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Structural and functional prediction analysis of mosquito Ninjurin protein: Implication in the innate immune responses in *Anopheles stephensi*

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Abstract

Ninjurin, identified as a two-pass transmembrane protein is induced upon nerve injury in vertebrates. Recent studies demonstrate that ninjurins are cell adhesion molecules, capable of regulating many cellular functions viz. embryogenesis, injury, inflammation, signals etc. However, their structural and functional properties controlling these cellular responses, especially innate immune responses have not been investigated in detail. Through comprehensive molecular and functional genomics approach, here we characterize and predict the possible role of mosquito ninjurin (*AsNinj*) in hemocyte mediated cellular immune response. Molecular modeling analyses provide crucial information about the key residues of ninjurin proteins, for further *in vitro* and *in vivo* functional analysis.

Keywords: Mosquito, Ninjurin, Malaria, Hemocyte, Innate Immunity

1. Introduction

Ninjurins are small transmembrane proteins of ~16–27 kDa, first identified as a result of their up-regulation in response to the peripheral nerve injury [1]. Ninjurin transcripts encode proteins having one ectodomain and two predicted transmembrane domains. They are believed to work as adhesion molecules, probably via ectodomain [2] or through direct homophilic binding on the cell surface [1-5]. Studies indicate that Ninjurins are known to regulate multiple biological processes including injury, infection, immunity etc. [1]. Although several *in vitro* studies suggest their conserved structure and function in diverse species e.g. humans, mice and *Drosophila* [6-11] but *in vivo* role has not been implicated in greater detail. Furthermore, our current knowledge about their role, especially in relation to immune regulation is very limited. Recent study [12], demonstrate that one of the three *Drosophila* Ninjurins, 'NijA' protein, induces non-apoptotic cell death and is redistributed to the cell surface in larval immune tissues after septic injury.

Like all other insects, mosquitoes elicit both local and systemic innate immune responses against foreign microbial pathogen exposure including *Plasmodium*, virus etc. Mosquito hemolymph components, especially hemocytes not only control injury specific responses but also participate in the regulation of humoral and cellular immune responses [13-14]. A recent *in vitro* RNAi screen analysis in the hemocyte-like cells, identify several novel regulators including ninjurin like proteins in mosquito *Anopheles gambiae* [15]. However, whether these proteins have a direct involvement in the regulation of cellular immune response against microbial exposure remains uncharacterized [16]. Currently, we are initiating NGS based pilot gene discovery projects to identify and predict putative innate immune genes which are likely to be activated in response to microbial pathogen exposure in the hemocytes of the mosquito *Anopheles stephensi*, a primary urban malarial vector in Indian subcontinents. During our ongoing annotation of the hemocyte transcriptome database, we identified a unique contig encoding putative ninjurin-like protein. Here, we report the initial characterization of the mosquito ninjurin *AsNinj* and predict its possible structural/functional relationships in the hemocyte mediated innate immune response.

2. Materials & Methods

2.1 Mosquito rearing and maintenance

Mosquito *Anopheles stephensi* were reared and maintained at 28 °C and 75% relative humidity, with a 12 hr light/dark cycle according to standard insectary protocols followed at NIMR. All protocols for rearing, maintenance of the mosquito culture were approved by ethical committee of the institute.

2.2 Bacterial Culture and Injections

Escherichia coli and *Bacillus subtilis* were grown overnight in LB medium, precipitated, washed and re-suspended in PBS. Bacterial suspension of 100 nl (*E. coli* at O.D 600 = 0.59 and *B. subtilis* at O.D600 = 0.52) were injected into the thorax of cold anesthetized mosquito with a nano injector. 100 nl of sterile PBS was injected into the control mosquitoes.

2.3 Hemocyte collection

Hemocytes were collected from 3-4 day old healthy adult female mosquitoes post 30 mins, 2hour and 24 hour of injection using some modifications to the high injection/recovery method as described earlier [17]. Briefly 2-3 µl of anticoagulant buffer: Schneider's/RPMI (60): FBS (10): Citrate buffer (30), was injected into the lateral wall of mesothorax of cold anesthetized mosquitoes, followed by flushing out the diluted hemolymph with additional 3-5 µl of anticoagulation buffer, by clipping of the last abdominal segment. The diluted hemolymph was collected onto a clean glass slide and transferred to an eppendorf having 100 µl TRIzol for further RNA isolation.

2.4 Gene expression analysis

Total RNA was isolated from hemocytes and/or other desired tissues, collected from 20-25 adult female mosquitoes using standard Trizol method, followed by first-strand cDNA synthesis using Oligo-dT primers as described earlier [18]. For differential expression analysis, routine RT-PCR and agarose gel electrophoresis protocols were performed, however, relative gene expression was assessed by qPCR (DyNAmo HS; New England Biolabs, Beverly, MA) in Eco-Real Time PCR (Illumina, CA). PCR involved an initial denaturation at 95 °C for 5 min, 40 cycles of 10 s at 95 °C, 15 s at 52 °C, and 22 s at 72 °C. A final extension at 72 °C for 5 min was completed before deriving a melting curve (70–95 °C) to confirm the identity of the PCR product. Following primers were designed for PCR based amplification Forward Actin: 5'TGCGTGACATCAAGGAGAAG3' Reverse Actin: 5'GATCCATACCCAGGAACGA3'; Forward Ninjurin: 5'ACAAACGGCATCCGTAATTC3', Reverse Ninjurin: 5'TGCCGATCGTGATGAGATTA3'. To better evaluate the relative expression, each experiment was performed in three independent biological replicates. The relative quantification results were normalized with internal control Actin gene and analyzed by $2^{-\Delta\Delta Ct}$ method [20]. Differential gene expression was statistically analyzed using student 't' test.

2.5 Bioinformatics Analysis

The putative *AsNinj* was identified as a partial cDNA from the hemocyte cDNA library sequence database search analysis against non-redundant database (Unpublished). Multiple BLAST analysis against mosquito draft genome and other transcript database was done using analysis tools available at

www.vectorbase.org. Domain prediction, multiple sequence alignment and phylogenetic analysis was done using multiple softwares as described earlier [19]. For 3D-Insilico analysis an iterative trial-and-error procedure using the comparative modeling program MODELLER 9V9 was done. The structure evaluation is done on the basis of DOPE (Discrete Optimized Protein Energy) Score that is a statistical potential used to assess homology models in protein structure prediction. The following structural validation programs Procheck, Prosa, Verify 3D and WHATIF are also used for Model verification.

3. Results & Discussion

3.1 Identification, annotation and molecular characterization of *AsNinj*

Our ongoing annotation and comparative analysis of mosquito hemocytes transcriptomes i.e. naïve vs. immune activated (unpublished data), revealed a 1057 bp long unique contig_922 transcript encoding putative ninjurin like protein (207 aa), from the immune challenged cDNA library. To examine and confirm the putative signature of ninjurin, first we performed BLASTX analysis against NR database at NCBI, predicting a putative Ninjurin domain towards C-terminal of the transcript (S1). Additionally, comparative alignment analysis also indicated that the putative *AsNinj* transcript lacks 5-prime sequences.

Therefore, to reconstruct a full length cDNA sequence, we performed BLASTN analysis against the available draft genome of the mosquito *A. stephensi* at www.vectorbase.org. Unexpectedly, this analysis showed two transcripts ASTE010358-RA (1855bp) and ASTE108643-RA (861bp) from the available database. Interestingly, we noticed that most of the mosquitoes carry a single gene for ninjurin with three exons and two introns, Our comparative analysis suggested that gene duplication of smaller (*AsNinjS*) may have resulted in an additional Ninjurin gene with five exons and four introns (*AsNinjL*) in *Anopheles stephensi*. Further analysis of the longer transcript ASTE010358 (*AsNinjL*) showed complete alignment (99.4% identity) with contig_922 originally identified from mosquito hemocyte, except seven nucleotides, out of which 4 nucleotides changed in the coding region and 3 nucleotides changed in the 3'upstream region (Supplemental data/S1). Manually, we verified that these changes did not alter any single amino acid sequence encoded by the putative transcripts, allowing us to reconstruct a full length transcript structure for further characterization.

A comprehensive and comparative genomic analysis revealed that mosquitoes carry similar genetic structure (Fig. 1a) with two introns and three exons, although length of the introns may vary among different species of mosquitoes (Supplemental data/S2). A detailed primary structural analysis of this full length 1855bp long transcripts (*AsNinjL*) revealed that it encodes a (423 aa) long peptide carrying all features of ninjurins, including the presence of ecto domain as well as two trans membrane domains (Fig 1b). To better characterize the molecular relationship of *Anopheles stephensi* ninjurin (*AsNinjL*), we performed multiple sequence alignment as well as phylogenetic analysis of the selected mosquito and insect ninjurins. This analysis revealed a high degree of conservation within the mosquito and other insect species (Fig. 1C; Supplemental data/S3), probably for similar functions.

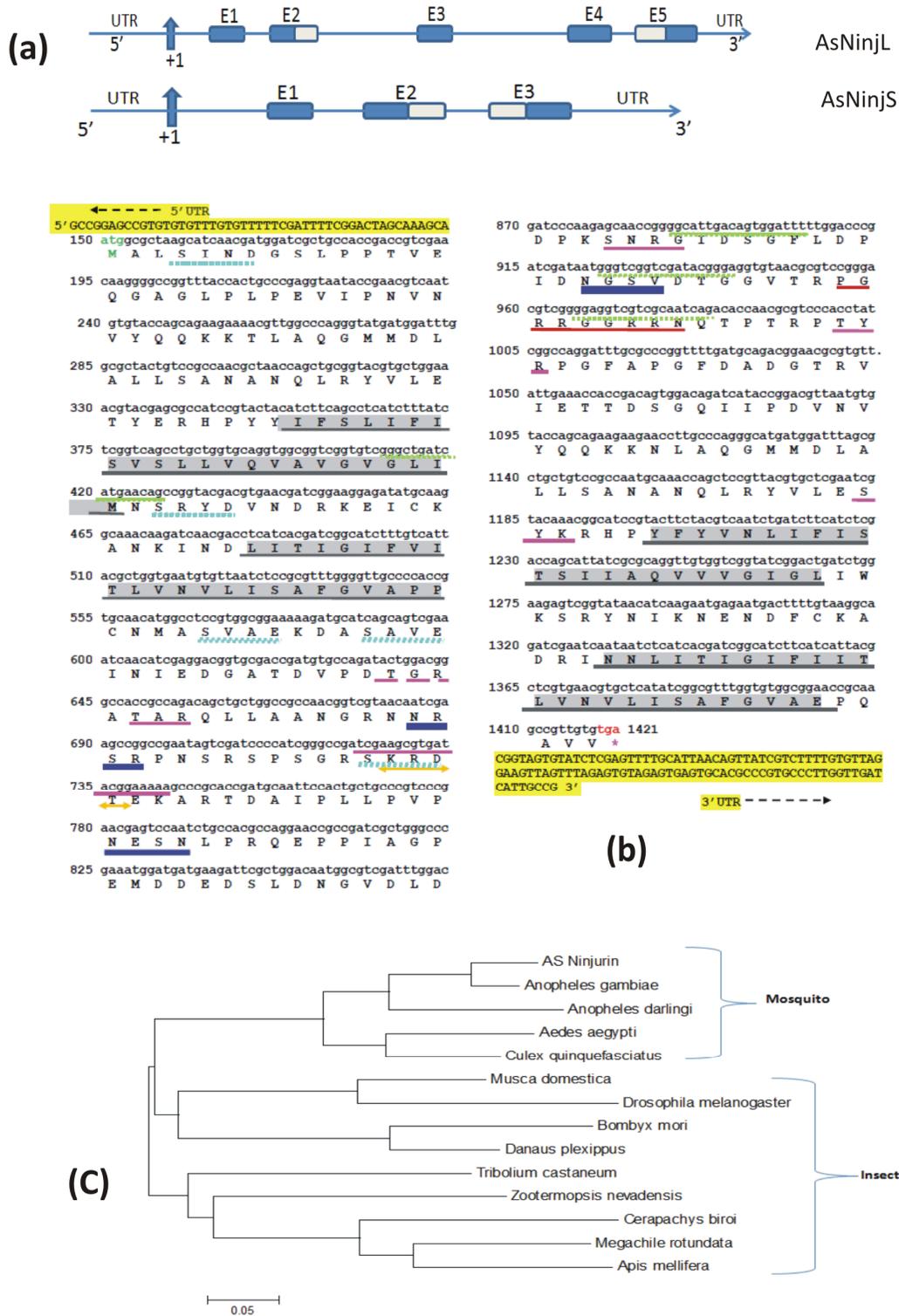


Fig 1: Genomic and molecular Gene Organization of mosquito Ninjurin: (a) Schematic representation of the genomic architecture of the mosquito *Anopheles stephensi* ninjurins. Grey regions indicates the predicted transmembrane of the ninjurin domain, and +1 mark the transcription initiation site; (b) Gene organization and molecular features of complete CDS of *AsNinjL*: The gene contains 1854 bp, encoding 423 AA long peptides with two ninjurin domains, whereas *AsNinjS* contains only one ninjurin domain. Both 5' and 3'-UTR regions are shown in bold & capital letters. The complete CDS region of 423 amino acids starts from ATG/Methionine/green color, ending with TGA/Red/* . The different predicted motifs viz. N-Glycosylation site (dark blue); PKC (Pink); CKP (sky blue); cAMP phosphorylation site(dark yellow arrow); Amidation site (dark red); Mrystoylation site (green dotted) are underlined. Other additional structural features like transmembrane domains are underlined by dark grey lines; (c) Phylogenetic relation of mosquito and insect ninjurins. *AsNinjL* & *AsNinjS* represents longer (ASTE010358) and smaller (ASTE108643) *Anopheles stephensi* ninjurin transcripts, respectively; E1-E5: no. of exons; UTR: Untranslated region.

3.2 Implication of *AsNinjL* in Mosquito Innate Immune response

Our initial RT-PCR analysis revealed that *AsNinj* constitutively express in all the developmental stages of mosquito, suggesting its role in embryonic development of the mosquito (Fig. 2a). Although, ninjurin proteins are believed to play an important role as cell-cell adhesion molecules during embryonic development, organogenesis, and tissue regeneration after injury [3], but their role during the innate immune responses are yet to be determined. Recent *in vitro* RNAi study in mosquito hemocyte like cell line suggests that ninjurin may be an important immune regulator during any microbial exposure [2]. In *Drosophila*, three putative Ninjurin transcripts have been predicted, where Ninjurin A (*DsrNinjA*) has been shown to mediate cellular adhesion during an altered immunity response in the larva [16]. Based on these findings and our own observation, we hypothesized that *AsNinjL* may participate in hemocyte mediated cellular innate immune responses.

To test this hypothesis, first we examined the relative

expression of *AsNinj* in the hemocytes, fat body and midgut collected from 3-4 days old naïve adult female mosquitoes. *AsNinjL* was found to be naively expressed in the hemocytes (Fig. 2b). Septic injury by PBS injection resulted in a slight up-regulation ($p<0.05$) within 30 min post injection, followed by a significant down regulation ($p<0.0005$) after several hours of injury (Fig. 2c.). Together these data suggest that *AsNinjL* may be participating in injury-specific responses viz. coagulation, wound sealing etc. Further evaluation of the hemocyte mediated role of ninjurin in the systemic immune response, was carried out by comparing the relative expression of *AsNinjL* in response to gram negative and gram positive live bacterial injection. A significant ($p<0.001$) and consistent up-regulation of *AsNinjL* was observed in response to both type of bacterial challenge (Fig.2d). These data clearly indicate that irrespective of the nature of microbial exposure, *AsNinjL* may play an important role in the regulation of hemocyte mediated cellular immune responses. However, a functional validation is required to further strengthen the above proposition and observations.

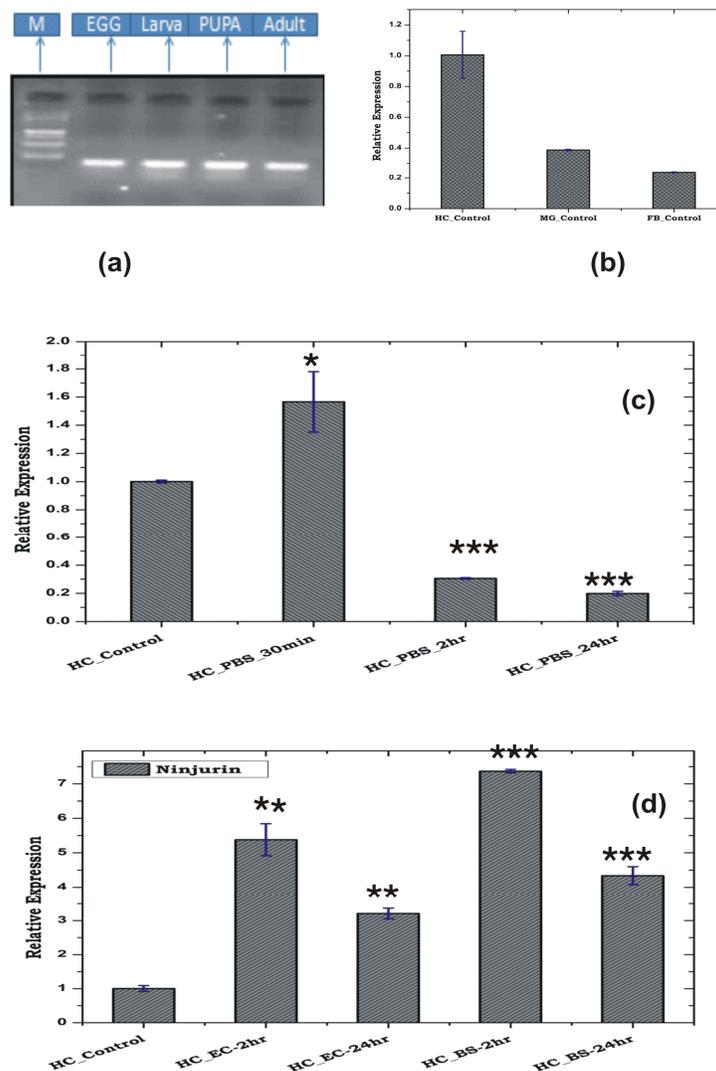


Fig 2: Transcriptional behavior of mosquito ninjurin protein: (a) *AsNinj* expression during the development of mosquito. Developmental stages include Egg, Larva, pupa, adult female; M= 500bp Ladder; (b) Tissue specific relative expression of *AsNinj*, HC: Hemocyte; MG: Midgut; FB: Fat body; (c) Injury specific response of (PBS: phosphate buffer saline); (d) Up regulation of *AsNinj* in response to microbial challenge.

3.3 *In Silico* 3-D and Structural functional Prediction analysis

The above observation prompted us to predict the structural/functional relationship of *AsNinj*, using *In Silico* 3D structural prediction analysis. Due to limited knowledge of the solved structure of ninjurin protein and a poor template match, we applied a random hit and trial error method through comparative molecular modeling approach. In this analysis we selectively included a stretch (110 aa) containing the full Ninjurin domain towards the c-terminal of the coding peptide. Based on initial secondary structure prediction analysis by JPRED, first we selected the best suitable template carrying three long and one short helix through THREADING approach (Fig. 3a). To validate the predicted molecular model, we comprehensively performed multiple analysis viz. Ramachandran Plot, Prosa Energy profile and residue packing quality. Ramachandran Plot analysis showed that 86.1 % of the amino acid residues have Ψ/ϕ angles falling in the most favored regions, while 10.9 % in the allowed region (Fig. 3b) and no residue lie in the unfavorable region. The Energy profile and residue packing analysis qualified the statistical/quality criteria thus validating the model (Fig. 3c). To know the nature of functional residues, we also calculated solvent accessibility, residue conservation score of *AsNinj* model using SAS and ConSurf server based programs (**Supplemental data/S4**). Our analysis predicted that lower solvent accessibility is associated with higher residue conservation score, whereas buried residues (ALA17, LEU18,

SER49, ASN95) were found to be highly conserved among aligned Ninjurins (Fig. 3d). Further analysis of the alignment using the Conseq server showed a scaffold of residues that are expected to be essential for the function of Ninjurin, and overall stability of the system (Fig. 3e).

Lastly, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis of *AsNinj* initially showed closest homology to a transcript (AGAP006745) encoding *Anopheles gambiae* ninjurin (*AgNinj*). Further analysis revealed that *AgNinj* (AGAP006745) may interact with at least three possible network partners (Fig. 3f). Among these three partners AGAP012523, encoding (720 aa) a putative protein named DEAD Box helicase is an ATP-dependent RNA unwinding family protein which participates in a variety of cellular processes including splicing, ribosome biogenesis and RNA degradation showed closest relationship at high confidence score of 0.656. Other two proteins were AGAP004064, encoding RNA binding Nob1 like protein (confidence score of 0.640), and AGAP007279, encoding putative SUMO (small ubiquitin modifier) ligase proteins (with confidence score of 0.536). The STRING analysis did not provide any statistically significant correlation of *AsNinjL*, which might be due to the lack of experimental data and limited knowledge on the structural/functional relationship of ninjurin family proteins. However, this analysis predicts that *AsNinjL* may have a direct coordination with these cascade partners, knowledge for future functional validation.

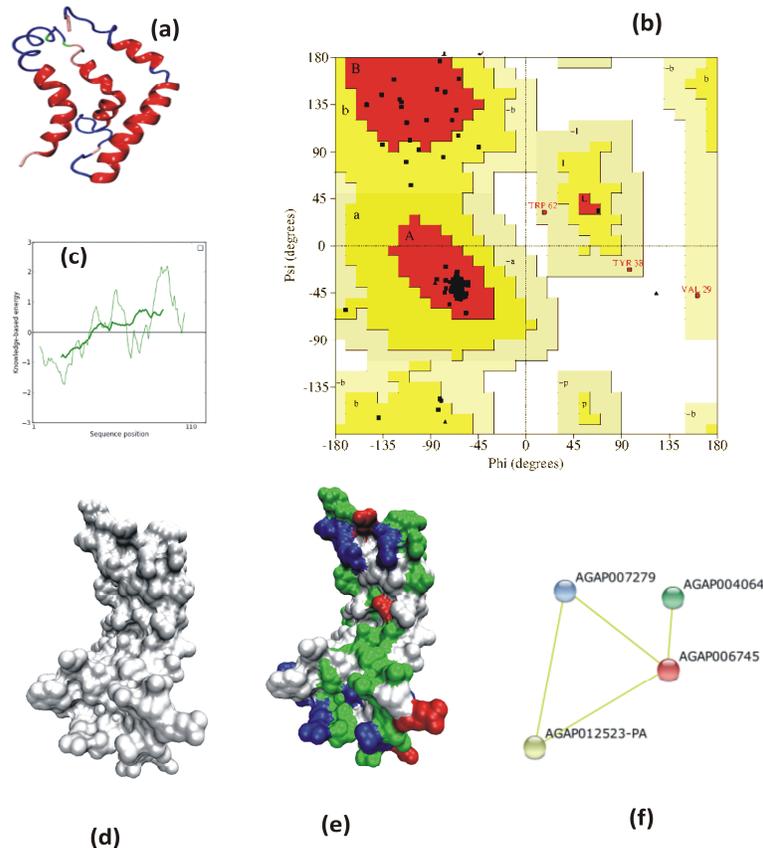


Fig 3: 3D- *In silico* analysis of mosquito Ninjurin: (a) Secondary structure prediction of *AsNinjL*; (b) Ramachandran Plot analysis; (c) Prosa energy distribution of the molecular modeled mosquito Ninjurin; (d,e) Surface representation of the Ninjurin: two front views (d) one side view (e). Polar, negatively and positively charged atoms are shown in green, red and blue respectively; (g) STRING based network prediction analysis of *AsNinjL*.

4. Conclusion

Through comprehensive molecular and functional genomics approach, we provide molecular evidence that mosquito ninjurin proteins may play key role in the regulation of the hemocyte mediated cellular innate immune response. Molecular modeling and functional prediction analysis provide crucial information of the key residues of the ninjurin proteins, for further *in vitro* and *in vivo* functional analysis. We believe these findings may guide our future investigations to clarify the role of ninjurin proteins in the immune regulation of microbial pathogens including malaria causing *Plasmodium* development and its transmission.

5. Acknowledgement

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6. Author's Contribution

Conceived and designed the experiments: RD, TT, and KCP. Performed the Experiments: TT, TDD, PS, SV, SR. Analyzed the data: RD, TT, TDD. Contributed reagents/ materials/ analysis tools: RD, KCP. Wrote the paper: RD, TT, and KCP. Maintenance of the mosquito culture were approved by ethical committee of the institute.

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