Computational Approach for Identification of *Anopheles gambiae* miRNA Involved in Modulation of Host Immune Response

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Abstract MicroRNAs (miRNAs) are small, noncoding RNAs that play key roles in regulating gene expression in animals, plants, and viruses, which involves in biological processes including development, cancer, immunity, and host–microorganism interactions. In this present study, we have used the computational approach to identify potent miRNAs involved in *Anopheles gambiae* immune response. Analysis of 217,261 *A. gambiae* ESTs and further study of RNA folding revealed six new miRNAs. The minimum free energy of the predicted miRNAs ranged from −27.2 to −62.63 kcal/mol with an average of −49.38 kcal/mol. While its A + U % ranges from 50 to 65 % with an average value of 57.37 %. Phylogenetic analysis of the predicted miRNAs revealed that aga-miR-277 was evolutionary highly conserved with more similarity with other mosquito species. Observing further the target identification of the predicted miRNA, it was noticed that the aga-miR-2304 and aga-miR-2390 are involved in modulation of immune response by targeting the gene encoding suppressin and protein prophenoloxidase. Further detailed studies of these miRNAs will help in revealing its function in modulation of *A. gambiae* immune response with respect to its parasite.

Keywords miRNA · *A. gambiae* · Immune response · miRNA target · EST sequence

Introduction

Small RNAs are nonconventional, noncoding, functional, or regulatory RNAs that directly function at RNA level in cells, but they are not messenger, transfer, or ribosomal RNAs.

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RNA-based regulation of gene expression is an evolutionarily conserved mechanism regulated by two kinds of small RNAs [1]. Small interfering RNAs, termed as siRNA, are produced by cleavage of double stranded RNA, which gives rise to two classes of siRNA based on their length (20 to 22 and 24 to 26 mers). The other type of small RNA, termed as MicroRNAs (miRNAs), are endogenous~22 nucleotide (nt) RNAs, some of which are known to play important regulatory roles by serving as guide RNAs for the posttranscriptional repression of protein coding genes [2]. As the first step miRNA genes are transcribed as long pre-miRNAs, RNase III enzyme Drosha processes them to ~70 nt precursors (pre-miRNA) with stem-loop. Then ~22 nt mature miRNAs from pre-miRNA are released by another RNase III enzyme Dicer [3, 4]. Finally, RNA-induced silencing complexes are formed to regulate the expression of target genes via complementary base pair interactions [5].

Direct cloning, forward genetics, and computational approaches using bioinformatics tools are the three main approaches globally used in detection of miRNA. In direct cloning, small RNA were isolated from biological samples and cloned to make a cDNA library for small endogenous RNAs [6]. In direct cloning approach, low expressed miRNAs are hard to clone and are limited to highly-expressed miRNAs. Forward genetics approach involves identification of mutation responsible for a certain phenotype. But this approach has not been used very much to detect miRNA families due to certain limitations like small size of miRNA and the "seed" sequence, which doesn't mutate easily [7]. Bioinformatics approaches and genome sequence analysis could be potentially used to overcome the limitations of the above two methods. Bioinformatics approach is based on the information about certain miRNA features generated by cloning approach. Computational approach in identification of miRNAs is widely used because they are evolutionary conserved. Bioinformatics predictions with experimental validation indicate that the total number of miRNAs is significantly higher than previously estimated [8].

Mosquitoes of the genera *Aedes*, *Anopheles*, and *Culex* transmit serious human diseases such as malaria, yellow fever, West Nile fever, and chikungunya. Every year, 500 million people are affected by malaria, transmitted by *Anopheles* sp. with above 2 million death cases. In the absence of an effective control strategy, these diseases have a major impact on the public health, environmental safety, and the socioeconomic development of a country [9]. Discovery of miRNAs *lin-4* and *let-7* started in *Caenorhabditis elegans* [10] and later, several hundred miRNAs with posttranscriptional regulatory functions have been reported from viruses, plants, and animals. The recent availability of the complete genome and development of large scale expressed sequence tag (EST) of the malaria mosquito *A. gambiae* [11] has enabled the identification of miRNAs from its genome. In spite this vast study about miRNA, research in the area of insect host–microorganism interactions has been limited, accumulating evidences in vertebrates suggests that miRNAs play major roles, including antipathogen responses targeting the microorganism directly or altering the expression of host genes that are beneficial to the microorganism [12–14]. Pathogens are also able to manipulate host miRNA to facilitate its replication [15] and the manipulation will be very parasite-species-specific as one miRNA might be downregulated by one parasite while upregulated by another closely related species [16].

In the present study, we have utilized all miRNAs reported from different species for the identification of its conserved miRNA homologues from *A. gambiae* ESTs. Here, we reported six new miRNAs from *A. gambiae* EST, identified its targets, and discussed in detail. We believe that the approach employed here will help in the prediction of miRNA in distantly-related species as well.

Materials and methods

Collection of reference miRNA, nucleotide, and EST sequences

We used EST analysis method for the identification of miRNAs from *A. gambiae*. All known pre-miRNA (18,226 as of June 2012) and mature miRNA (21,623 as of June 2012) were retrieved from the miRBase (http://www.mirbase.org/). After elimination of homolog, rests of the miRNAs were used for searching *A. gambiae* miRNA. EST sequences (217,261 as of June 2012) of *A. gambiae* were downloaded from the Vectorbase EST database (http://www.agambiae.vectorbase.org/). Local nucleotide database was created after eliminating redundant and poor quality sequences.

Identification of miRNAs and their precursor sequences

Instead of mature miRNA sequences of *A. gambiae*, pre-miRNAs were used as reference material for the search of its homolog in *A. gambiae* transcriptome. Reference miRNA sequences were used as a query for homology search against our local *A. gambiae* nucleotide sequence database at e-value threshold <0.01 using BLAST 2.2.22+ program with all other parameters as default [17]. The FASTA formats of all the candidate sequences were saved. The repeated ESTs from the same gene were removed by BLAST against the *A. gambiae* ESTs Database using blastn with default parameters and single tone EST was created for each miRNA. Reference miRNA sequence was aligned against the corresponding singleton ESTs using ClustalW [18] multiple sequence alignment tool and initial miRNA candidate was created. Target sequences with not more than four mismatches were validated for their nonprotein encoding phenomenon using BLAST against protein database at NCBI using blastx with default parameter [19].

Validation of precursor candidate miRNAs

The candidate pre-miRNA satisfying the above conditions were considered for prediction and validation of secondary structure using Mfold v 3.2 (http://www.mfold.rna.albary.edu/).The precursor sequences were selected from 100 nucleotides upstream or downstream from the location of mature miRNAs. While selecting a RNA sequence as a candidate miRNA precursor, the following criteria were used according to Zhang et al. [20]: (a) RNA sequence is able to fold into an appropriate stem-loop hairpin secondary structure, (b) mature miRNA sequence site in one arm of the hairpin structure, (c) miRNAs should have less than seven mismatches with the opposite miRNA* sequence in the other arm, (d) loop or break in miRNA sequences should not be noticed, (e) had less than seven mismatches with the opposite miRNA sequence in the other arm, (4) no loop or break in miRNA sequences, (5) predicted secondary structures had higher negative energy MFEs ($\leq$−18 kcal/mol) and 40–70 % A + U contents, (6) predicted secondary structures should have higher negative energy MFEs ($\leq$−18 kcal/mol) and 40–70 % A + U contents.

Prediction of *A. gambiae* miRNAs targets

The identified *A. gambiae* miRNA query sequences were used for their target prediction using NCBI blastn program [17]. The parameters such as Database (Nucleotide collection), organism (*A. gambiae*—taxid: 3328) and program selection (highly similar sequences—

megablast) were selected for analysis. The sequences showing 60 % query coverage were selected and subjected to RNA-hybrid, a miRNA target prediction tool (http://www.bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

Nomenclature of predicted miRNAs

Names for the predicted miRNAs were assigned as per the pattern of miRBase Griffiths-Jones et al., [21]. The mature sequences were designated "miR" and the precursor hairpins were labeled as "mir" with the prefix "aga" for *A. gambiae*.

Phylogenetic analysis

Phylogenic relationship of *A. gambiae* miRNA conservation with their orthologues was analyzed. Candidate *A. gambiae* miRNAs was searched for their homolog against all miRNAs using NCBI standalone BLAST [17] allowing a maximum of three mismatches and e-value <0.001. The corresponding precursor sequences of homolog miRNA's were identified, collected, and aligned with homolog *A. gambiae* miRNA using ClustalW [18]. Phylogenetic analysis of the aligned miRNA sequences of the selected was performed with Mega5. The evolutionary distances were computed using the maximum composite likelihood method [22] and are in the units of the number of base substitutions per site. Finally, the evolutionary analyses were conducted in MEGA5 [23].

Results and discussion

Here in this study, we employed computational prediction of *A. gambiae* miRNA from its EST resource (Fig. 1). miRNAs have been shown to regulate diverse and important processes such as B-cell differentiation [24], adipocyte differentiation [25], cardiogenesis [26], insulin secretion [27], antiviral defense [28], and the development of cancer [29]. A total of 217,261 *A. gambiae* EST sequences were retrieved from the Vectorbase database and used for prediction of miRNAs from *A. gambiae* genome. From the present study on in silico search for novel miRNAs, we have noticed that out of the whole EST sequences, only 105 sequences were recorded with less than four mismatches. Further analysis of the sequence for their protein encoding nature using BLAST against protein database at NCBI using blastx revealed the presence of 63 nonprotein encoding ESTs. Six new *A. gambiae* miRNAs was reported after a careful evaluation of the secondary structure analysis. Though miRNA research in the area of insect host–microorganism interactions has been limited, accumulating evidences in vertebrates suggests that miRNAs play major roles, including antipathogen responses targeting the microorganism directly or altering the expression of host genes that are beneficial to the microorganism [13, 14]. For the predicted miRNAs, Table 1 provides details such as source sequences, length of precursor sequences, and their minimum folding free energies and A + U content. Though Chatterjee and Chaudhuri, [30] had reported 41 *A. gambiae* using *Drosophila melanogaster* miRNA dataset, they failed to predict the miRNA which are absent in *D. melanogaster*.

Among the predicted miRNAs from *A. gambiae* EST, only two were noticed in direct strand and the rest of the predictions were observed in indirect strand. The predicted miRNAs had minimum free energy ranging from −27.2 to −62.63 kcal/mol with an average of −49.38 kcal/mol. While considering the A + U percentage in the predicted miRNAs, it
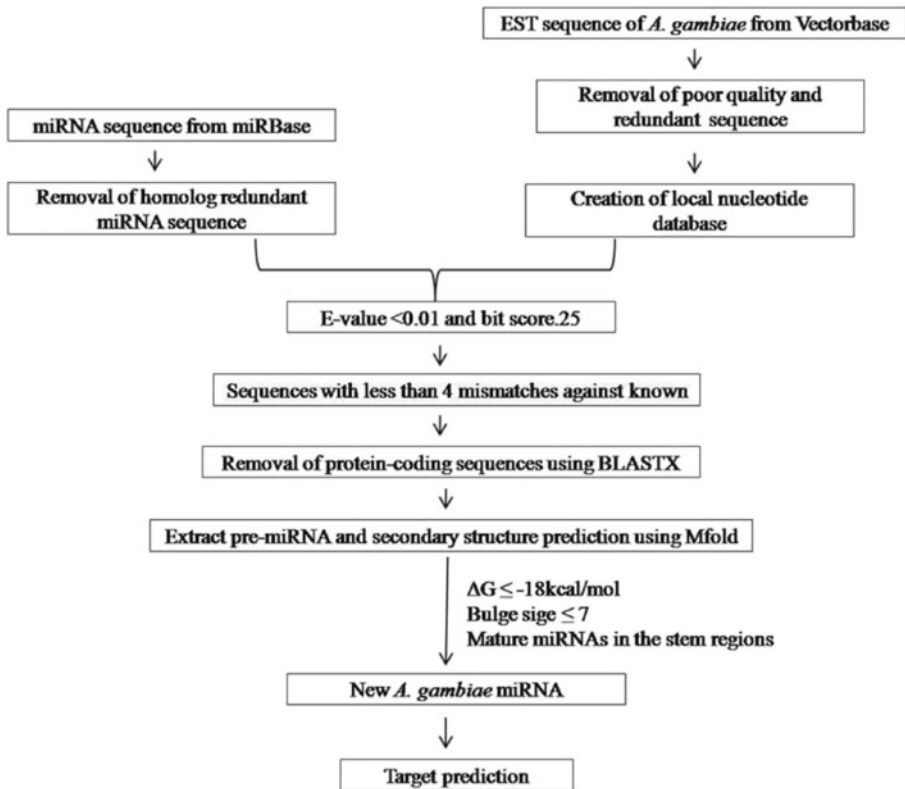
Fig. 1 A schematic illustration of different steps involved in miRNA and their target prediction

ranges from 50 to 65 % with an average value of 57.37 % (Table 1). Hackl et al. [31] extracted pre-miRNA from reference genomes folded in silico to verify correct structures from Chinese hamster ovary cell lines. Among the miRNAs predicted, only two (aga-miR-277 and aga-miR-466i) sequences were matched 100 % with their homologue in miRNA database. Analysis of RNA folding using Mfold revealed that, all the mature *A. gambiae* miRNAs that were found in the stem portion of the hairpin structures are broken or loopless inside the sequences (Fig. 2 a-f) and contains less than seven mismatches in the other arm.

Phylogenetic analysis of the predicted miRNAs revealed miRNA (aga-miR-277) was highly conserved among the insect species, with more similarity with other mosquito species. Conservation of miRNA sequence was observed among the mosquito *A. gambiae* and *Anopheles stephensi* species [32]. Phylogenetic tree revealed their evolutionary development among the insect species (Fig. 3 a-b). For the six predicted miRNAs, we have identified 20 potent target genes. Among the miRNAs, a maximum of six targets were identified for the aga-miR-466b.

Genes encoding Netrin-B, starry night, and visceral mesodermal armadillo-repeats were predicted as targets of aga-miR-277. Thus, the predicted aga-miR-277 could modulate the expression pattern of genes involved in biological process such as axon guidance and rhabdomere development. The aga-miR-466b are able to target the expression of genes encoding proteins such as N-acetyl transferase, N-formylmethionylaminoacyl-tRNA

Table 1 Details of predicted *Anopheles gambiae* miRNA

New gambiae miRNA	Source miRNA	Source organism	PL	MFE ΔG	MS	ME	SE	Strand	A + U%
aga-miR-277	ame-miR-277	*Apis mellifera*	223	−54.08	UAAAUGCACUAUCUGGUACGACA	23/23	BM634284	3'	60.87
aga-miR-466b	mo-miR-466b	*Rattus norvegicus*	220	−54.80	UAUGUGUGUGUGUAUCCUAUG	22/23	BX614132	3'	54.09
aga-miR-466i	mmu-miR-466i-5p	*Mus musculus*	221	−62.63	UGUGUGUGUGUGUGUGUGUG	20/20	BX622948	3'	50.00
aga-miR-1638	gga-miR-1638	*Gallus gallus*	220	−27.20	GUAGUUUGUGUGUUUGUGUGUG	20/21	CR528498	3'	61.90
aga-miR-2304	bta-miR-2304	*Bos taurus*	197	−57.17	UCUUGUGUGGUUGUGUGUGUG	19/21	CR530271	5'	52.38
aga-miR-2390	bta-miR-2390	*Bos taurus*	186	−40.44	AGUGUGUGUUUUUUGUUUUCC	18/20	CR535150	5'	65.00

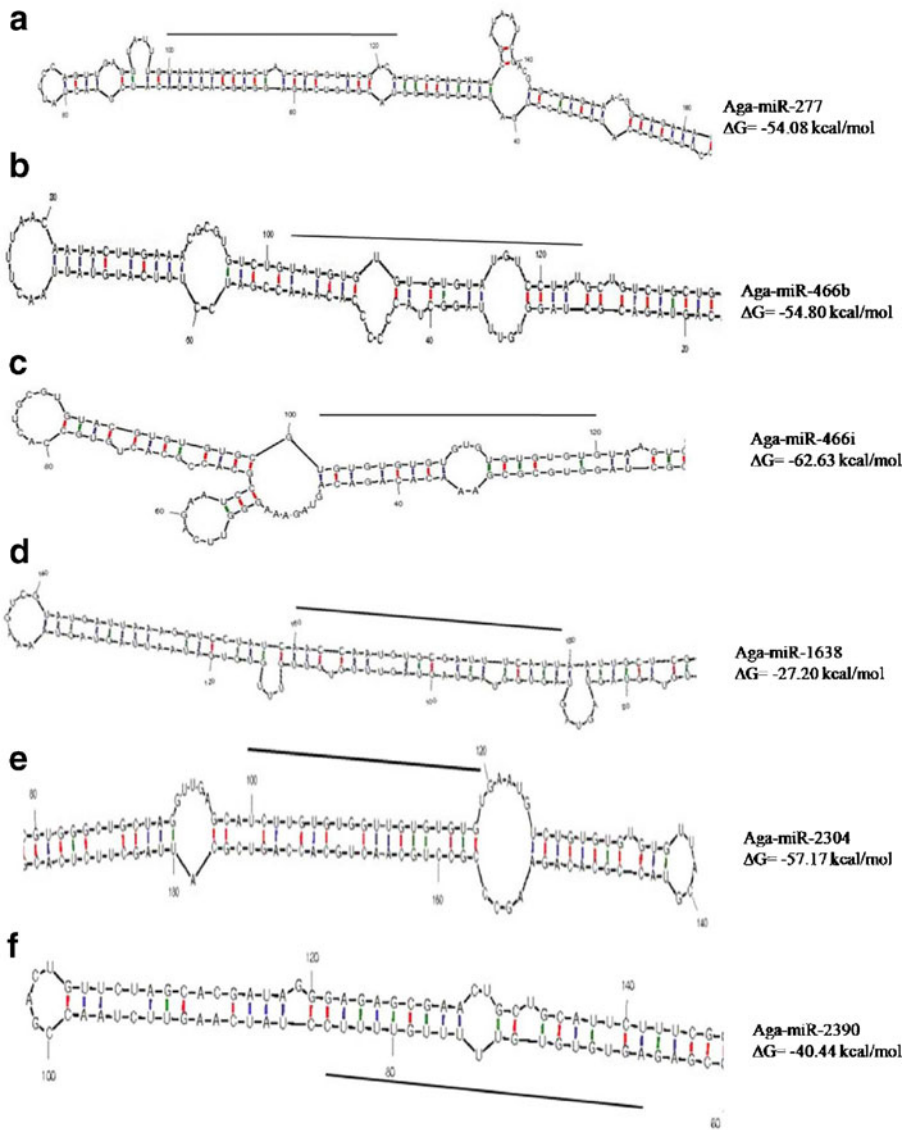*PL* pre-miRNA length, *MEF* minimal free energy, *MS* mature sequence, *ME* match extent, *SE* source EST

Fig. 2 Hairpin structure of predicted *Anopheles gambiae* miRNA. **a** aga-miR-277, **b** aga-miR-466b, **c** aga-miR-466i, **d** aga-miR-1638, **e** aga-miR-2304, and **f** aga-miR-2390

deformylase, rho guanine dissociation factor, Lipase, rap GTPase-activating protein, and innexin. Thus, aga-miR-277 could be involved in modulation of biological process such as cell proliferation, lipid metabolism, signal transduction, and response to light stimulus. Genes encoding protein like ceramidase, glial maturation factor, G-protein coupled receptor, and cytoplasmic dynein intermediate chain are targeted by aga-miR-466i and modulates the hatching behavior, gliogenesis, G-protein coupled receptor-signaling pathway, and microtubule cytoskeleton organization biological process (Table 2). The other miRNAs, such as aga-miR-1638, 2304, and 2390, are involved in biological process such as transcription, protein
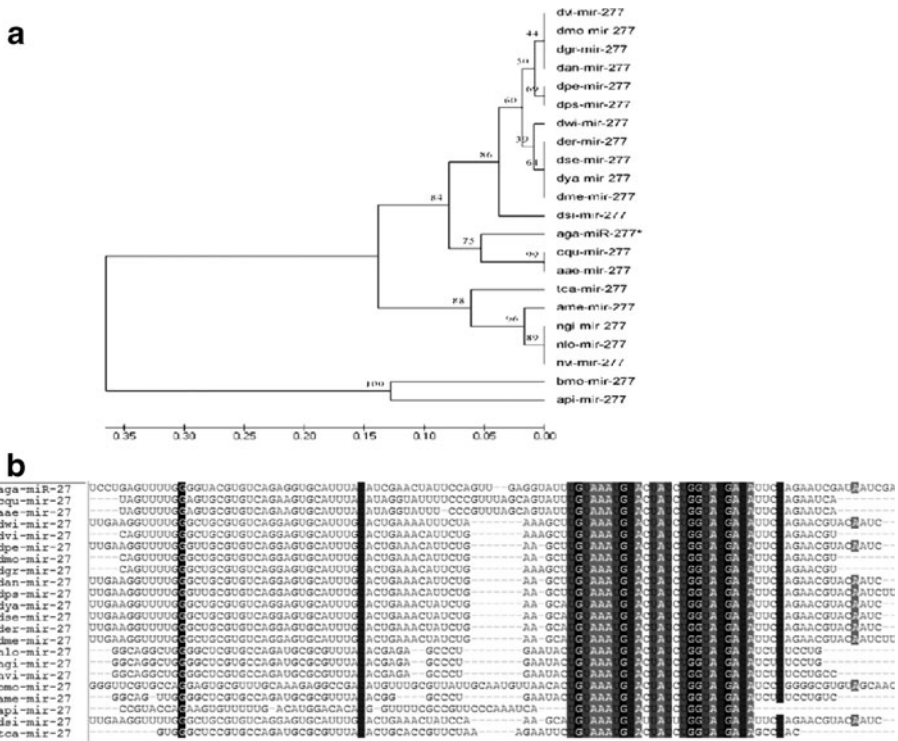
Fig. 3 a Phylogenetic tree showing evolutionary conservation of aga-miR-277 among insect species **b** Multiple sequence alignment of aga-miR277 with its related species showing conserved region

phosphorylation, immune response, and signaling cascade by targeting the genes encoding protein phosphatase, cyclin-dependent kinase, suppressin, monooxygenase, and protein prophenoloxidase. Pathogens are also able to manipulate host miRNA to facilitate its replication [9] and the manipulation will be very parasite-species-specific as one miRNA might be downregulated by one parasite while upregulated by another closely related species [16]. Recent report of Hussain et al., [33] demonstrated that *Wolbachia* could use host miRNA to manipulate host gene expression for its colonization in dengue vector *Aedes aegypti*. Differential expression of miRNA in response to the malaria parasite *Plasmodium berghei* was reported from the midgut of the mosquito following parasite invasion [34]. Results from the study of Winter et al., [34] suggested that host miRNAs might play key roles in antiparasitic responses and resistance of *A. gambiae* to *P. berghei*, perhaps by regulating defense-related host genes. Though Skalsky et al. [35] discovered that only small portion of miRNAs was found to show variation in *Culex quinquefasciatus* mosquitoes infected with the West Nile virus.

As conclusion, the present study reported six new miRNA candidates from *A. gambiae* using EST resource of the target organism. Further analysis of the miRNA targets revealed that biological process such as immune response, signaling cascade, cell proliferation, and post translation modification are controlled by the identified candidate miRNAs. Further future studies are to be carried out with wet lab to unravel the importance of the identified miRNA in response to the host–parasite interaction and developmental pattern of the vector mosquito.

Table 2 Targets of predicted *Anopheles gambiae* miRNA

Sl. No	A. gambiae miRNA	Target protein	NCBI acc. no.	Molecular function	Biological process
1	aga-miR-277	Netrin-B	NM_078600	Unknown	Axon guidance
		Starry night	NM_165794	Receptor-signaling protein activity	Axon guidance
		Visceral mesodermal armadillo-repeats	NM_057957	Ral GTPase binding	Rhabdomere development
2	aga-miR-466b	N-acetyl transferase	XP_001866158	Fatty acid binding	Mitotic sister chromatid cohesion
		N-formylmethionylaminoacyl-tRNA deformylase	XP_001654759	Peptide deformylase activity	Positive regulation of cell proliferation
		Rho guanine dissociation factor	XP_001663181	Rho GDP-dissociation inhibitor activity	Unknown
		Lipase	XP_001846790	Triglyceride lipase activity	Lipid metabolic process
		rap GTPase-activating protein	XP_002427167	GTPase activator activity	Regulation of small GTPase mediated signal transduction
3	aga-miR-466i	Innexin	XP_001649755	Photoreceptor activity	Response to light stimulus
		Ceramidase	XP_001847780	Ceramidase activity	Hatching behavior
		Glial maturation factor	XP_001652689	Actin binding	Gliogenesis
		G-protein coupled receptor	XP_001651852	G-protein coupled receptor activity	G-protein coupled receptor signaling pathway
		Cytoplasmic dynein intermediate chain	XP_001842242	Microtubule motor activity	Microtubule cytoskeleton organization
4	aga-miR-1638	KH domain containing, RNA-binding, signal transduction associated 2	NM_152688	Poly(U) RNA binding	Transcription, DNA dependent
		Protein phosphatase 3	NM_000944	Phosphoprotein phosphatase activity	Calcineurin-NFAT-signaling cascade
		Cyclin-dependent kinase 19	NM_015076	ATP binding	Protein phosphorylation
5	aga-miR-2304	Suppressin	XP_001663599	sequence-specific DNA binding transcription factor activity	Regulation of immune response
6	aga-miR-2390	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	NP_998310	protein domain specific binding	Negative regulation of protein dephosphorylation
		Protein prophenoloxidase	AAC27383	monophenol monooxygenase activity	Defense response

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