

Computational Profiling of Non-coding RNAs–viable Targets and Restorative Candidates of Influenza Viral Infection with Bioconductor Packages

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ABSTRACT

The advent of recent small non-coding RNA research enabled investigations of various non-coding factors like miRNAs, siRNAs and lncRNAs as post transcriptional modulators of viral pathogenesis either on a positive or negative note. Influenza A virus is responsible for contagious respiratory infection that invades the host cellular machinery to stimulate pathogenesis. The original public gene expression dataset GSE89008, from the Gene Expression Omnibus was analysed using the edgeR package and normalized log counts per million normalization. A variety of screening procedures were performed to understand the targets, miRNA transcripts, lncRNAs and the pathway enrichment. The interaction networks of the host mRNA-miRNA and lncRNA and viral siRNA- mRNA- host sense strands were constructed using the cytoscape. The study demonstrated a short list of miRNAs, which appear to be important regulators in virus–host interactions. Among them, hsa-mir-484, hsa-mir-1-3p, hsa-mir-149-5p, hsa-mir-615-3p, hsa-mir-34a-3p and hsa-mir-324-3p, were shown to be highly capable of suppressing influenza infection. Transcription factors KLF41, NFYB, PLAG1, FOXO1, CEBPA and STAT1 were enhanced and may present as markers of influenza A infections. Viral-siRNAs -1534, 1537, 1538, 1540 and 1658 had great inhibitory efficacy and were predicted to silence NA, M and PA segments of viruses and suppress the viral pathogenesis by silencing the host genes most predominantly, the STAT, ELF and MAP3K genes. Elucidation of this unexpected antiviral facet of noncoding RNAs, will contribute to a better understanding of influenza–host interactions and can be established as biomarkers, or targets or therapeutic agents upon extensive research in future.

Key words: Influenza A virus, Gene expression profiles, mRNA, Noncoding RNA, Interaction network, Transcription factors.

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INTRODUCTION

Influenza A virus (IAV) has been a major reason of pandemic and seasonal outbursts all over the world. Many variants of IAV are evolving due to its high mutation rate, which elicits a major challenge in both

diagnosis and treatment in spite of considerable medical advances.^[1] Viruses generally depend on the host genome to replicate and hijack the host cellular functions in a complicating way.^[2] A recent advancement in the molecular mechanism, called competing endogenous RNA (ceRNA) composed of miRNA, siRNA and lncRNAs has made the genetic mechanisms understandable. They are all non-coding but interact with the transcript mRNA, thereby either repressing the mRNA or increasing them.^[3] In many eukaryotic organisms, the mechanism of RNA interference is an important antiviral defense mechanism. Micro RNAs (mirNA) are non-coding endogenous RNAs and are either intragenic

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(Processed from introns and very few exons) or intergenic (transcribed and regulated by their own promoters).^[4] They are initially long precursors which are processed by type III ribonuclease Dicer into a ~22nucleotide long miRNA duplex along with the effectors Argonaute and in turn activate the microRNA Induced Silencing Complex (miRISC). MiRISC target the viral mRNA or genome with its sequence complementarity to a guide RNA strand called MicroRNA Response elements (MRE) and mediate the destruction of viral genes.^[5] Depending on the targets of miRNA, the agronaute guides either viral transcripts or host Cellular transcripts involved in antiviral responses exhibiting a dual action mechanism.^[6] Understanding of miRNA targeting viral transcripts can be extensively studied to design new antiviral therapies. The miRNAs are circulated to the extracellular space and act as autocrine, paracrine, or endocrine regulators to target cellular activities.^[7] Long non-coding RNAs (lncRNA) are transcripts of ~200 nucleotides which do not code proteins but are reported to interact with mRNA, Proteins, DNA and other ncRNAs.^[8] In viral infections, lncRNAs demonstrated interactions with the host miRNA or mRNA directly, thereby modifying a multitude of functions including an influential roles in the virus – host interactions.^[8] During viral infections, there are certain miRNAs circulating which bind to 3' untranslated regions of mRNA thereby involved in post transcription regulation.^[9] The miRNA acts like sponges to lncRNA and acts on the host mRNA to either upregulate or downregulate the genes necessary for viral survival and pathogenicity.^[10]

Mutual interference between viruses and host-cell's miRNA machinery has generated a more favorable cellular environment. The viruses are capable of regulating their own miRNAs, by (i) evading cellular miRNAs, (ii) damaging the miRNA pathway by interacting with some key proteins (iii) synthesizing their own miRNA (iv) utilising cellular miRNAs to favor them.^[11] Conversely, host-cell's endogenous miRNAs are also able to target viral mRNAs either by repressing or increasing viral replication.^[12]

Viral non-coding RNAs –v-miRNAs and v-siRNAs

Research investigations have unraveled that certain viral genomes encode viral miRNAs (v-miRNAs) which are expressed inside the host cells upon infection. The predominant functions of v-miRNAs are viral persistence, survival and pathogenesis. They are capable of inducing metabolic and cellular processes either by hijacking the host transcripts or suppressing them. The biogenesis of v-miRNAs is similar to those of eukaryotes and is

processed by either DROSHA or DICER complexes.^[13] siRNA are silencing RNAs, which are similar to miRNA but differ in their exogenous nature and occurrence in the cytoplasm. The mechanisms of action of siRNAs is transient and have perfect complementarity to the sense strand, whereas miRNAs are endogenous, cause translational repression and have limited complementarity.^[14] The present study involved RNA sequence analysis of Human tracheobronchial epithelial (HTBE) cells that serve as an excellent associate of influenza virus infection in the human respiratory tract. There is demonstrated evidence of infection and defense responses in human respiratory epithelial cells due to influenza infections.^[15] The mRNA sequence data set from Gene Expression Omnibus with the ID GSE89008 included the cellular transcriptome of HTBE cells at multiple time points in response to infection with influenza A/California/04/09 (H1N1), A/Wyoming/03/03 (H3N2) and A/Vietnam/1203/04 (H5N1) HALo virus.^[16] This data set was analysed for the differentially expressed genes and the competing endogenous RNA (ceRNA) network. The study enables the elucidation of the molecular components and genes that are activated due to tracheobronchial influenza infection. These ceRNAs will enable the easy identification of the infection at an earlier stage and lncRNAs can also act as therapeutic RNA candidates to suppress the overly expressed genes.^[17]

METHODOLOGY

Raw data and Signature Generation

Raw RNA-seq data for publically available GEO dataset GSE89008 was downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89008>) using the Bio-conductor package of R language and quantified to gene-level counts.^[12] The sequence data was generated from Human tracheobronchial epithelial (HTBE) cells isolated from healthy donor airway epithelial tissue. The tissues were cultured and infected with influenza A/California/04/09 (H1N1), A/Wyoming/03/03 (H3N2) and A/Vietnam/1203/04 (H5N1) and updated to GEO by Steel, *et al.* The HTBE transcriptome had 52 samples with mock control and H1N1, H3N2 and H5N1 infected samples that were collected at 3, 6, 12 and 18 hr post infection.^[13] The data set was chosen based on three criteria 1) there were enough samples to understand the molecular mechanisms 2) Functional, target and non-coding gene enrichment analysis were not performed on the transcriptome and 3) samples had three strains of IAV which enabled the comparison of the disease states. The

gene expression signature was generated by comparing gene expression levels between the control group and the perturbation group using the edgeR package of Bioconductor.^[14]

Identification of differentially expressed genes

EdgeR package of Bioconductor was used to identify the differentially expressed genes of the influenza affected HTBE dataset. The raw gene counts were normalized using the log CPM method (Log Counts Per Million) and filtered by selecting the 2500 genes with the most variable expression and finally transformed using the Z-score method.^[15] The transformed genes were subjected to Principal Component Analysis and a heatmap was generated for the normalized and transformed gene counts.^[16] Volcano plot scaled the significant values to fold change. Average gene expression was identified by calculating the mean of the normalized gene expression values and displayed on the x axis; P-values were corrected using the Benjamini-Hochberg method,^[17] versus fold-change estimated from the differential expression analysis on the Y-axis.

Gene ontology Analysis and Functional enrichment analysis

Gene Ontology analysis is a unifying attribute to represent differentially expressed genes based on their Biological, molecular and cellular component functions. The genes were screened against the KEGG database and were filtered based on the scores for both upregulated and down regulated genes.^[18] Enrichment results were generated by analyzing the up-regulated and down-regulated gene sets using the libraries of ENCODE and TargetScan, databases. Significant results were determined by using a cut-off of p -value < 0.05 after applying Benjamini-Hochberg correction.^[19] Transcription Factors (TFs) are proteins involved in the transcriptional regulation of gene expression. Associations between TFs and their transcriptional targets that are over-represented in the up-regulated and down-regulated genes were identified by comparing two groups of samples and miRNA, which play a key role in the post-transcriptional regulation of gene expression.

Construction of host mRNA- miRNA- lncRNA network and viral ceRNA network

The miRNA gene targets were identified through mirTarBase and Target scan databases. The more predominant targets were filtered based on the rank and P value < 0.1. The microRNAs associated with the regulation of the differentially expressed genes were then identified by entering the list of filtered genes into mirsystem software. The list of miRNAs along with the

corresponding genes was obtained. The Corresponding long non-coding RNA was identified from the Diana Lnc Database.^[20] The predicted targets (mRNA), miRNA and lncRNA were pooled together and the interaction network was created using cytoscape based on the interaction scores. The higher nodes and degrees of the interacting network were computed.^[21] The viral siRNA (v-siRNA) and target genes were identified from the ViSiRNA database. The detailed information such as the structure, silencing efficacy, viral targets, human targets and human 3' seed match regions about each v-siRNA were explored. The viral mRNA, miRNA and siRNA were used to construct the ceRNA network in cytoscape software.

RESULTS AND DISCUSSION

Differentially expressed genes

The transcriptome dataset of HTBE infected with H1N1, H3N2 and H5N1 downloaded from GEO database with ID GSE89005 was quantified and the meta data was split into two groups control samples that were uninfected and perturbation samples that were infected with strains of influenza. The differentially expressed genes were selected based on the criterion fold change > 2.0, P < 0.05 and FDR < 0.05. There were 35,238 differentially expressed genes, among which 24,427 (70.4%) genes were upregulated and the remaining 10,811 (30.6%) genes were down regulated. Most upregulated genes were interferon's, chemokine ligands, antiviral host proteins like IFIT and many more. Volcano plots are scatter plots that represent the differential genes based on \log_2 FC (fold change), which establishes a genes expression when in control and during infection.^[22] The Volcano plots (Figure 1A) with \log_2 FC on X-axis and \log_{10} P on Y-axis, plotted for influenza infected HTBE cells revealed the upregulated (red) and down regulated (blue) genes. Heat map of the dataset is represented in Figure 2, in which the top 50 genes were filtered based on variance and are clustered based on average linkage representing genes that are upregulated during infection and downregulated in the control. H5N1 viral infection had significantly over represented genes and appears red on the heatmap, while H3N2 and H1N1 have less upregulated genes. The genes like zinc finger10, IST, BCIL, INF2, etc were upregulated immediately after the infection of H5N1, whereas they were over expressed after 24 hr and 12 hr during H1N1 and H3N2 infections respectively.

Gene Ontology Enrichment Analysis

The experimentally validated and predicted associations between various gene attributes enabled the identifica-

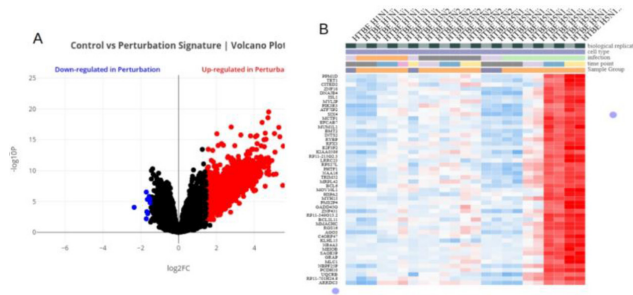


Figure 1A: Volcano plots of upregulated (red) genes and downregulated (blue) genes during influenza infection. **B** Heatmap displaying gene expression for each sample in the RNA-seq dataset. Rows of the heatmap represent a gene, columns represent a sample and cell represents normalized gene expression values.

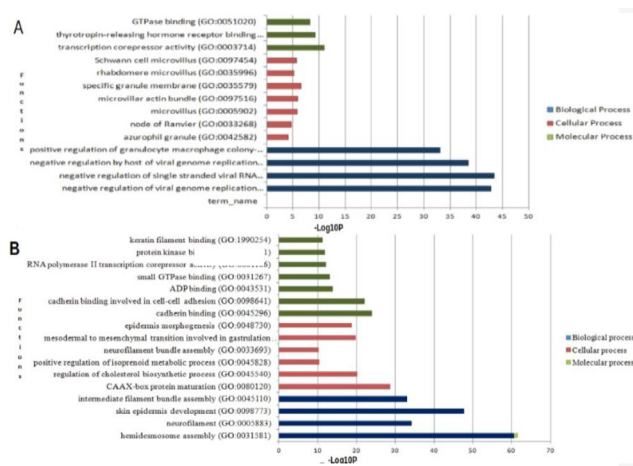


Figure 2: A GO of the upregulated genes **B.** GO of the downregulated genes.

tion of biological processes, molecular functions and cellular components for the DEGs. Gene ontology (GO) predictions (in Figures 2A and 2B) were screened based on $-\log_{10}P$.^[23] The biological process of the host genes responsible for negative regulation of ssRNA viral replication were highly upregulated and gene responsible for hemidesmosome assembly and skin epidermis development was downregulated. Molecular functions like GTPase binding and thyrotropin releasing hormone receptor genes were upregulated and genes responsible for cadherin binding involved in cell adhesion were downregulated during infection. Cellular component genes that are responsible for Interleukin production are upregulated and all the filament formation genes were downregulated during viral infection.^[24]

Functional Enrichment Analysis

The functional enrichment analysis revealed that there were numerous transcription factors (TF) inactivated by the miRNAs during the infection. Their function and

tissue specificity were identified. Table 1 represents the TFs of genes filtered based on p -value less than 0.05 and suppressed by miRNAs.^[25,26]

These transcription factors are all found to activate cellular signaling and regulation of various cellular factors at a rapid rate. They are involved in balancing the normal cell functions to resume quickly compensating the viral cellular functions. These transcription factors such as KLF41, STAT1, NFYB, PLAG1, FOXO1 and CEBPA were suppressed by miRNAs and are inactivated. STAT1, KLF41 and NFYB were significantly over-expressed in H1N1. PLAG1 was upregulated in H3N2 and FOXO1, CEBP and STAT1 were significantly upregulated in H5N7.

Enrichment of human miRNAs targeting host and viral genes

The differential gene expression counts data from edgeR resulted in the miRNA sequences which were ranked and filtered based on p value < 0.05 and FDR < 0.05 . Human targets for the miRNA were identified from mirBase and Targetscan.^[27] The viral-mir-Db (viral miRNA database) and mirtap database maintain the viral targets for the host miRNAs. The best 10 human miRNA ranked based on the lowest binding energy and site accessibility percentage > 75 were filtered. Table 2 represents the predominant miRNA, their sequence and viral miRNA targets from viral-mir-db and mirtap.^[28]

Nucleoprotein NP, Polymerase PA, Neuraminidase NA, Polymerase PB2, Hemagglutinin HA, Matrix protein 1 M1, Polymerase PB1 were the predominant viral targets that were inactivated by the host miRNAs. MiRNAs such as hsa-mir-492, hsa-mir-149-5p significantly suppressed both the viral and host targets.

Identification and construction of host ceRNAs

The top seven host miRNAs were filtered based on the highest site accessibility percentage above 90%. The filtered miRNAs enabled the identification of valid mRNA targets from targetscan and the corresponding lncRNAs predicted from the Diana Lncbase. The total mRNA-miRNA and lncRNA interaction was plotted using the cytoscape and a network representing a higher score of 23.54 was constructed using MCODE module and is represented in Figure 3A. MiRNAs such as hsa-mir-124-3p, hsa-mir-484, hsa-mir-615-3p, hsa-mir-1-3p, hsa-mir-149-5p, hsa-mir-34a-5p, hsa-mir-324-3p were found predominant in suppressing the host genes. Long non-coding RNAs such as LINC01410, RP11-764K9.1, XLOC_011494, LINC01410, CTC-360G5.9, RP11-473M20.9, with higher degrees of interaction to

Table 1: Top 6 Transcription Factors Filtered based on the P-value.

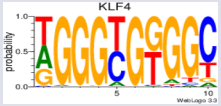
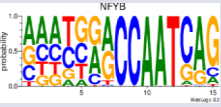


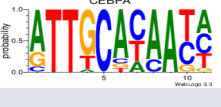
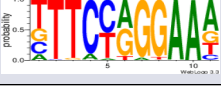
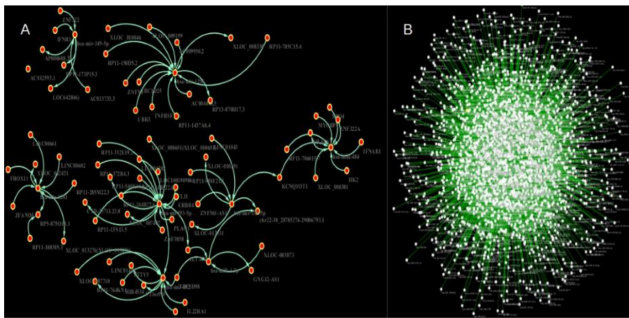
TF	PWM OF MOTIFS	FUNCTION	p-value
KLF41 Kruppel like factor (Zinc finger family)		Hematopoietic TF. Increases expression of adult alpha globin and erythroid genes	0.027944
NFYB Nuclear Transcription Factor Y – beta subunit		Nuclear TF Y subunit regulates transcription and protein dimerization	0.017407
PLAG1 Pleomorphic adenoma gene 1 (zinc finger protein)		Found on salivary glands of pleomorphic adenoma cells.	0.020101
FOXO1 Forkhead box 1		Triggers apoptosis	0.000605
CEBPA CCAAT/enhancer-binding protein beta		Transcription of other regulatory genes	0.004286
STAT 1 Signal transducer and activator of transcription 1		Multiple immune system functions including defense	0.01296

Table 2: Identified miRNAs and Targets.

Rank	Viral mRNA Targets	miRNA	Energy kJ/mol	Site Acceibility Percentage	Human mRNA Targets
1	Nucleoprotein NP Polymerase PA Neuraminidase NA	hsa-mir-484*	-25.4	97	46 upregulated targets
2	Polymerase PB2 Hemagglutin HA Matrix protein1 M1 Polymerase PB1	Hsa-mir-615-3p	-32.1	96	43 downregulated targets
3	Segment 1 Polymerase PB2 Segment 6 Neuraminidase N1	hsa-mir-1-3p*	-31.6	98	44 upnregulated targets
4	PA- X protein Hemagglutin HA	hsa-mir-149-5p*	-24.5	97	24 downregulated targets
5	Polymerase PB2	Hsa-mir-34a-5p	-27.8	94	38 downregulated targets
6	Nucleoprotein NP Polymerase PB1 PA-X PBI- F2	Hsa-mir-324-3p	-26.2	97	30 Downregulated targets
7	Polymerase PB1 Matrix Protein M1	hsa-mir-1296-5p	-26.2	82	40 downregulated targets
8	Polymerase PA	hsa-mir-933	-25.4	79	39 downregulated targets
9	Polymerase PB2, PA-X	hsa-mir-1281	-26.3	80	45 downregulated targets
10	Nucleocapsid Protein Nucleoprotein Matrix Protein M1	hsa-mir-492	-26.2	82	65 downregulated targets

Table 3: Identification of viral siRNA and host sense strands.

S. NO	Si RNA	Silencing efficacy	Virus type	Target gene-virus	Human mrna-3' seed matches
1	virsi1534	99	H3N2, H5N1	PA, segment 3	EDA2R, FUT9, MTHFR, C9ORF122
2	VIRSI1658	100	H5N1	NP	CAB39, GK5, RBM33, PRR8, HSDL1, ADAMTSL3, MCPH1, FUT9, MSR1
3	VIRSI1537	99	H2N2, H3N2, H5N1, H1N1	NP, segment 5	SPANXN1, NTRK2, GABPA, SH3TC2, PGM2L1, SPANXN5, PGAP1, ABI2, ARHGAP26
4	VIRSI1538	99	H9N2, H2N2, H5N1, H1N1	M, segment 7	PRX, ERGIC1, ZNF69
5	VIRSI1540	99	H9N2, H2N2, H5N1, H1N1	M, segment 7	SPIN3, ZNFR1, JRK, KLF13, TOM1, GARNL4, FZR1, NF2, SORCS2, NAT9, PPP1R16B, C16ORF28, SH3TC, ENTPD1, MCFD2, ZNF445, PDPK1, RANBP10, MAPKAPK3, PERLD1, SPN, THSD4, WHSC1, AQP2, KLHL21, SLC6A17, PRELP, SLC12A7, LUZP1, PAPLN, MTF1, MCART6, KIAA1909, IL16, KNK5, SRGAP3, OVOL1, STK24, RXRA, MLLT6, NFAM1, LOC653808, AMZ1, KHK, BTBD14B

**Figure 3A: Interaction of host ceRNA network and B Network of viral siRNA-viral mRNA- host seed RNA.**

miRNA can be suggested to suppress the miRNAs and release the host mRNA for translation.

Identification of viral siRNA and Human targets

Viral siRNAs are predominantly involved in the silencing of host genes. SiViRNA^[29] database housed experimentally validated siRNA targets of Influenza A viruses. From the 96 siRNAs reported, the top 20 siRNAs were filtered based on the silencing efficacy and identity percentage of the complementary strands. The genes for the guide strand and genes for the sense strand were filtered based on the high specificity score (>60%) corresponding to each siRNAs.^[30] The genes with > 4 hits matching 3' seed region of siRNAs^[31] were filtered and the results of top 5 siRNA are tabulated in Table 3. The interacting viral siRNAs on viral genes and on the host gene is represented as Figure 3B.

Viral siRNAs were capable of inactivating the viral genes themselves. Here we predicted 20 vi-siRNAs that had good silencing efficiency and targeted the viral genes such as polymerase, nucleoprotein, matrix protein, segments -3,5 and 7. VIRSI1538 and VIRSI1540 might be significant in targeting the matrix protein of four influenza viruses -H9N2, H2N2, H5N1, H1N1. So these two viruses can be claimed as good small RNA based therapeutic option for Influenza infection.

DISCUSSION

Generally, viruses either utilize host cellular pathways or encode miRNAs to repress host genes. Predicting potential viral non-coding RNA would be a valuable tool to investigate viral pathology and host interactions.^[32,33] The target region of these non-coding RNAs is 'seed' complementarity sequence ranging from 2-8 nucleotides from 5'-3' of miRNA that perfectly complements the 3' Un Translated Region of mRNA.^[34] The siRNAs are duplex RNA structures that are cleaved into a single stranded siRNA upon the activated RISC complex. The single strand siRNA acts complementary to guide mRNA strands and silences the gene from forming proteins. The real havoc is that in some cases, the siRNA can act as a miRNA and attach to a limited complementary mRNA sequences thereby exhibiting off-target effects.^[35] In the present study, the signature genes of influenza infected samples were understood along with the underlying miRNA, transcription factors and long non coding RNAs. The best results were filtered based on the p -value <0.05, False Discovery Rate (FDR) of

<0.05 and Fold change, that is, \log_2 ratio of infected to control samples.^[36]

The genes like zinc finger10, IST, BCIL, INF2, etc were upregulated immediately after the infection of H5N1, whereas they were over expressed after 24 hr and 12 hr during H1N1 and H3N2 infections respectively. The gene ontology analysis predicted the upregulation of biological, cellular and molecular functions genes as regulation of ssRNA viral replication, Serine type endopeptidase activity and calcium ion binding component and interleukin receptor complexes respectively. The down regulated genes of biological, cellular and molecular gene functions were identified as hemidesmosome assembly, skin epidermis development, cadherin binding and filaments production, respectively. The most significantly enriched transcription factors were KLF41, NFYB, PLAG1, FOXO1, CEBP and STAT1. KLF41 and NFYB were significantly over expressed in H1N1. PLAG1 was upregulated in H3N2 and FOXO1, CEBP and STAT1 were significantly upregulated in H5N7. All of these are important components of normal cellular functions. These transcription factors can be valid biomarkers for influenza infections.^[33] V-siRNAs, were explored and also the host target sites, 3' seed match, guide strand, sense strand responsible in the influenza infections. V-siRNAs with codes 1534, 1537, 1538, 1540 have a strong predicted inhibitory efficacy of 99 % and that of V-siRNA 1658 is 100%. VIRSI1538 and VIRSI1540 might be significant in targeting the matrix protein of four influenza viruses -H9N2, H2N2, H5N1, H1N1. So these two viruses can be claimed as good small RNA based therapeutic option for Influenza infection. VIRSI 658 is specific to H5N1. VIRSI1534 is specific to H1N1 and H3N2. They are more prone to target viral NP, M and PA genes of all strains.^[37] STAT, ELF, MAP3K, ZNF are the top genes that are silenced by the top v-siRNAs with accurate predictions. The predominant micro RNAs that were found to be involved in the up-regulation and downregulation of various targets were identified as hsa-mir-484, hsa-mir-1-3p, hsa-mir-149-5p, hsa-mir-615-3p, hsa-mir-34a-3p and hsa-mir-324-3p. Genes that were extremely upregulated and downregulated based on the miRNAs were found to be responsible for zinc finger proteins, Nuclear transcription factor Y, Interleukins, Forkhead box proteins and Ubiquitin ligases.

The current study has enabled the prediction of vital miRNA sequences that are prevalent during H1N1, H5N1 and H3N2 influenza infections. These miRNAs can be biomarkers and provide useful insights into the pathogenesis of influenza infections.

CONCLUSION

There is a balance maintained between complex regulatory systems for the normal functioning of cellular and molecular processes. Investigation of the balance in normal physiological conditions and its alteration during infection will enable the understanding of the versatile genomic elements.^[38] Recent studies have unveiled the abundant roles of non-coding RNAs (ncRNAs) especially microRNAs (miRNAs) and long non coding RNAs (lncRNAs) involved in a plethora of biological processes affecting cell homeostasis.^[39] miRNAs are considered post-transcriptional gene regulators enabling translational repression, mRNA degradation and gene silencing, thus playing a major role in gene expression.^[40] The exact underlying mechanism is still a puzzle to be solved. However, extensive studies on these least concerned genomic elements will unravel the mystery and prove to be therapeutic targets and restorative therapy as well in the near future.

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CONFLICT OF INTEREST

There were no conflicts of interest between the authors

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