP, Kumar BA, Balaraman K. Transferrin in the mosquito, Culex quinquefasciatus say (Diptera: Culicidae), up-regulated upon infection and development of the filarial parasite, Wuchereria bancrofti (Cobbold) (Spiroirida: Onchoceiridae). Parasitol Res 2007;101(2):325–330. DOI: 10.1007/s00436-007-0474-2.
18. Magalhaes T, Oliveira IF, Melo-Santos MAV, Oliveira CMF, Lima CA, Ayres CFJ. Expression of defensin, cecropin, and transferrin in Aedes aegypti (Diptera: Culicidae) infected with Wuchereria bancrofti (Spiroirida: Onchoceiridae), and the abnormal development of nematodes in the mosquito. Exp Parasitol 2008;120(4):364–371. DOI: 10.1016/j.exppara.2008.09.003.
19. Kauffman E, Payne A, Franke MA, Schmid MA, Harris E, Kramer LD. Rearing of Culex spp. and Aedes spp. mosquitoes. Bio Protoc 2017;7(17):e2542. DOI: 10.21769/BioProtoc.2542.
20. Kumar BA, Paily KP. Up-regulation of lipophorin (Lp) and lipophorin receptor (LpR) gene in the mosquito, Culex quinquefasciatus (Diptera: Culicidae), infected with the filarial parasite, Wuchereria bancrofti (Spiroirida: Onchoceiridae). Parasitol Res 2011;108(2):377–381. DOI: 10.1007/s00436-010-2075-8.
21. Paily KP, Null A, Kumar BA, Balaraman K. Changes in the haemocyte population of the mosquito, culex quinquefasciatus, following infection with the filarial parasite, Wuchereria bancrofti. Med Vet Entomol 2005;19(5):116–118. DOI: 10.1111/j.0269-283X.2005.00549.x.
22. Balakrishna Pillai A, Nagarajan U, Mitra A, Krishnan U, Rajendran S, Hoti SL, et al. RNA interference in mosquito: Understanding immune responses, double-stranded RNA delivery systems and potential applications in vector control. Insect Mol Biol 2017;26(2):127–139. DOI: 10.1111/immb.12282.
23. Beerntsen BT, Severson DW, Christensen BM. Aedes aegypti: characterization of a hemolymph polypeptide expressed during melanotic encapsulation of filarial worms. Exp Parasitol 1994;79(3):312–321. DOI: 10.1006/expr.1994.1094.
24. Kucharski R, Maleszka R. Transcriptional profiling reveals multifunctional roles for transferrin in the honeybee, Apis mellifera. J Insect Sci 2003;3(1):27. DOI: 10.1093/jis/3.1.27.
25. Yun EY, Kang SW, Hwang JS, Goo TW, Kim SH, Jin BR, et al. Molecular cloning and characterization of a cDNA encoding a transferrin homolog from Bombyx mori. Biol Chem 1999;380(12):1455–1459. DOI: 10.1515/BC.1999.188.

26. Seitz V, Clermont A, Wedde M, Hummel M, Vilcinskas A, Schlatterer K, et al. Identification of immunorelevant genes from greater wax moth (Galleria mellonella) by a subtractive hybridization approach. Dev Comp Immunol 2003;27(3):207–215. DOI: 10.1016/S0145-305X(02)00097-6.

27. Thompson GJ, Crozier YC, Crozier RH. Isolation and characterization of a termite transferrin gene up-regulated on infection. Insect Mol Biol 2003;12(1):1–7. DOI: 10.1046/j.1365-2983.2003.00381.x.

28. Shapiro AB, Wheelock GD, Hagedorn HH, Baker FC, Tsai LW, Schooley DA. Juvenile hormone and juvenile hormone esterase in adult females of the mosquito Aedes aegypti. J Insect Physiol 1986;32(10):867–877. DOI: 10.1016/j.jinsphysiol.2003.12.003.

29. Jamroz RC, Gasdaska JR, Bradfield JY, Law JH. Transferrin in a cockroach: Molecular cloning, characterization, and suppression by juvenile hormone. ProcNatl AcadSci USA 1999;90(4):1320–1324. DOI: 10.1073/pnas.90.4.1320.

30. Hirai M, Watanabe D, Chinzei Y. A juvenile hormone-repressible transferrin-like protein from the bean bug, riptortus clavatus: CDNA sequence analysis and protein identification during diapause and vitellogenesis. Arch Insect Biochem Physiol 2000;44(1):17–26. DOI: 10.1002/(SICI)1520-6327(200005)44:1<17::AID-ARCH3.0.CO;2-O.

31. Ampasala DR, Zheng S-C, Retnakaran A, Krell PJ, Arif BM, Feng Q-L. Cloning and expression of a putative transferrin cDNA of the spruce budworm, Choristoneura fumiferana. Insect Biochem Mol Biol 2004;34(5):493–500. DOI: 10.1016/j.ibmb.2004.03.002.

32. do Nascimento AM, Cuvillier-Hot V, Barchuk AR, Simões ZLP, Hartfelder K. Honey bee (Apis mellifera) transferrin-gene structure and the role of ecdysteroids in the developmental regulation of its expression. Insect Biochem Mol Biol 2004;34(5):415–424. DOI: 10.1016/j.ibmb.2003.12.003.