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Initial function of microRNAs as a defence mechanism against invading organisms

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Abstract

Background: Although microRNA (miRNA) regulation is widely considered as a mechanism to regulate gene expression in metazoans, plants and viruses, there are recent reports that show the interaction between viruses and their mammal hosts via miRNAs produced from both sides. MiRNAs are highly conserved among mammals, whereas the early miRNAs seem to be more diverse, implying a dynamic functional evolution of miRNAs in the early species. To obtain an evolution landscape of miRNA function and elucidate the initial function of miRNAs, we investigated the targets of miRNAs in the viral system and among metazoan species.

Methods: The targets in a set of 5361 viral genomes for all the miRNAs encoded by 17 metazoan species that occur at the key evolutionary nodes on metazoan phylogeny were calculated according to base matching of miRNAs to their target sites and the free energy of miRNA-mRNA duplex.

Results: The results showed that sponge miRNAs had the high targeting potential against viral systems, whereas those in other early metazoans showed lower targeting potential. The miRNAs of ancient species tended to have more targets in double-stranded DNA viruses and bacteriophages than in other viruses. The metazoan miRNA targets on self-genomes showed an increased tendency along with evolution.

Conclusions: The results of miRNA target analysis for 17 metazoan and virus genomes suggest that the initial function of miRNAs was predominantly antiviral, as evolution proceeded, miRNAs acted more specifically on self-genomes. This may imply the origin of microRNAs as a defensive rather than a regulatory strategy.

Keywords: microRNA, Initial function, Antiviral, Evolution, microRNA target

Background

MicroRNAs (miRNAs) are a type of small (19–24 nucleotides [nt]), noncoding RNA and traditionally considered to be factors that post-transcriptionally regulate gene expression by binding to their target messenger RNAs (mRNAs). miRNAs were firstly identified in *Caenorhabditis elegans* and have since been shown to be expressed in all metazoans and plants, and in several DNA viruses. Mature miRNAs typically bind to complementary sequences in the 3′ untranslated regions of their target mRNAs, and they regulate several cellular processes, including cell apoptosis, stress responses, homeostasis, growth, differentiation, development, and immune activation, by repressing translation and/or inducing mRNA degradation [1, 2]. miRNAs mainly act when nucleotides (nt) 2–7 bases from the 5′ end of the mature miRNA, designated the 'seed sequence', bind to the 3′ end of a complementary mRNA. Perfectly complementary targeted mRNAs in plants are usually endonucleolytically cleaved. When the sequences are not perfectly complementary, which is observed most often in mammalian and viral miRNA targets, transcription is usually repressed [3]. Although these kinds of regulatory mechanisms are traditionally considered to involve an interaction between cellular miRNAs and their own cellular mRNAs, increasing evidence indicates that the interaction of miRNAs and mRNAs also occurs between viruses and their hosts, with both the host and the virus producing miRNAs that mediate the host–virus interaction [4]. miRNAs have been reported to interact in several ways with viral

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genes. For example, miR-32 restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells [5]. It has also been reported that mice deficient in Dicer-1 (and therefore deficient in mature miRNAs) are more susceptible to vesicular stomatitis virus (VSV) infection [6]. It is interesting to consider why a virus with a high mutation rate would retain several target sites for host miRNAs that are upregulated during the infection process and inhibit viral replication [7]. One suggestion is that some viruses take advantage of the host's conserved miRNA regulatory mechanism to downregulate its own replication to facilitate a persistent infection. For example, human miR-122a induces hepatitis C virus (HCV) replication by targeting the 5′ noncoding region of the viral genome [8]. These facts imply a complex role for miRNAs during the coevolution of viruses and their host species.

miRNAs have been present since the dawn of animal life [9]. Sixteen miRNAs have been identified in *Amphimedon queenslandica*, a poriferan of the subkingdom Parazoa that is considered to represent the earliest animal lineage [10]. However, another Parazoa lineage branching sister to the clade Bilateria is the phylum Placozoa, which includes *Trichoplax adhaerens* [11], within which no miRNAs have been found [9]. The pre-miRNAs of the phyla Porifera and Cnidaria and the clade Bilateria are different sizes [9], and the conservation of the miRNAs of these early species is not as strong as in mammals. These facts suggest that the evolution of metazoan miRNAs was very dynamic [9], indicating their possible diverse functions. The functions of the miRNAs of early species have not yet been fully explored. Many miRNAs reported in mammals regulate the expression of self-genes (genes in the same species as the miRNAs are called 'self-genes'), but contribute to immune system defence against viruses [5]. It is interesting to speculate upon the functions of miRNAs in ancient species. Theoretically, the generation of a new kind of molecular or regulatory mechanism may occur in response to an environment stressor, and this mechanism may play an important role in survival. From this perspective, the need to regulate self-gene expression may not have been the factor instigating the evolution of miRNAs. Gene knockout studies have demonstrated that miRNAs are not essential for the viability of animals [12, 13], which suggests that some miRNAs merely act as subtle regulators to balance gene expression. The evolution of protein-based immune responses may date back to *Branchiostoma lanceolatum* [14]. In an analysis of four complete invertebrate genomes (*Drosophila melanogaster*, *Anopheles gambiae*, *C. elegans*, and *Ciona intestinalis*), no homologue of vertebrate interferon (IFN) has been found. Therefore, it seems clear that invertebrates lack an antiviral system [15]. Several lines of evidence support the notion that the RNA interference (RNAi) pathway plays a role in the antiviral immunity of the Metazoa. The possibility that RNAi has an antiviral function was first raised by plant researchers [16], and in animals, antiviral RNAi was first identified in *Drosophila* and subsequently in nematode worms [17, 18]. RNAi commonly functions to defend the host against harmful nucleic acids, such as the RNA of exogenous viruses or endogenous transposons [19]. However, RNAi does not seem to play an antiviral role in most mammalian cells. Nevertheless, some components of the RNAi machinery seem to protect mammalian cells against transposons [20]. Prokaryotes use clustered regularly interspaced short palindromic repeats (CRISPRs) to defend against foreign nucleic acids, and CRISPRs can be thought of as adaptive immune responses that protect the host against plasmids, transposons, and phages. Some bacterial CRISPRs use double-stranded RNA (dsRNA) to cleave the targeted DNA [21]. In bacterial lineages, some CRISPR machinery has been lost, similar to the loss of RNAi in some eukaryotic lineages. Some bacteria have also evolved to use the CRISPR machinery to regulate self-genes [22]. Similarly, eukaryotic lineages have evolved to use the RNAi machinery to regulate the expression of protein-coding self-genes with miRNAs [23]. To investigate the possible evolution of miRNAs in the interactions between viral systems and metazoan hosts, we systematically analysed the miRNA targets in a set of 5361 viral genomes for all the miRNAs encoded by 17 species that occur at key evolutionary nodes on the metazoan phylogeny. We found that the miRNAs from *A. queenslandica*, which represents the earliest animal, showed high targeting potential against viral systems (meaning the potential targeting intensity of sponge miRNAs on viruses), whereas those of other early Metazoa showed lower targeting potential. The types of viruses that are targeted by different host miRNAs have changed through evolution, and the role of miRNAs in regulating self-gene expression has increased with evolution. These lines of evidence suggest that the initial function of miRNAs was mainly as a defence mechanism against invading organisms.

Methods

In this work, we collected all the viral genomes from the National Center for Biotechnology Information (NCBI) database [24] (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10239). These viruses could be grouped into 14 categories based on the hosts they infect and into 15 classes based on their genome types. Seventeen representative species from the evolutionary tree of the animals were selected as research subjects. The argonaute proteins and miRNA family information were used to calculate the evolutionary distances among

these 17 species. By predicting the targets of the miRNAs of these species in different viruses and self-genes, we hoped to determine the initial functions of the miRNAs and the evolution of their functional traits. The binding sites of the miRNAs on mRNAs were predicted with imperfect complementarity. BLASTn [25] was first used to find potential binding sites, and RNAhybrid [26] was then used to calculate the minimum free energy of the hybridization between the miRNAs and their potential target mRNAs. According to a previous study [27], a value for the free energy of a miRNA–mRNA duplex below − 25 kcal/mol constitutes a relatively stringent threshold. Therefore, binding sites with free energy less than − 25 kcal/mol were considered potential miRNA targets. The procedure used was the same as that in a previous study on *trans*-acting small interfering RNAs (siRNAs) [28].

Results

MiRNAs of the oldest animal phylum, Porifera, target viruses

The marine sponges (phylum Porifera) are among the oldest multicellular invertebrate organisms [29]. In the nineteenth century, the remarkable similarity between porifera-specific choanocytes and free-living choanoflagellates was recognized, which prompted the proposition that sponges evolved from choanoflagellate-like protist ancestors and are the most primitive metazoans [30]. They are also the oldest animals for which there are sequenced miRNAs in miRBase [31]. To analyse the regulatory mechanism of sponge miRNAs directed against viruses, the targets of 16 *A. queenslandica* miRNAs from miRBase were predicted in 5361 viral genomes. The number of targets per miRNA in one species against all the viral genomes was designated as the 'functional potential' of the miRNAs of this species to regulate viral systems. The distribution of the numbers of target sites against all viral sequences is shown in Fig. 1. A randomization was performed using viral genomic sequences that were shuffled in such a way as to preserve their nucleotide compositions. We computed 100 randomizations and calculated the *p* value for all the viruses. Overall, the *p* value was 6.87e− 91 for all the viruses, indicating the significance of the sponge miRNAs targeting viruses. As a control, the p value of human miRNAs targeting all the human-infecting viruses recorded in the NCBI database was 3.04e− 146, whereas the p value for human miRNAs targeting the viruses that infect Protozoa was 1, confirming the accuracy of the method.

To further investigate the viruses that were significantly targeted by sponge miRNAs, we set the p value threshold at 0.01. Those viruses containing the top 5% target sites among all viruses were deemed to be efficiently targeted by miRNAs. In other words, there were at least 10 target sites in each of these selected viral sequences, as shown in Fig. 1. We ultimately identified 154 viruses that may be targeted by sponge miRNAs. The hosts of these viruses are shown in Fig. 2. Interestingly, approximately 66.2% of the viruses were bacteriophages, which account for only 25% of all viruses. These bacteriophages can be regarded as ancient viruses. They are more likely to be targeted by sponge miRNAs, or similar viruses may infect sponges and therefore also be targeted by sponge miRNAs.

Of the 16 *A. queenslandica* miRNAs investigated, nine have significant targets in viruses (aqu-miR-2017-3p, aqu-miR-2019-5p, aqu-miR-2015-3p, aqu-miR-2020-5p, aqu-miR-2018-3p, aqu-miR-2016-3p, aqu-miR-2021-3p, aqu-miR-2016-5p, and aqu-miR-2021-5p). Some of the nine miRNAs show a degree of sequence similarity to vertebrate miRNAs based on the results predicted with miRBase [31] using default parameters. For example, aqu-miR-2017-3p has a similar sequence to those of *Branchiostoma floridae* bfl-miR-2064, *B. belcheri* bbe-miR-2064-5p, *Homo sapiens* hsa-miR-619-3p, *Pan troglodytes* ptr-miR-619, and *Pongo pygmaeus* ppy-miR-619, as shown in Fig. 3. The homologous miRNAs derive from animals ranging from cephalochordates to mammals, implying the evolutionary conservation of aqu-miR-2017-3p. The homologues of other sponge miRNAs are listed in Additional file 1.

Evolutionary distances of the 17 representative species

Because the argonaute (AGO) protein is an important factor in miRNA function, we downloaded 80 argonaute protein family sequences from Ensembl [32] and Ensembl Metazoa [33] based on gene trees ENSGT00760000119148 and EMGT00840000133527. We then constructed a phylogenetic tree using ClustalW [34] and a maximum likelihood algorithm [35, 36] (Additional file 2). We found that the sequences were classified into two distinct groups, AGO subfamily and piwi subfamily that were evolved independently. Pfam [37] and the CD-search Tool [38] were further used to predict the featured domains of these AGO proteins (Additional file 3). Combined the phylogenetic tree and the featured domains, the sequences of PIWI subfamily could be clearly separated from AGO subfamily. Then, the sequences of AGO subfamily were taken and the protein records with incomplete sequence were excluded through the following steps.First, the proteins required at least five of the six domains predicted by Pfam (PF02170.20, PF02171.15, PF08699.8, PF16486.3, PF16487.3, and PF16488.3) and four of five domains predicted by CD-search (215,631, 239,212, 285,861, 293,095, and 240,015). Second, the within-group mean distance of each species should exceed 1. Finally, we used 48 proteins to construct a phylogenetic tree. By calculating the between-group mean distances, we determined the representative evolutionary distances of each species (Fig. 4a).

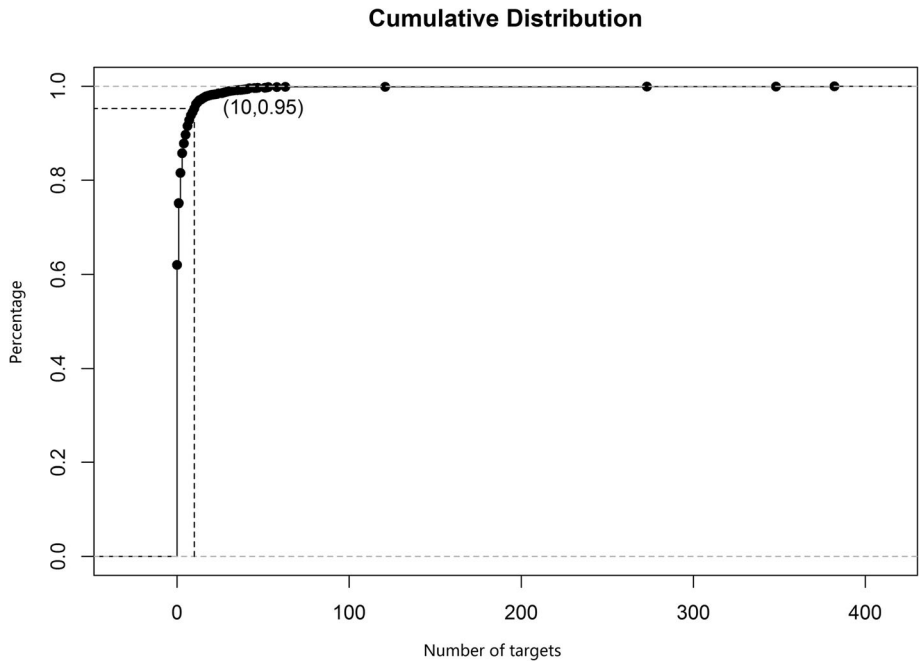

Fig. 1 Cumulative distribution of the numbers of viral sequences targeted by phylum Porifera miRNAs. The X-axis represents the target number, and the Y-axis represents the cumulative percentage of viruses

Because miRNAs can be used as excellent phylogenetic markers [39], we downloaded miRNA family information from miRBase [31] and calculated the number of miRNA families (n) shared by any two species. The evolutionary distances were calculated as 1/n. Because only a few miRNA families are annotated in *A. queenslandica* or *Nematostella vectensis*, we calculated the evolutionary distances of only 15 species. The results were similar to the evolutionary tree calculated with the argonaute proteins, with a correlation coefficient (R^2) of 0.817 (Fig. 4b). Figure 5 shows a heatmap drawn from the calculated evolutionary distances. Based on these results, it is reasonable to treat the evolutionary distances calculated from the AGO proteins as a timeline representing the functional transition of the miRNAs.

Targeting on viruses by miRNAs of 17 species

To systematically analyse the functions of miRNAs during evolution, we firstly focused on the species with known infecting viruses. Among three viruses infecting *C. elegans*, one is significantly targeted by *C. elegans* miRNAs ($p < 0.01$). Six of eight viruses infecting *D. melanogaster* are significantly targeted by *D. melanogaster* miRNAs. Six of 11 *Gallus gallus* infecting viruses are significantly targeted by

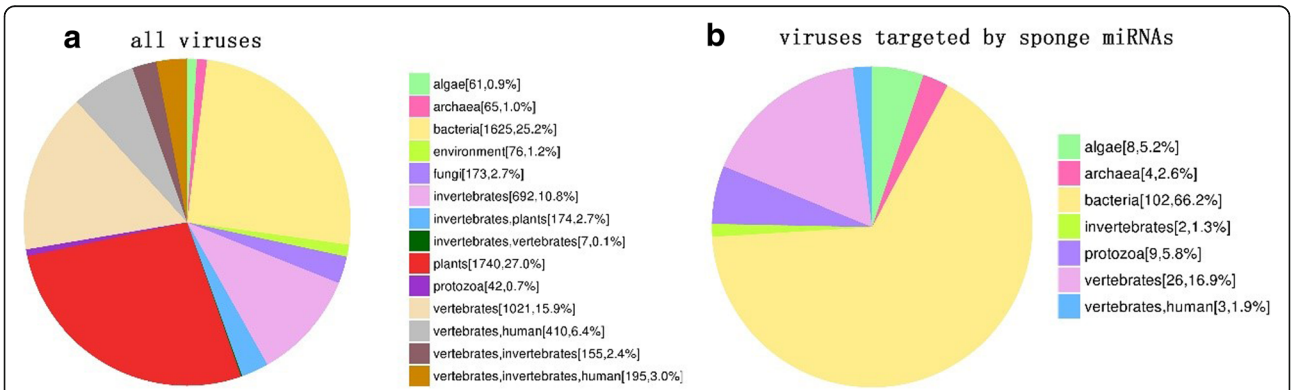

Fig. 2 Proportions of viral hosts. **a**. Hosts of all the included viruses, showing that the majority of viruses infect vertebrates and humans; **b**. Viruses significantly targeted by sponge miRNAs, showing that the majority are viruses that infect bacteria

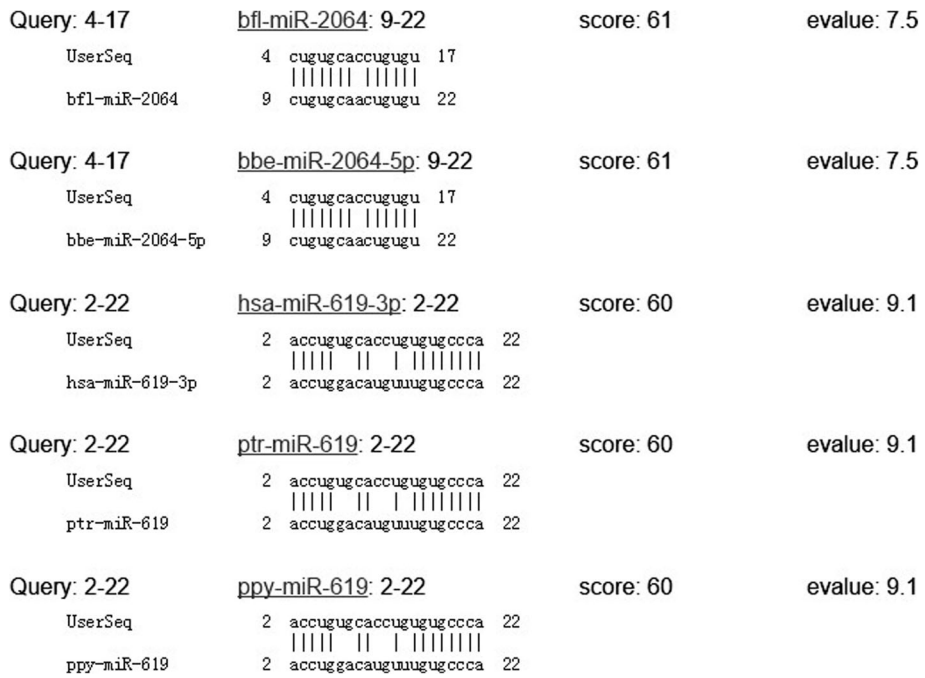

Fig. 3 Homologues of aqu-miR-2017-3p in vertebrates. "score" and "evalue" are calculated by blastn. "score" quantifies the similarity of two sequence and "evalue" quantifies the reliability of the score

G. gallus miRNAs. Detailed viral information is given in Additional file 4. Of the 675 viruses that infect humans, approximately 89% (599) are significantly targeted by human miRNAs (Fig. 6).

We then predicted the targets of miRNAs of the other 16 species in all viruses and compared them with the targets of sponge miRNAs. Figure 7 shows the proportions of viruses infecting different hosts that are significantly targeted by *H. sapiens, G. gallus* and *N. vectensis* miRNAs. This demonstrates the obvious differences between *H. sapiens, G. gallus*, *N. vectensis*, and the phylum Porifera. Of all the viruses that can be significantly targeted by *N. vectensis* miRNAs, approximately 77% are bacteriophages. Of all the viruses that could be significantly targeted by *G.*

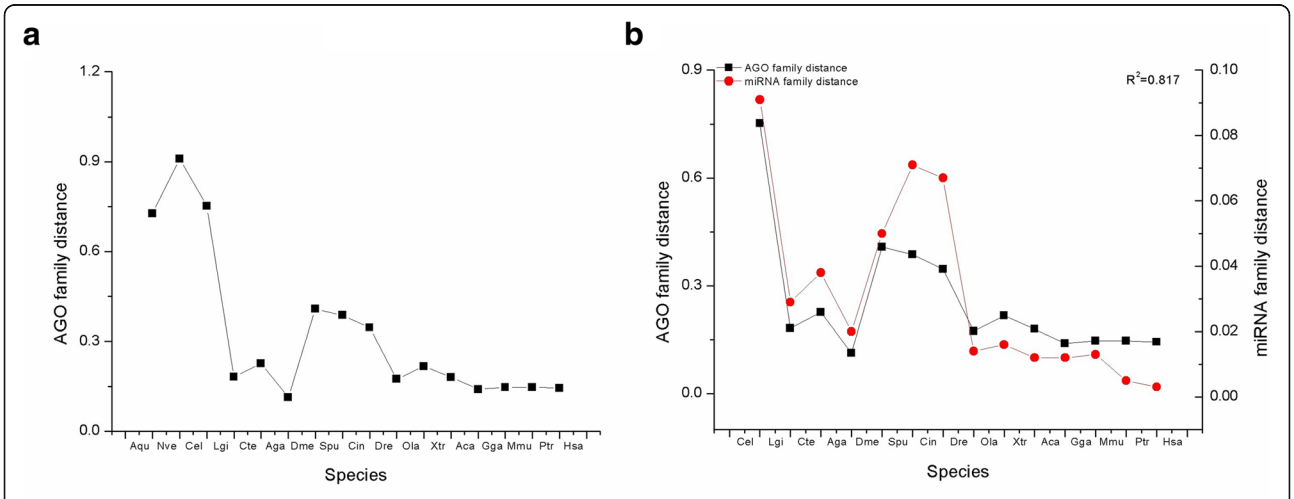

Fig. 4 Evolutionary distances of species. **a**. Evolutionary distances of 17 species calculated with AGO proteins. The X-axis shows the different species, and the Y-axis shows the evolutionary distances of two adjacent species; **b**. Evolutionary distances of 15 species calculated with AGO proteins and miRNA families. The red line indicates the distances calculated with the miRNA family information, and the black line indicates the distances calculated with AGO proteins

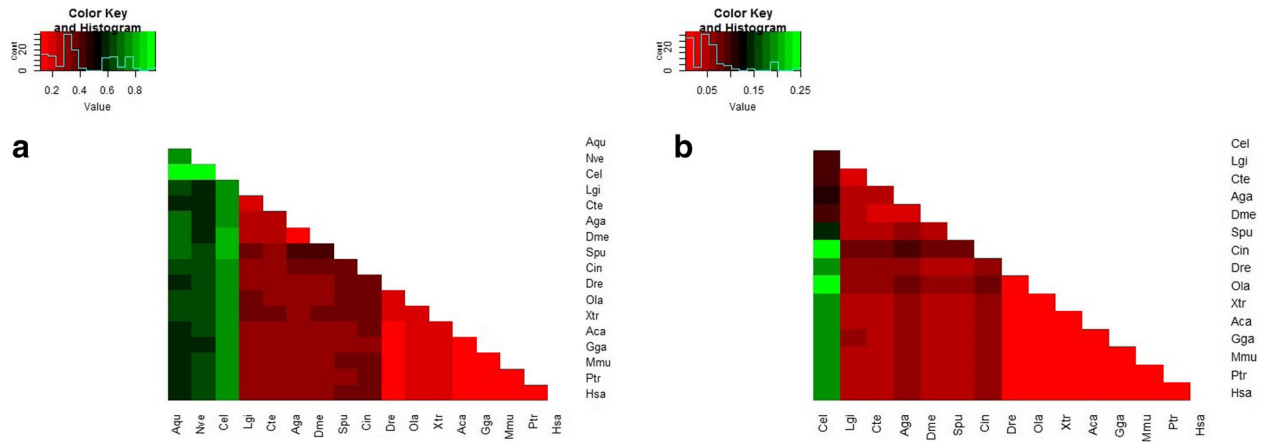

Fig. 5 Heatmaps drawn using the evolutionary distances derived with. **a**. AGO proteins; **b**. miRNA family information

gallus miRNAs, only 52.6% are bacteriophages and approximately 31% are viruses that infect vertebrates. Only 41.2% of the viruses targeted by human miRNAs are bacteriophages, and 20.7% are viruses that infect vertebrates, showing a change in the distribution of miRNA targets during evolution. The results for other species are given in Additional file 5. Another interesting result is that the genome types of viruses that are targeted by different species have changed in the course of evolution. Figure 8 shows the results for representative ancient and modern species. Other species are listed in Additional file 6. These results show that the miRNAs of ancient species tended to target double-stranded DNA (dsDNA) viruses. Among the invertebrates, RNA viruses can infect *C. elegans* and *D. melanogaster*. The types of infecting viruses suddenly exploded in *G. gallus*, and the infecting RNA viruses increased greatly. Studies have shown that the majority of viruses in prokaryotes have dsDNA genomes. In contrast, RNA viruses constitute most of the eukaryote virome, although DNA viruses are also common [40]. These results reflect the evolution of viruses and support the hypothesis that miRNAs target infecting viruses.

Based on phylogenetic analyses, we predicted the targets of the miRNAs of 17 species in all viruses. The number of targets of miRNAs of each species was normalized by being divided bythe number of miRNAs to study the evolutionary features of miRNA functions (Fig. 9). The results showed that the number of targets of an average miRNA in *A. queenslandica* was approximately 489, more than in *G. gallus* and later species, which had approximately 400 targets per miRNA. However, the average number of targets per miRNA in the species between *A. queenslandica* and *G. gallus* on the evolutionary tree was lower, at approximately 200. These

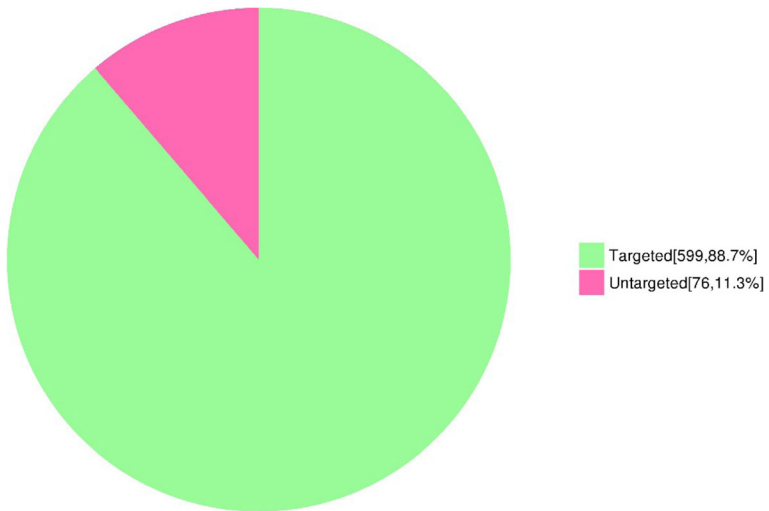

Fig. 6 The targeting of human miRNAs to human infection viruses. The green portion represents the viruses that are significantly targeted by human miRNAs and the pink portion represents the viruses that are not significantly targeted by human miRNAs

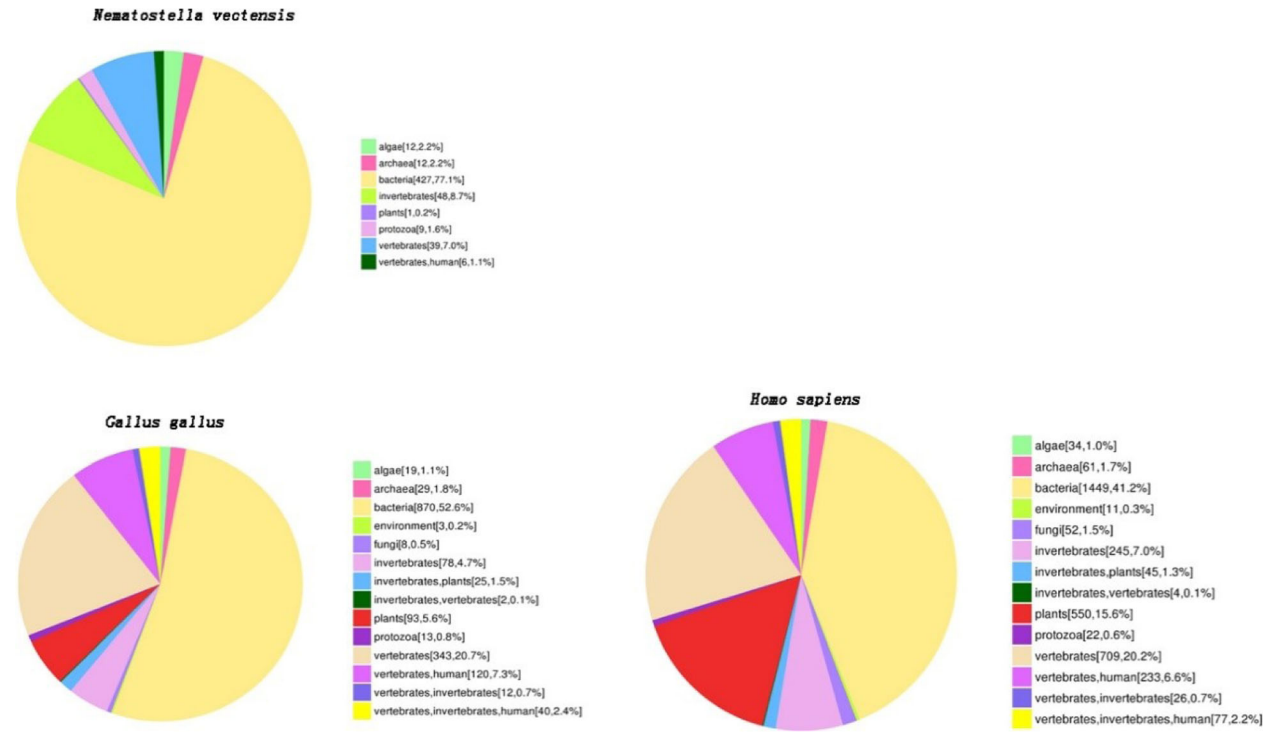

Fig. 7 Distribution of the categories of viruses significantly targeted by *N. vectensis*, *G. gallus*, and *H. sapiens* miRNAs. Viruses were categorized according to the infected hosts, and were represented by different colours

results imply that miRNAs defended ancient species against viruses, but this function was weakened during the evolution of both viruses and their hosts. Viruses and their hosts then evolved more strategies to compete with one another, and the host miRNAs either inhibited viral infection or took advantage of the viral infection.

MiRNA targets in host genomes

The miRNA targets in self-genes of the 17 species downloaded from Ensembl [41] were also predicted. The results were compared with random gene sequences to calculate the *p* values. Unsurprisingly, the miRNAs of all 17 species had significant p values (< 0.01) because the miRNAs were directed towards self-genes. However, the p values differed between different species (Fig. 10). During evolution, the p value decreased, indicating that the significance of miRNAs targeting self-genes increased. Combined with the results for viruses, it is clear that *A. queenslandica* miRNAs have more targets in viruses than in self-genes. In contrast, the miRNAs of *H. sapiens* have targets in both viruses and self-genes.

Discussion

The innate immune system constitutes the first line of defence against inherent and environmental threats, and therefore plays a vital role in the early recognition of invading organisms [42]. However, no vertebrate-like immune system exists in simple multicellular animals or unicellular organisms. They may protect themselves from invading organisms by producing secondary metabolites or small RNAs. Prokaryotes use CRISPRs to protect themselves from foreign nucleic acids. This is a nucleic-acid-based defence mechanism, such as RNAi, which uses dsRNA and RNAse III enzymes to silence gene expression. Several lines of evidence indicate that RNAi plays a role in the antiviral immunity of invertebrates, such as *C. elegans* and *D. melanogaster*. miRNA also functions as a kind of RNAi, and because invertebrates have an RNAi system, miRNAs may also function as an antiviral mechanism, as they do in mammals. Although miRNAs are commonly thought to regulate the balanced expression of genes, this may not have been their original function because numerous miRNAs are not essential for organismal viability [12]. Increasing numbers of miRNAs in mammals have been found to defend against viruses, so it is reasonable to speculate that miRNAs may have retained this function from very early in the evolution of animals or that the initial function of miRNAs was antiviral because viruses exerted a more powerful evolutionary pressure than the need for balanced gene expression.

Understanding the initial function of miRNAs and their evolution will improve our understanding of the evolutionary relationship between viruses and miRNAs. The

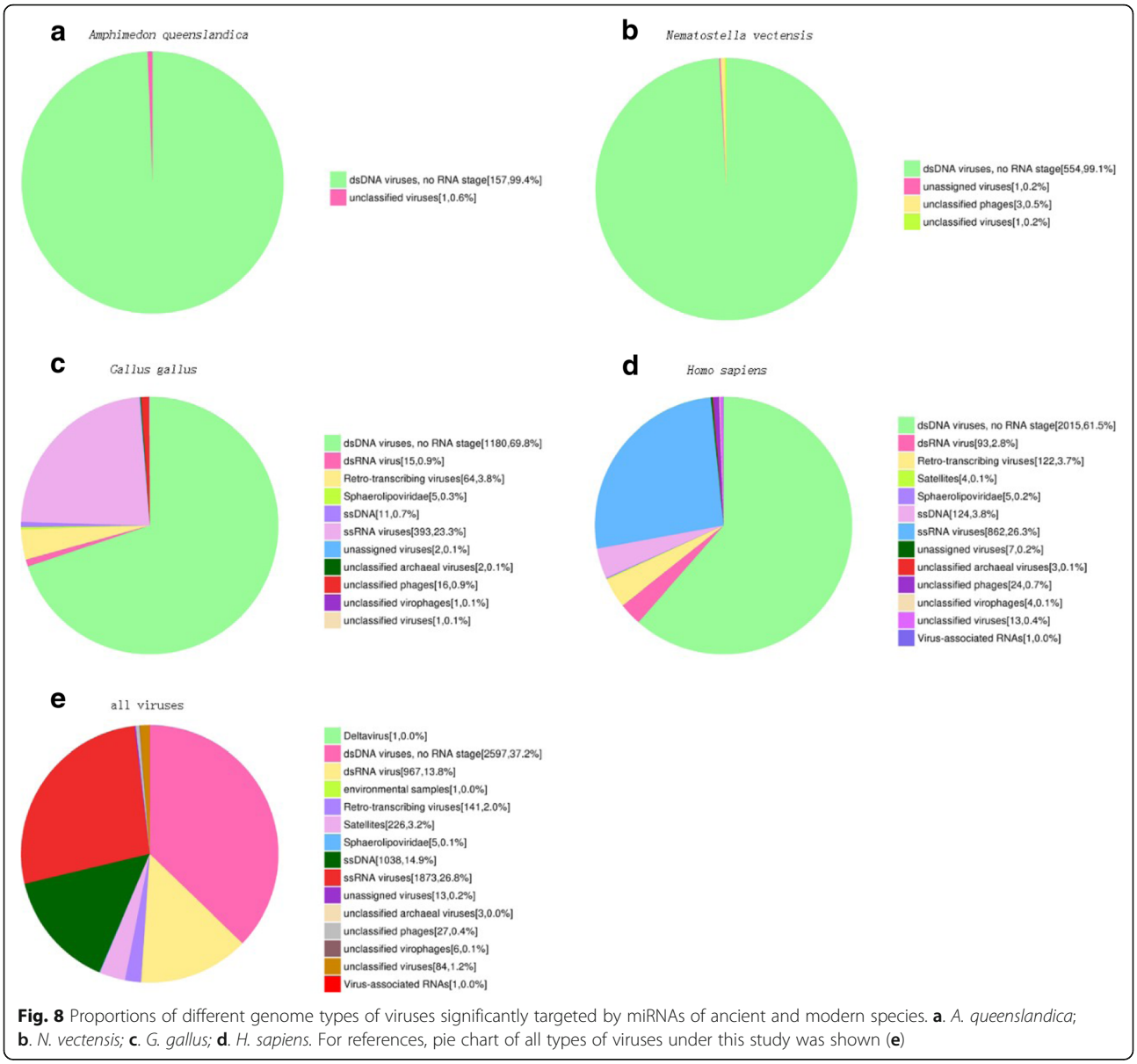

Fig. 8 Proportions of different genome types of viruses significantly targeted by miRNAs of ancient and modern species. **a**. *A. queenslandica*; **b**. *N. vectensis*; **c**. *G. gallus*; **d**. *H. sapiens*. For references, pie chart of all types of viruses under this study was shown (**e**)

predicted targets of 16 *A. queenslandica* miRNAs in all viruses showed more significant associations than random sequences. This suggests that miRNAs function as a defence mechanism to resist the invasion of viruses. This function was weakened slightly as evolution proceeded, and a more complex regulatory mechanism was ultimately generated. The details are difficult to discern because little information is available regarding viruses, such as their evolution and their infection of different hosts. Despite this, we have used big data to identify the trends in the antiviral functions of miRNAs. The computational results for the miRNAs of 17 representative species against all viruses showed that the average target number per miRNA has changed with evolution in a parabolic way. This implies that the targeting of viruses by miRNAs was strong in the early stages of evolution, but weakened slightly as viruses evolved more rapidly; then, it strengthened again, which may be attributable to the coevolution of the host and its viruses. This detailed study has shown that the miRNAs of ancient species target more sites in dsDNA viruses and viruses that infect bacteria than do later species, implying that dsDNA viruses and bacteriophages infected ancient species.

We also studied the functions of miRNAs on self-genes. By comparing self-gene targets with random sequences, we found that the targeting specificity of the miRNAs against self-genes increased during evolution. Because different species have different numbers of genes and miRNAs, we compared species by calculating p values, which also showed the targeting

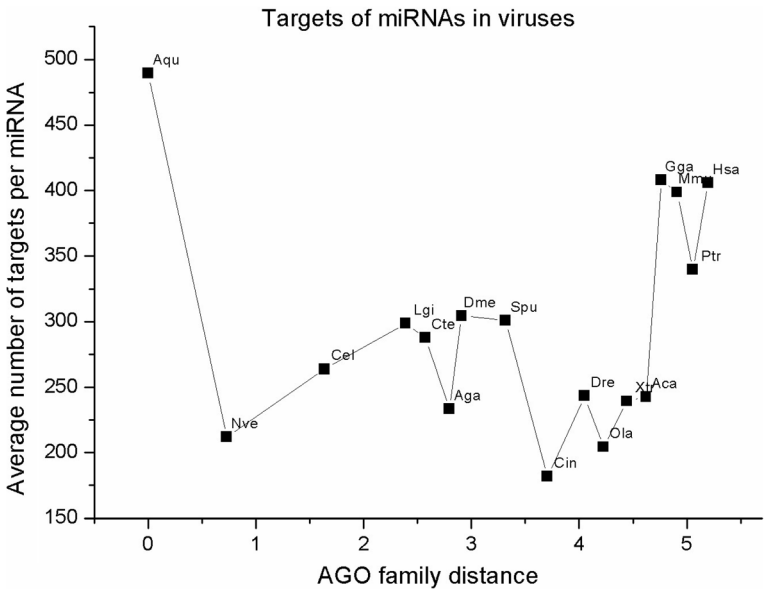

Fig. 9 Average numbers of targets in all viruses per miRNA of the 17 different species investigated. The X-axis shows the evolutionary distances of the species, and the Y-axis shows the average numbers of targets per miRNA

specificity of genes by miRNAs. It is clear that *A. queenslandica* miRNAs tend to target viruses more often than self-genes. *Amphimedon queenslandica* miRNAs have more targets in viral genomic sequences and fewer targets in self-genes than other species. Compared with random sequences, the p value was smaller for viruses than for self-genes, which confirms our hypothesis that the initial function of miRNAs was in defending the host against invading organisms, such as viruses.

Other studies into enzymes such as Dicer are consistent with our results. Five *Dicer* genes are present in *A. queenslandica*, more than in other metazoan phyla [43]. One function of the Dicer protein is to generate miRNAs, implying that the miRNAs of *A. queenslandica* are more efficient in their defence against viruses.

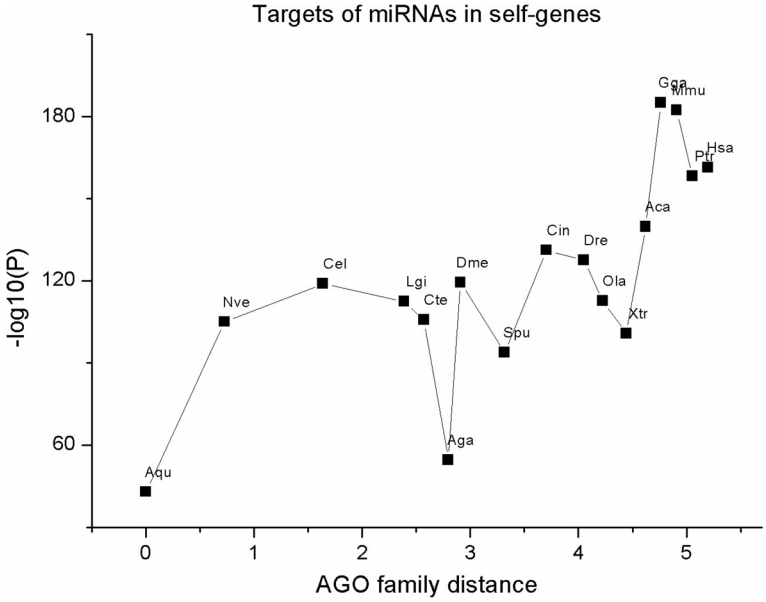

Fig. 10 Targets of miRNAs in the self-genes of the 17 species investigated. The X-axis shows the evolutionary distances of the species, and the Y axis shows –log10(P) of the different species compared with random sequences

Conclusions

Based on the miRNA target investigation in virus genomes and 17 metazoan genomes, we proposed that the initial function of miRNAs in early species was predominantly antiviral. During the evolution, later species evolved miRNAs that target more specifically on their own genomes. This may suggest that the origin of miRNA could possibly be defensive relevant.

Additional files

Additional file 1: The homologues of A. queenslandica miRNAs. The vertebrate miRNAs which show sequence similarity to the A. queenslandica miRNAs are listed in this file. (DOCX 14 kb)

Additional file 2: The phylogenetic tree of ago proteins. The phylogenetic tree of all ago and ago-like proteins of the 17 species investigated. (PNG 84 kb)

Additional file 3: The predicted domains of all ago and ago-like protein of the 17 species investigated. The domain IDs along with the *p* values of the predicted domains were listed in this file. (XLSX 23 kb)

Additional file 4: Viruses that infect *Caenorhabditis elegans*, *Drosophila melanogaster* and *Gallus gallus*. (DOCX 14 kb)

Additional file 5: The distribution of categories of viruses significantly targeted by miRNAs of 17 species. Viruses were categorized according to the infected hosts, and were represented by different colours. (DOCX 1317 kb)

Additional file 6: The distribution of genome types of viruses significantly targeted by 17 species. (DOCX 1116 kb)

Abbreviations

AGO: Argonaute; CRISPRs: Clustered regularly interspaced short palindromic repeats; dsRNA: Double-stranded RNA; HCV: Hepatitis C virus; IFN: Interferon; miRNA: microRNA; NCBI: National Center for Biotechnology Information; PFV-1: Primate foamy virus type 1; RNAi: RNA interference; siRNAs: small interfering RNAs; VSV: Vesicular stomatitis virus

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Availability of data and materials

All data generated during this study are included in this published article and its Additional files 1, 2, 3, 4, 5, 6.

Authors' contributions

XL, XZ and HF carried out the computation and drafted the manuscript; CY conceived the study; JW and QZ supervised the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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