Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 6, 2015

Original Article

IN SILICO PREDICTION AND VALIDATION OF MICRORNAS FROM JAPANESE ENCEPHALITIS VIRUS (JEV)

NEEMA TUFCHI[1], KUMUD PANT[1*], BHASKER PANT[2], SYED MOHSIN WAHEED[1*]

[1]Department of Biotechnology, Graphic Era University, 566/6 Bell Road, Clement Town, Dehradun, Uttarakhand, India, [2]Department of Information Technology, Graphic Era University, 566/6 Bell Road, Clement Town, Dehradun, Uttarakhand, India
Email: pant.kumud@gmail.com

Received: 15 Nov 2014 Revised and Accepted: 02 May 2015

ABSTRACT

Objective: MicroRNAs are endogenous, small, single stranded, non coding RNAs having 19-25 nucleotides. These miRNAs are complementary to their target messenger RNAs that bind principally to its 3' un translated regions (3' UTRs). Small RNAs play crucial roles in the regulation of gene expression in many eukaryotes; therefore it is important to predict potential viral miRNAs which might be involved in an establishment of Japanese Encephalitis virus (JEV) disease. Different computational approaches and methods were used for predicting viral microRNAs from the JEV genome in this work.

Methods: In the present study, the use of genome-wide computational approach has been demonstrated to predict miRNAs and their target(s) in JEV genome. Two freely accessible softwares, MiPred and Genscan were used to predict the secondary structures of the potential miRNAs.

Results: In all, 36 miRNAs were predicted and characterized by conducting genome-wide homology search against all the reported miRNAs. These miRNAs were further validated by performing phylogenetic analyses and using statistical tools.Further, attempt was made to predict the 3′ untranslated regions of mRNAs from whole genome of JEV which may prove helpful in finding putative targets of these miRNAs.

Conclusion: This is the first study to identify and validate miRNAs in JEV which is an important step in identifying putative JEV miRNAs that utilize host cell machinery, and may play a crucial role in neuroinflammation and silencing of host genes, thus demonstrating the role of viral miRNAs in establishing viral pathogenesis.

Keywords: MicroRNAs, Japanese encephalitis virus, Secondary structure prediction, MiPred, Genscan, Mirbase, RNApred, RNAfold, Mfold and minimum free energy.

INTRODUCTION

MicroRNAs (miRNAs) are small non coding RNA molecules which contain about 22 nucleotides. MiRNAs are found in all plants, animals and viruses which function in RNA silencing and post-transcriptional regulation of gene expression [1]. Moreover, miRNAs play role in regulation of many cellular and developmental processes such as cell proliferation, neurogenesis, endocrine function and apoptosis [2]. Genes that encode for the miRNAs are mostly found in the intronic regions, their transcription generates the precursor miRNA known as primary miRNA (pri-miRNA).Drosha enzyme cleaves the long fold-back hairpin precursor, and the cleaved pri-miRNA generates 80-100 nucleotide stem-loop precursor-premature miRNA (pre-miRNA).These pre-miRNAs are exported to the cytoplasm by Exportin5 proteins, where the ribonuclease III Dicer-like (DCL) enzyme cleave the pre-miRNAs, to release the miRNA/miRNA* duplex. The miRNA released after the Dicer activity binds to the complementary mRNA forming the RNA Inducing Silencing Complex (RISC). The miRNA-mRNA duplex is degraded. The miRNA is designated as the guide strand, while miRNA* as the passenger strand [3]. In animals, target genes are recognized by miRNAs through miRNA-complementary sites located at the 3 - untranslated regions (UTR), whereas plant miRNAs usually recognize one motif in the coding region of their targets [4].MiRNAs were first discovered in 1993, when the migraine Lin-4 was determined to down regulate expression of the gene Lin-14 in *Caenorhabditiselegans* by the Ambrose and Ruvkun laboratories [5]. Since Lin-4 homolog is absent in other species, this finding was considered unique [6]. About one thousand miRNAs have been identified in humans and are assumed to regulateexpression of more than half of the protein coding genes. A single type of miRNAperhaps may regulate hundreds of such genes [7].

Japanese encephalitis virus (JEV) is one of the major causes of viral encephalitis in humans. JEV is a mosquito borne disease which causes inflammation of the brain. The main vectors of this disease are *Culextritaeniorhynchus* and *Culexvishnui*[8] and transfer this virus to humans. The disease is most prevalaent in East Asia (countries of western Pacific region) and Southeast Asia, wading birds and domestic pigs are the reservoirs of this virus [8]. This disease was first recognized in India in 1955 [9]. JEV belongs to the family Flaviviridae and has an incubation period of 5-15 days. In most JEV infections, symptoms are mild like fever and headache, but in severe cases, high fever, headache, neck stiffness, seizures, coma, paralysis and death may follow. The persons who survive with this virus suffer permanent intellectual, behavioral or neurological problems like aphasia (speech impairmenet), recurrent seizures and paralysis[10]. The persons who live in or travel to the JE endemic areas are more vulnerable to get infected. There are currently three vaccines available: SA14-14-2, IC51 (marketed in Australia and New Zealand as JESPECT and elsewhere as IXIARO) and ChimeriVax-JE (marketed as IMOJEV). All these vaccines are based on the genotype III virus [8]. In spite of these three vaccines that too against only one geneo type of the virus, there is no treatment for this disease only preventive measures can be taken to safeguardagainst this deadly disease. In the present study, we identified and characterized potential viral miRNAs, which may be useful in predicting their target(s), and elucidated their phylogenetic relationship with those reported from other viruses.

In-vitro identification of miRNAs and their targets is difficult and complicated. Thus, several computational methods have been developed and employed for reliable and rapid identification of miRNA genes. There are many approaches that are being used for the prediction of miRNAs, but the one which is based on phylogenetic conserved sequences across multiple species is reported to provide more reliable predictions of functional miRNAs [1]. In this study, we attempted to find novel miRNAs from JEV genome that may serve as potential targets for anti-viral drug therapies by inhibiting the binding of these molecules in the host target genes. The current work is the first report to predict and

validate JEV genome derived miRNAs. Work carried out by other researchers focused solely on host miRNAs which get expressed in response to host-virus interactions during neuroinflammation in microglial cells [11-13]. Potential JEV miRNAs reported here may prove more potent drug targets than any anti-viral therapy hitherto reported. Since these miRNAs may have targets in host genome, or may have protective role to prevent viral genome from degradation by host nucleolytic degradation,thus may be conserved compared to other viral genome sequences that are more prone to mutations and are targets of therapies currently being tried[(12,14].

MATERIALS AND METHODS

Sequence Retrieval

The complete set of genome of JEV was retrieved from NCBI (National Center for Biotechnology Information) [15]. Mirbase release (version 20) database contains 24 521 microRNA loci available from 206 species that after processingproduce 30 424 mature MicroRNA products (http://www.mirbase.org/) [16].

Homology search and Multiple Sequence alignment

A BLASTn search of all the 24521 miRNA sequences with the whole genome sequence of JEVwas carried out with the evalue < 0.01 and the default parameters were used, including low complexity filter. The two criteria employed to screen the BLAST results were: (1) more than 80% identity between the compared potential and the corresponding miRNA in data set (i.e., JEVmiRNA and the corresponding miRNA in the reference set-a known murine homolog); (2) the length difference between the compared dataset(JEVmiRNA and the corresponding miRNA) is not more than three bases. The whole genome of JEV was then aligned with all the miRNAs which were predicted after BLASTn by clustalW. Clustal W is nucleic acid and protein sequence alignment program for three or more sequences. There are three main steps involved inclustalW: (1) Pairwise alignment; (2) creation of phylogenetic tree; and (3) use of the guide tree to carry out multiple sequence alignment.

Secondary Structure Prediction

Extracted miRNA precursor sequences were checked for their secondary structure. There are many types of softwares for prediction of the secondary structures of RNA or DNA. Predicted miRNA sequences were then submitted to RNAfold [17] and Mfold [18] for checking and generating of the fold-back secondary structure. The RNAfold web server predicts the secondary structure of a single stranded DNA or RNA sequences associated with the minimum free energy of the RNA sequences. It also calculates the partition function (pf) and base pair probability matrix. It generates a "dot plot" of the base pairing matrix and produces files with plots of the resulting secondary structure. The dot plot depicts a matrix of squares having an area proportional to the pairing probability in the upper half, and one square for each pair in the minimum free energy structure in the lower half of the plot[17]. The 'mfold' RNA folding software was developed in the late 1980s [18]. The 'm' simply refers to 'multiple'. The core algorithm predicts a minimum free energy,δG, as well as minimum free energies for foldings that must contain any particular base pair [17].

Mi Pred

Mired is a web server that distinguishes between the real and pseudo micro RNA precursors (http://www.bioinf.seu.edu.cn/miRNA/). It uses a random forest prediction model for the classification of real and pseudo micro RNAs. To differentiate the real pre-miRNAs from other hairpin sequences that might be pseudo pre-miRAS having similar stem-loops, a hybrid feature consisting of local contiguous structure, sequence composition, minimum free energy (MFE) of the secondary structure and *P*-value of randomization test is used in Mired[19].

Genscan

Genscan is a program that identifies complete gene structures in the genomic DNA sequences from a variety of organisms, including human, other vertebrates, invertebrates and plants [19, 20]. It is used to predict the locations of genes and their exon- intron boundaries within genes of a genomic sequence,and also to predict multiple genes in a sequence, consistent sets of genes occurring on either or both DNA strands and to deal with partial as well as complete genes**.** GENSCAN is shown to have substantially higher accuracy than existing methods when tested on standardized sets of human and vertebrate genes, with 75 to 80% of exons identified exactly [22, 23]. For each of the selected genes, the region from the end of the stop codon until the beginning of poly-A was assigned as 3′UTR and was extracted to predict exon and intron boundaries. The overview of the steps involved in 3' UTR extraction is shown in fig. 1.

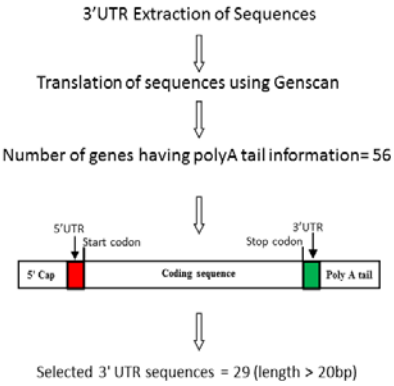

Fig. 1: An overview of the steps followed for extracting 3'UTR sequences in JEV

RESULTS AND DISCUSSION

Prediction of miRNAs

For prediction of miRNA from the JEVgenome, in this approach mature miRNA sequences from viruses were taken as a reference database. The JEV genome was matched with the reference database using BlastN and the hits obtained were further used for prediction of miRNA precursors. Blast N was used with default settings for this purpose. In total 36 sequences were predicted as putative miRNA from the JEV genome by this search method. The sequences were then selected for secondary structure prediction. We used two online web servers for secondary structure prediction ie; RNAfold and Mfold. Fig. 2 shows the result page of RNAfold web server for secondary structure prediction which indicates that the secondary structure of miRNA has the minimum free energy of-43.10 kcal/Mol. In addition to the minimum free energy (MFE) of the structure, it gives a coarse representation of the base pairing probabilities in the form of a pseudo bracket notation, followed by the ensemble free energy, as well as the centroid structure derived from the pairing probabilities together with its free energy and distance to the ensemble. Fig. 3 illustrates the graphical output of 4 predicted miRNA candidates. Figure 3 illustrates the graphical output of 4 predicted miRNA candidates. M Fold program calculates the minimum free energy (MFE) contributed by various probable secondary structures. Free energy (δG) values of all the 36 miRNA sequences and their secondary structure prediction by Mfold are listed below in table 1.

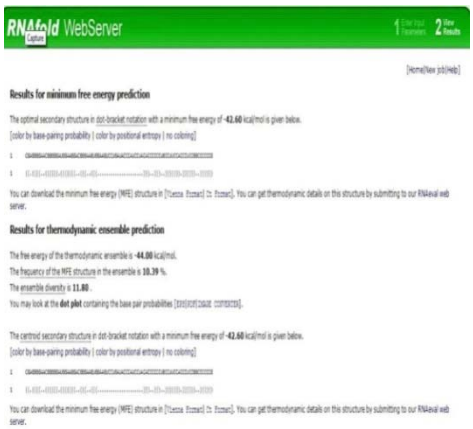

Fig. 2: Screenshot of RNAfold Webserver for RNA secondary structure prediction

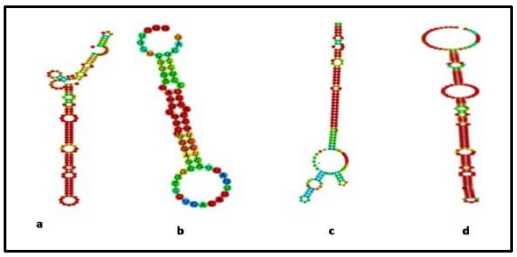

Fig. 3: Graphical output generated by RNAfold web server (http://rna. tbi. univie. ac. at/cgi-bin/RNAfold. cgi): a) RNA folds of sequence 4; b) RNA folds of sequence 3; c) RNA folds of sequence 7; d) RNA folds of sequence 10

In animals, miRNAs primarily target the 3'UTRs of target mRNA(s) [24, 25]. There are only a few recent reports indicating that target mRNAs are also repressed by miRNA-binding sites on 5 ' UTR or coding regions as efficiently as in the 3 'UTR [26, 27]. Hence, we restricted our search for targets only to the 3 ' UTRs of mRNAs.

Thus, using the whole genome of JEV, we extracted the 3'UTR sequences. In plants, miRNAs bind their targets with complete or nearly complete complementarity [28, 29]. By contrast, animal miRNAs are partially complementary to their target mRNAs. This has rendered computational approaches for target identification based merely on reverse complementary searches quite challenging in animals [30, 31]. Subsequently the sequence which shows the appropriate secondary structure and minimum free energy was selected and further classified as real or pseudo precursor sequences using the Mipred web server as shown in fig. 4. It is based on the statistical calculation of reference dataset, which predicts whether the candidate precursor miRNA is real or pseudo.

Table1: δG values for secondary structure in Mfold

Sequence number	δG value (kcal/Mol)	Length of sequence
Sequence 1_JEV	-28.00	64 bases
Sequence 2_JEV	-17.02	24 bases
Sequence 3_JEV	-44.00	26 bases
Sequence 4_JEV	-46.80	64 bases
Sequence 5_JEV	-32.04	22 bases
Sequence 6_JEV	-18.40	19 bases
Sequence 7_JEV	-58.38	60 bases
Sequence 8_JEV	-39.09	26 bases
Sequence 9_JEV	-53.70	62 bases
Sequence 10_JEV	-41.79	65 bases
Sequence 11_JEV	-29.20	21 bases
Sequence 12_JEV	-43.09	33 bases
Sequence 13_JEV	-12.75	17 bases
Sequence 14_JEV	-24.40	21 bases
Sequence 15_JEV	-40.60	39 bases
Sequence 16_JEV	-28.70	22 bases
Sequence 17_JEV	-14.57	19 bases
Sequence 18_JEV	-49.70	34 bases
Sequence 19_JEV	-27.56	26 bases
Sequence 20_JEV	-33.30	29 bases
Sequence 21_JEV	-16.20	20 bases
Sequence 22_JEV	-32.50	22 bases
Sequence 23_JEV	-62.60	42 bases
Sequence 24_JEV	-52.50	39 bases
Sequence 25_JEV	-33.10	26 bases
Sequence 26_JEV	-35.10	22 bases
Sequence 27_JEV	-20.36	17 bases
Sequence 28_JEV	-33.80	25 bases
Sequence 29_JEV	-14.31	12 bases
Sequence 30_JEV	-50.30	30 bases
Sequence 31_JEV	-22.19	26 bases
Sequence 32_JEV	-29.40	19 bases
Sequence 33_JEV	-37.70	26 bases
Sequence 34_JEV	-28.70	29 bases
Sequence 35_JEV	-19.70	27 bases
Sequence 36_JEV	-28.00	32 bases

Fig. 4: Snapshot of MiPred result: Classification of real and pseudo miRNA precursors using Random Forest Prediction model with combined features (http://www. bioinfo. seu. edu. cn/miRNA/)

CONCLUSION

The main objective of the present study was to perform computational analysis of the JEV genome for identifying probable miRNA and its precursor sequences. MiRNA analyses were done by using both sequence based and structure based prediction algorithms through various web based tools. In this approach Mirbase was used as a reference database to analyze small subsequences from the JEV genome that were used as queries for the subsequent analyses.

Then, analyses of sequences were done by RNAfold and Mfold web servers for determining thermodynamic stability and prediction of RNA secondary structure. Further, the precursor miRNA sequence classified in the Mipred web server was analyzed using statistical approach by random forest method into real or pseudo precursor miRNA sequence. MiRNAs pair partially with the target mRNAs in order to block translational expression. Since no research has been done till date in predicting the miRNAs in JEV viruses. Thus, the predicted miRNAs reported in the present study will serve as potential resource to initiate their experimental validation. By using this approach we can use miRNAs to counteract this virus and its replication. In other words, these miRNAs can be used for adopting therapeutic approachusing antisense oligonucleotides and other drugs against JEVto block expression of these viral miRNAs that may have number of targets in host genome to silence and exploit host responses,and establish pathogenesis. Antisense oligonucleotides may work as competitive inhibitors by annealing miRNAs, possibly to the mature miRNA guide strand, thus inducing degradation or stoichiometric duplex formation [32]. Using antisense oligo nucleotides may prove helpful in two ways, firstly, that it may block the JEV miRNAs. Secondly,once the viral miRNAs are blocked, host may be able to develop its immune response against the JEV infection, which is normally suppressed by the virus. This strategy may work in combination with other drug therapies or even alone.

ACKNOWLEDGEMENT

This work was carried with the aid of UCOST project UCS&T/R&D/lS_18/12-13/6141. We are highly thankful to UCOST, Dehradun for funding this project.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Reference. Available from: http://en.wikipedia.org/wiki/ MicroRNA.
2. Singh J, Nagaraju J. *In silico* prediction and characterization of micro RNAs from red flour beetle (*Tribolium castaneum*). Insect Mol Biol 2008;17:427-36.
3. Kamarajan BP, Sridhar J, Subramanian S. *In silico* prediction of MicroRNAs in plant mitochondria. Int J BIOautom 2012;16:251-62.

4. M Zhou, Q Wang, J Sun, X Li, L Xu, H Yang, *et al. In silico* detection and characteristics of novel microRNA genes in the *Equuscaballus* genome using an integrated *ab initio* and comparative genomic approach. Genomics 2009;94:125–31.
5. Lau NC, Lim NP, Weinstein EG, Bartel DP. An Abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditiselegans*. Sci 2001;26:858-62.
6. Kalaria R, Patel N. In silico candidates miRNA prediction from genome *Dictyosteliumdiscoideum*. Int J Pharm Biol Res 2012;3:41-50.
7. Yue D, Meng J, Lu M, Chen C, Guo M, Huang Y. Understanding microRNA regulation: a computational perspective. Signal Processing Magazine IEEE 2012;29:77–88.
8. Reference. Available from http://en.wikipedia.org/ wiki/ Japanese_encephalitis.
9. Reference. Available from: http://www.icmr.nic.in/pinstitute/ niv/JAPANESE%20ENCEPHALITIS.pdf.
10. Reference. Available from: http://www.who.int/mediacentre /factsheets/fs386/en/.
11. Thounaojam CM, Kundu K, Kaushik DK, Basu A. MicroRNA-29b modulates Japanese encephalitis virus-induced microglia activation by targeting tumor necrosis factor alpha-induced protein 3. J Neurochem 2014;129:143-54.
12. Wu Z, Xue Y, Wang B, Du J, Jin Q, Broad-spectrum antiviral activity of RNA interference against four genotypes of japanese encephalitis virus based on single MicroRNA polycistrons. PLoS ONE 2011;6:e26304.
13. Thounaojam CM, Kundu K, Kaushik DK, Swaroop S, Mahadevan A, Shankar SK, *et al.* MicroRNA 155 regulates japanese encephalitis virus-induced inflammatory response by targeting src homology 2-containing inositol phosphatase 1. J Virol 2014;88:4798-10.
14. Sharma N, Verma R, Kumawat KL, Basu A, Singh SK. miR-146a suppresses cellular immune response during Japanese encephalitis virus JaOArS982 strain infection in human microglial cells. J Neuroinflammation 2015;12:30.
15. Reference. Available from: http://en.wikipedia.org/wiki/ National_Center_for_Biotechnology_Information.
16. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 2014;42:D68-73.
17. Reference. Available from: http://bibiserv.techfak.uni-bielefeld.de/rnafold/manual.html.
18. Reference. Available from: http://www.bioinfo.rpi.edu/ applications/mfold/rna/form1.cgi.
19. Zuker M. On finding all suboptimal foldings of an RNA molecule. Sci 1989;244:48–52.
20. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406-15.
21. Jiang P, Wu H, Wang W, Ma W, Sun X, Lu Z. MiPred: classification of real and pseudo microRNA precursors using random forest prediction model with combined features. Nucleic Acids Res 2007;35:W339–44.
22. Reference. Available from: http://en.wikipedia.org/wiki/ GENSCAN
23. Reference. Available from: http://icb.med.cornell.edu/ crt/GENSCAN/index.xml.
24. Burge C, Samuel K. Prediction of complete gene structures in human genomic DNA. J Mol Biol 1997;268:78-94.
25. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. Cell 2003;113:25–36.
26. Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, Gamberi C. The *C elegans* hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev Cell 2003;4:639–50.
27. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. Cell 2002;110:513–20.
28. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci 2007;104:9667–72.
29. Jones-Rhoades MW, Bartel DP. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 2004;14:787–99.
30. Bartel B, Bartel DP. MicroRNAs: at the root of plant development. Plant Physiol 2003;132:709–17.
31. Rajewsky N. MicroNA target predictions in animals. Nat Genet 2006;38:S8–13.
32. Vella MC, Choi EY, Lin SY, Reinert K, Slack FJ. The *C. elegans* microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. Genes Dev 2004;18:132–7.