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Investigation of Potential Human Micrornas in Influenza Virus

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Abstract: Influenza A virus (IAV), a negative segmented single-stranded RNA virus belonging to the family Orthomyxoviridae, IAVs causes global pandemics and are highly infectious with symptoms such as high temperature, headache, fever, and body pains; in some severe cases, the infection may lead to death due to changes in significant pathways and target genes, change in expression of some miRNAs are associated with several diseases such as cancer, miRNAs are hypothesized as significant players during viral infections. This study aimed to identify potential human miRNAs like sequences in the whole genome sequence of 12 Influenza A virus subtype and adopt the use of various bioinformatics databases and servers. The study identifies 83 human miRNA like sequence, some of which were found within various subtypes and were involved in several functions, among these, 3 significant miRNA (hsa-miR-5009-5p, hsa-miR-3200-5p, hsa-miR- 3145-3p) were found within the genome of 5 different Influenza A virus subtypes each and were co involved in TGF-beta signaling pathway, Estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells and proteoglycans in cancer. Although this study identifies potential human miRNAs in IAV, affected pathways and target genes, and demonstrate the significant contribution of miRNAs to IAV infection, further research is required to fully expound on how miRNAs can be used to diagnose, monitor the pathogenesis, and develop therapeutic strategies.

Keywords: Influenza A virus (IAV), Micrornas, bioinformatics, pluripotent, proteoglycans.

1. Introduction

1.1 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are post-transcriptional regulators that contribute to various biological processes and control of inflammatory pathways Arisan et al. (2020). Change in the expression of various miRNAs is associated with illnesses such as cancer and infarction. Meanwhile, miRNAs are hypothesized to be significant players during viral infections. Several DNA viruses encode their miRNAs, this is important in maintaining an internal environment conducive for viral replication. miRNAs are non-immunogenic unlike viral proteins, advance quickly, and precisely target mRNAs. Until recently about 400 viral miRNAs have been discovered. In the course of infection, the expression of host cellular miRNAs can be greatly affected. Izzard and Stambas (2015)." MicroRNAs are a class of short non-coding single-stranded RNA sequences of about 20 bp reported a few years ago to control gene expression in eukaryotes. These small RNAs are transcribed as long hairpin primary RNAs (pri-miRNAs) by RNA polymerase II. In the nucleus, pri-miRNAs are cleaved by the microprocessor complex including Drosha ribonuclease III and the

RNA-binding DGCR8 protein to form hairpin precursor miRNAs (pre-miRNAs, ~70 bp). Pre- miRNAs are exported to the cytoplasm by exportin-5 protein, belonging to the Ran-dependent nuclear transport receptor family, and are further cleaved by cytoplasmic endoribonuclease Dicer and trans-activation response element RNA binding protein (TRBP) to form mature RNAs Buggele et al. (2012). Each miRNA gene forms two mature miRNAs that are designated as – 3p miRNA and -5p miRNA. Both of these can coexist and is functional by associating with the RNAInduced Silencing Complex (RISC). Mature miRNAs often are known to bind to 3-untranslated regions (UTRs) of target mRNAs to regulate gene expression. Most miRNA:mRNA interactions involve nucleotides 2–7 of miRNAs, a region called 'seed'. Seed-based interactions lead to mRNA destabilization and/or translation inhibition miRNA and -5p miRNA. Both of these can coexist and is functional by associating with the RNAInduced Silencing Complex (RISC). Mature miRNAs often are known to bind to 3-untranslated regions (UTRs) of target mRNAs to regulate gene expression. Most miRNA:mRNA interactions involve nucleotides 2–7 of miRNAs, a region called 'seed'. Seed-based interactions lead to mRNA destabilization and/or translation inhibition.

1.2 Influenza A Virus

Influenza A virus (IAV), a negative segmented single-stranded RNA virus belonging to the family Orthomyxoviridae, comprising of 8 segments of which each segment encodes one or two proteins, The 8 segments include RNA polymerase complexes (PB2, PB1, and PA), Hemagglutinin (HA), Nucleoprotein (NP),

Neuraminidase (NA), Matrix proteins (M1, M2) and Non-structural proteins (NS1 and NEP). The HA and NA surface protein determine the IAV subtype. IAVs cause global pandemics and are highly infectious with symptoms such as high temperature, headache, fever, and body pains; in some severe cases, the infection may lead to death. During infection, the virus uses the host cell to promote replication and entry into the host cell; however, this activates immune responses from the host against the virus. "In the fight between IAVs and host cells, miRNAs play a necessary and regulatory role" Zhang et al. (2018)."

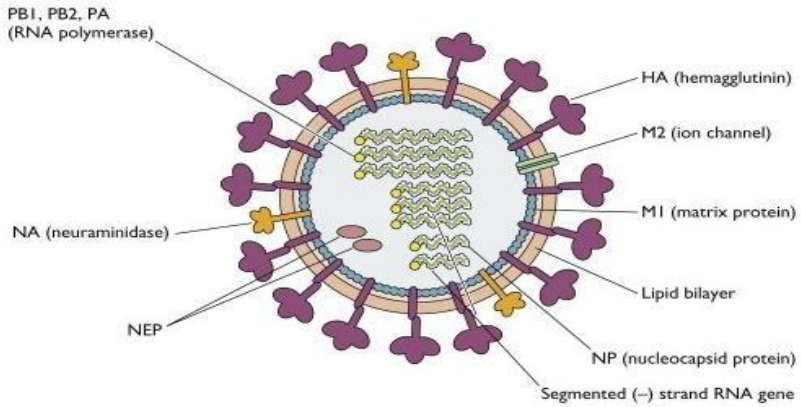


Fig 1: Labelled diagram of Influenza Virus (Vincent Racaniello, 2009)

The spherical virus contains an outer membrane called the Lipid bilayer, inserted in the bilayer are M2 protein and the glycoproteins (Hemagglutinin (HA) and Neuraminidase (NA)) which play critical roles in immune response. Underneath the lipid layer contains the M2 protein, which provides toughness to the bilayer envelope, the inside of the virus contains viral RNA (PB1, PB2, PA NP, and NEP) which are genetic materials of the virus (Nwokocha & Grove, 2021; Vincent Racaniello, 2009). Studies have shown that expression of about 100 host miRNAs is changed during infection; miRNAs either promote or prevent viral replication. Some host miRNAs target IAV genes directly to inhibitreplication, other host uses various intracellular signaling pathways to control the viral infection. Large populations are normally infected during Influenza virus pandemics, this affects both humans and domestic animals, such as swine and poultry, thereby leading to economic loss and public health concerns. During the pH1N1 pandemic which lasted for about a year, the mortality rate was about 284,000 people. Zoonotic strains such as H5N1, may cause significant losses in the poultry, and has been described to cause death in humans (>60%) (Iqbalet al., 2023; Izzard &Stambas, 2015). The host genes can respond in various ways to infection which is determined by the strain of the influenza virus.

It has been reported in recent studies that influenza virus utilizes the host cells for replication in various ways, one of the mechanisms is to regulate gene expression using miRNA (Makkoch et al., 2013). About 1000 miRNA have been identified in the human genome, it has also been reported that the genome of about 150 species of organisms as well as viruses contains mature miRNAs sequences. It has been true by various findings that the host and virus miRNAs play an important role in virus propagation. Influenza virus is also reported to modulate the miRNA mechanisms of infected cells and animal models (Geer et al., 2010). In contrast, miRNAs can also react with the influenza virus replication process. Recent findings suggested that cellular miR-323, miR-491, miR-654, and miR- 146a particularly binds to the PB1 gene resulting in the inhibition of influenza virus replication and propagation. Various methods can be used to validate miRNA screening and target prediction e.g. qPCR however microarray techniques are the most used technique to study miRNA profiles. The advantage of the microarray technique is it provides a lot of data about miRNA expression; however, it cannot identify significant genes in the cellular mechanism which is not covered by the probe set (Makkoch et al., 2013). The high-throughput next-generation sequencing (NGS) solves this problem as NGS analysis data shows expression profiles of infected host cells.

1.3 Research Question

1. Are there potential human microRNAs in Influenza A virus?

1.4 Aims and Objectives

This study aims to:

- identify potential human miRNA that shows similarity in sequence to specific strains of influenza A virus responsible for causing outbreaks in the human population; Avian-origin highly

pathogenic avian influenza (HPAI) H5N1, human pandemic (pH1N1), seasonal H3N2 influenza A virus infection, H1N2, H1N5, H2N2, H6N1, H7N2, H7N7, H7N9, H9N2, and H10N7.

- Predict target genes and affected pathway which might be useful for understanding the host defense mechanism in terms of regulating viral infection.
- Investigate evolutionary, structural, and functional relationships across the whole genome sequence of selected influenza A virus subtypes.
- Predict the secondary structure of Influenza A virus genome sequence.
- Determine the correlation between miRNA and the symptoms experienced by humans during infection.

2. Literature Review

According to Zhang et al. (2018). MicroRNAs (miRNAs) are a class of endogenous small (about 22 nucleotides (nt) non-coding RNAs that play important roles in the regulation of gene expression. The miRNA genes are first transcribed as primary miRNAs that are further processed into pre-miRNAs by Drosha, an RNase III enzyme. Then pre-miRNAs are exported to the cytoplasm and processed by Dicer, another RNase III enzyme, to generate a ~22-nt duplex consisting of a mature miRNA and its corresponding star miRNA. Finally, the duplex is unwound to give rise to mature miRNAs. Mature miRNA species may be generated from the 5′ and/or 3′ arms of the precursor duplex, and are called miRNA-5p and -3p, respectively. The mature miRNA is incorporated into a miRNA-induced silencing complex (miRISC), which then binds to the 3′- UTR of the target mRNA transcript, leading to translational inhibition or mRNA degradation." As per Buggele, et al. (2012), the significance of miRNAs in health and disease is still an unfolding story. A single miRNA can regulate hundreds of target mRNAs concurrently. Importantly, aberrant regulation of miRNAs plays a central role in pathological events underlying cancers and neurodegenerative diseases. Many researchers have demonstrated the potential role of miRNAs as non-invasive biomarkers of a variety of diseases. Targeting miRNAs provides an emerging opportunity to develop effective miRNA-based therapy. The rising body of advanced preclinical evidence on the biological significance of miR-221/222 in a variety of malignancies indicates that they will play a crucial role in the future of innovative therapeutic strategies, both as validated biomarkers and drug targets.

According to Cui and Sahi (2019), recent advances in next-generation sequencing (NGS) technologies have enabled the interrogation of genome-wide miRNA expression at high throughput and low cost. Deep sequencing of miRNA (miRNA-seq) has provided researchers an opportunity to catalog the repertoire of miRNA expression across various tissues and models and comprehensively study their dysregulation. Importantly, miRNA profiling by sequencing can better distinguish very similar miRNAs compared with other available methods, including microarrays and qPCR panels. The NGS approach is a powerful way of cataloging miRNAs and has led to an exponential increase in miRBase entries in the last few years. Arisan, et al. (2020) noted that MicroRNAs (miRs) are non-coding RNAs of length approximately 20–22 nucleotides; they post-transcriptionally regulate gene expression by binding to the 3′- untranslated regions of target mRNAs, leading to degradation or translational inhibition. Each miR can target hundreds of mRNAs within a given cell type, and a single mRNA is often the target of multiple miRs, and thus over half of the human transcriptome is predicted to be under miR regulation, embedding this post-transcriptional control pathway within nearly every biological process (Nwokocha & Grove, 2021).

Virally expressed miRs have recently been discovered, especially in viruses with DNA genomes. The best-known viral miRs are found mostly in herpesvirus families, where they enhance bilateral crosstalk between viral pathogenesis and host response mechanisms. Additionally, it has been shown that different virus families such as Viruses 2020, 12, 614 3 of 31 delta bovine leukemia virus, and foamy retroviruses could encode miRs. As previously shown, viral miRs are critical in the immune evasion mechanisms, affecting host immunity-related gene regulation networks. This bilateral effect results in a rapid increase during the virus resistance against host defense mechanisms and leads to their survival in host cells. The scope of miR generation from ssRNA viral genomes has been controversial, mainly due to the potentially deleterious effect of ssRNA viral genome cleavage into pre-/pri-miRs, making it unavailable for packaging into new virus particles. However, predictive studies on RNA viral genomes reveal RNA structures, which are conceivable Drosha and Dicer substrates."

Shu and McCauley (2017) stated that any groups have developed open-source tools for miRNA-seq data analysis, including mirTools, DSAP, miRNAkey, miRanalyzer, miRDeep2, Oasis, and miRge. These tools differ in the methods and algorithms used for various processing steps such as adapter trimming and sequence alignment. Despite the availability of these tools, many bioinformatics challenges remain. On the one hand, a miRNA-seq dataset is enriched for small RNA species between 19 and 23 nt, and short sequence lengths make it more likely that a read maps to a genomic locus or known miRNA simply by chance in a large and complex reference genome. On the other hand, a sequence read can map to more than one miRNA, and how to deal with

multiple mapping reads is still a challenge. This issue becomes more severe when miRNA-seq reads derived from multiple precursors are aligned to a reference genome directly. Therefore, for accurate miRNA quantification, it is especially important to introduce computational strategies to reduce or minimize potentially false mappings (Izzard and Stambas, 2015)."

According to Geer et al. (2010), early all miRNA-seq data analyses are performed using Linux clusters or workstations. However, analysis results in Linux are often hard to access for most bench scientists. Moreover, analyses of miRNA-seq datasets typically generate large amounts of data and a variety of result files that are difficult to interpret. Therefore, it is crucially important to organize and share miRNA-seq data analysis results in an efficient and user-friendly way.

Lowen (2017) asserted that the genome of influenza A virus (IAV) comprises eight segments of negative-sense RNA. Each segment encodes one or two proteins, and all eight are required to support the production of progeny virions. Genome segmentation has important implications for the mechanisms by which viral gene expression is controlled and viral RNAs are assembled into nascent virions. Segmentation also creates the potential for reassortment, the exchange of intact gene segments between viruses that coinfect the same cell. Reassortment is a type of recombination and is functionally similar to the intramolecular recombination seen in many monopartite, positive-sense RNA viruses. Reassortment outcomes are quantitatively different from those of intramolecular recombination, however, in that reassortment allows many distinct genotypes to emerge from a single coinfected cell (e.g., for IAV, 254 novel gene constellations can be produced from a cell coinfected with two unrelated parental strains). Thus, genome segmentation supports rapid genetic diversification and is highly important to the evolutionary biology of viruses with segmented genomes. Although some viruses carry segmented genomes and therefore undergo reassortment, IAVs are arguably the best characterized in this respect and are the focus of the following discussion.

2.1 Interhost Dynamics of Influenza A Virus Infection

According to Peng et al. (2016), there are two ways in which coinfection of an individual host can come about: via two independent transmission events or by co-transmission of a mixed virus population. Two independent transmission events. The likelihood of two independent transmission events resulting in reassortment is governed in part by their relative timing. In a guinea pig model, we found that animals inoculated intranasally with 103 PFU of A/Panama/2007/99 (H3N2) (Pan/99) virus could be superinfected with a variant of the same strain within 18 h of the primary infection. By contrast, time intervals of 24 h or longer resulted in no detectable growth of the second virus. Rambaut (2008) also observed that, where superinfection of the animal could be achieved reassortment was also detected; thus, the coinfection of individual cells proceeded until a block in superinfection developed at the whole-host level. We hypothesize that innate immune responses triggered by the first virus prevented the second virus from establishing infection in the same host.

Interestingly, when secondary infection was initiated at 12 h after primary infection, reassortment was increased relative to simultaneous coinfection. This trend was also observed in cell culture when primary infection was performed under low-multiplicity conditions and multiple rounds of replication were allowed to proceed. We propose that allowing time for the first virus to complete its life cycle and spread to additional cells increases the probability that, upon its introduction, the second virus will enter cells that are already infected. This mechanism requires that the host remain permissive at the time of the second inoculation. However, given a 12-h time interval, most individual cells are unlikely to be refractory because they will have been infected recently via a secondary or tertiary round of viral spread. Overall, in a guinea pig model of influenza virus coinfection, there was an ∼18-h time window during which secondary infection could take hold and lead to reassortment and staggering of infections by 12 h increased the probability of reassortment (Nwokocha & Grove, 2021; Ikpuri, 2018; Shu and McCauley 2017)."

2.2 Intrahost Dynamics of Influenza A Virus Infection

"The extent of viral diversity present within an individual host is determined by a combination of the effective population size, the rate of de novo mutation, and selection acting within the host. Whenmultipleviralvariantsarepresentinanindividual,theopportunityforreassortmentisthen reliant on the spatial dynamics of spread. Within-host diversity. Published estimates of the mutation rate for IAVs are in the range to 10−4 substitutions per site per replication. The breadth of this range indicates that further examination of this key property of IAV is needed. Nevertheless, the higher estimates of mutation rate suggest that, on average, one mutation is introduced into each genome copied. Such a high rate would give the potential for viral sequence diversity to accrue quickly within a single host (Iqbalet al., 2023; Othumpangat, et al., 2013).Though significant intrahost diversity has indeed been observed for IAV, selection acting within the host can limit the extent of this diversity. Even in cell culture, most mutations are deleterious and approximately one-third are lethal thus, the accumulation of variant genomes is often precluded by negative selection. Similarly, following the introduction

of a diverse viral population through inoculation or transmission, selective bottlenecks acting within a host have been observed. Highly biased reductions in diversity were seen with a human 2009 pandemic strain in eggs and with an avian-like H7N9 subtype virus in ferrets. Experimental inoculation of humans with virus passaged in egg and cell culture was also associated with strong purifying selection: Genetic diversity that had accumulated during laboratory passage of A/Wisconsin/67/2005 (H3N2) virus was greatly diminished following the reintroduction of this virus into humans. Thus, selection acting on co-infecting viral variants can limit the potential for those variants to meet within the same cell and reassort."

Where robust coinfection with multiple variant viruses occurs, however, reassortment in vivo can be highly efficient. Using wild-type and variant Pan/99-based viruses, in which the variant carried neutral mutations in each gene segment, we showed that up to 70% of viruses shed from coinfected guinea pigs are reassortant. These high levels of reassortment were observed within 24 h of co-inoculation when a high dose of 106 PFU was used. In animals co-inoculated with 103 PFU of each virus or infected via co-transmission, reassortment increased over time with similar kinetics to viral loads, again peaking at 70% on average. These observations indicate that multiple infections of individual cells are not rare in vivo, despite the presumed presence of large numbers of target cells. The increase in reassortment overtime after a low-dose inoculation furthermore suggests that viral spread increases the likelihood of coinfection. However, the features of IAV spatial dynamics within an infected host that enable frequent coinfection are unclear. Although focal spread would give rise to regions of high- multiplicity infection, these regions would tend to host clonal virus populations. The efficient mixing of two distinct genotypes most likely requires a dispersed spread. It will be of interest in future studies to define the characteristics of IAV spread in guinea pigs that support high levels of coinfection, and to test whether these properties extend to natural hosts

2.3 MicroRNAs Directly Target Influenza Viral RNAs

"Influenza A virus consists of eight gene segments that encode for 12 viral proteins including surface glycoprotein [hemagglutinin (HA) and neuraminidase (NA)], nucleoprotein (NP), two matrix proteins (M1 and M2), three polymerase complex proteins PB1, PB2, and PA, four non- structural proteins (NS1, NS2, PA-X, and PB1-F2). HA and NA proteins predominantly regulate virus entry and exit from host cells, and their genes are the major genetic segments for influenza antigen drift and shift by genetic mutation and reassortment to create new strains/subtypes. In contrast, other IAV viral proteins are more conservative, which is essential for IAV replication. For example, viral polymerase complex proteins (PA, PB1, PB2) and NP form a viral ribonucleoprotein (vRNP), a minimal functional unit for influenza virus replication. M1 forms a coat inside the viral envelope and binds to viral RNA. Therefore, exploration of those miRNAs that directly target those conservative viral sequences could uncover novel therapeutics to control influenza replication and propagation Izzard and Stambas (2015)."

Indeed,several lines of evidence have implied the feasibility of this concept. For example, miR 323, -491, and -654 destabilize PB1 mRNA by targeting the conservative region, as demonstrated in H1N1 infected cells that are treated with plasmids carrying those miRNA mimics or inhibitors, respectively. A similar investigation has shown that miR-485 directly binds to a conserved site of PB1 mRNA to regulate viral replication, in H5N1-infected HEK293T cells following miR-485 mimics or inhibitor treatment. Furthermore, multiple miRNAs may target the same seed sequence to regulate IAV replication. Khongnomnan and colleagues, through in silico analysis and aluciferase reporter assay have reported that the same conservative region of PB1 mRNAs of H1N1, H5N1, or H3N2 subtypes is targeted by miR-3145. Neutralization of this miRNA by using plasmid-encoded anti-miRNA oligonucleotides restored the expression of PB1 mRNA and miR3145 mimics treatment reduced PB1 expression in H5N1-, H1N1-, or H3N2-infected A549 cells (Iqbalet al., 2023; Zhang et al., 2018).

M1 is the most abundant protein in the IAV viral particle and regulates vRNP export, virus assembly and budding, and virus-host interactions. Ma and colleagues have reported that let-7c precursor diminishes H1N1 replication by binding to the 3′-UTR of M1 mRNA and that let-7c inhibitor reinstates the expression of M1 protein and influenza infection in A549 cells. Certain microRNAs have also been shown to inhibit the expression of IAV viral proteins, not only in a direct manner but also through regulations of other host factors that affect viral replication. For example, miR-33a mimic suppressed the expression of NP and M1 proteins by directly binding to the 3′-UTR of Archain 1 (ARCN1) RNA in HEK293T, A549, and Hela cells infected with H1N1, H9N2, or H3N2, resulting in greatly decreased virus replication. ARCN1 is an important component of the human coatomer protein complex, which regulates protein transport from the Golgi body to the endoplasmic reticulum and critically modulates influenza virus entry to host cells, viral membrane protein expression, and assembly) Buggele et al., 2012; Iqbalet al., 2023).

Treatment with miR-33a inhibitor recovered the expression of ARCN1, NP, and M1 proteins, and thus increased H1N1, H9N2, or H3N2 replication. In the same study, miR-33a has also been shown to attenuate the replication of H1N1, H9N2, or H3N2 by reducing vRNP activity through an ARCN1- independent pathway in

HEK293T cells, suggesting the multiple functions of this miRNA. A recent investigation by Zhang, et al, identified that miR-21 targets NP, PB1, PB2, PA, NA, and HA segments of H1N1, by using infected miR-21-deficient MDCK cells. It is promising that targeting the NP segment or combination of both PA and NA segments of IAV simultaneously reduced IAV replication greater than twofold, as compared to other treatments (e.g., targeting sole segment and a combination of PA and HA. Although the role of miRNAs in the pathogenesis of IAV infection should be further investigated, targeting these small viral RNAs may provide alternative approaches to reduce influenza infection by directly inhibiting expression of conserved viral proteins (e.g. PB1, NP, or M1), regardless of the viral antigen drift and shift.

3. Method

3.1 Genome Sequences

The genome sequences of 13 influenzas A virus subtypes (H1N1, H1N2, H1N5, H2N2, H3N2, H5N1, H6N1, H7N2, H7N7, H7N9, H9N2, H10N7, and H18N11) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). The genome sequence of H17N10 was obtained from the GISAID database (https://www.gisaid.org), and multiple sequence alignment using Clustal Omega (Sievers and Higgins, 2014) at EBI (www.ebi.ac.uk/Tools/msa/clustalo/) was carried out.

3.2. Identification of human MicroRNAs in 12 Influenza A Virus Subtype Genome

The fasta format of the whole genome sequence of H1N1 H1N2, H1N5, H2N2, H3N2, H5N1, H6N1, H7N2, H7N7, H7N9, H9N2, H10N7 were split into 14 parts, each part containing 1000bp and the last containing 588 bp for the H1N1 subtype. Each segment was analyzed with miRBase mature miRNA search tool (http://www.mirbase.org/search.shtml) and MiRNA's found were recorded along with their values, scores, and alignment; e-value <10 and score >70 were considered as significant.

3.3 Sequence Alignment of H1N1 and H1N5

Clustal Omega (Sievers and Higgins, 2014) at EBI (www.ebi.ac.uk/Tools/msa/clustalo/ was used to perform sequence alignment on the whole genome sequence of H1N1, and H1N5 to determine to which extent the sequences are conserved.

3.4 Multiple Sequence Alignment of 14 Influenza A Virus Subtypes

Clustal Omega (Sievers and Higgins, 2014) at EB I (www.ebi.ac.uk/Tools/msa/clustalo/) was used to perform multiple sequence alignment on the genome sequence of 14 Influenza A virus subtypes (H1N1, H1N2, H1N5, H2N2, H3N2, H5N1, H6N1, H7N2, H7N7, H7N9, H9N2, H10N7 H17N10, and H18N11)

3.5 MiRNApathwayandtargetcellprediction

Heat maps were created with DianaTools miRPath V3 (http://snf-515788.vm.okeanos.grnet.gr/) to determine pathways and target cells altered by selected microRNAs and functional characteristics. Using the microT-CDS version 5.0 database, the parameters for the analysis are as thus: the p- value threshold was 0.05, the microT threshold was 0.8, Fisher's Exact Test (Hypergeometric Distribution) was selected and Heatmap analysis was done with pathway union.

3.6 RNA secondary structure prediction

RNAfold webserver was used to predict the secondary structure (folding) of selected regions of the viral genome of interest from the alignment (http://rna.tbi.univie.ac.at//cgi- bin/RNAWebSuite/RNAfold.cgi).

4. Results

4.1 Human MicroRNAs identified in 12 Influenza A Virus Subtype Genome

A total of around 83 same human microRNAs were identified within the genome of influenza A virus subtypes in the current study. Of these, three (Hsa-miR-5009, Hsa-miR-3145-3p, Hsa-miR- 3200-5p) were found within the genome of 5 different Influenza A virus subtypes, and four (Hsa- miR-518c-3p, Hsa-miR-6841-3p, Hsa-miR-6768-3p, Hsa-miR-6780b-3p) were found within the genome of 4 different Influenza A virus subtypes. The human microRNAs found within the genome of influenza A virus subtypes are as under-Foundwithinthegenomeof5differentInfluenzaAvirus subtypes:

Hsa-miR-5009-5p (e value: 7.3, score: 72) for A/Puerto Rico/8/1934(H1N1), A/Japan/305/1957(H2N2), A/NewYork/392/2004(H3N2) and A/WildDuck/Guangdong/314/2004 (H5N1), e value: 2.3, score: 78 for A/swine/Oklahoma/010226-17/2008(H1N2)).

Hsa-miR-3145-3p (e value: 0.41, score: 87) for A/Puerto Rico/8/1934(H1N1), e value: 7.3, score: 72 for A/Japan/305/1957(H2N2), A/WildDuck/Guangdong/314/2004 and (H5N1), A/blue- winged teal/Ohio/566/2006(H7N9), e value: 3.4, score: 76 A/chicken/Shantou/2712/2001(H9N2));
Hsa-miR-3200-5p (e value: 7.3, score: 72) for A/swine/Oklahoma/010226-17/2008(H1N2), A/Japan/305/1957(H2N2), A/mallard duck/New York/6861/1978(H1N5), A/chukar/New York/11653-1/2005(H7N2), and A/blue-winged teal/Ohio/566/2006(H7N9));

Found within the genome of 4 different Influenza A virus subtypes:

Hsa-miR-518c-3p (e value: 6.2, score: 70) for A/Puerto Rico/8/1934(H1N1), e value: 6.7, score:70 for A/Japan/305/1957(H2N2), e value: 3.0, score: 70 for A/blue-winged teal/Ohio/566/2006(H7N9), e value: 8.8, score: 70 for A/chicken/Shantou/2712/2001(H9N2));Hsa-miR-6841-3p (e value: 2.8, score: 77) for A/Puerto Rico/8/1934(H1N1), A/Japan/305/1957(H2N2), A/New York/392/2004(H3N2) and e value: 8.8, score: 71 A/chicken/Shantou/2712/2001(H9N2));

Hsa-miR-6768-3p (e value: 5.0, score: 74) for A/mallard duck/New York/6861/1978(H1N5), A/WildDuck/Guangdong/314/2004 (H5N1), A/chukar/New York/11653-1/2005(H7N2), and A/blue-winged teal/Ohio/566/2006(H7N9));

Hsa-miR-6780b-3p (e value: 7.3, score: 72) for A/Japan/305/1957(H2N2), A/chukar/New York/11653-1/2005(H7N2), A/blue-winged teal/Ohio/566/2006(H7N9) and A/northern shoveler/California/HKWF592/2007(H10N7)

Many of the human microRNAs identified in the present study were found to play significant roles in various conditions influencing health as well as diseases; a few major ones are mentioned below-

- **Hsa-miR-5009-5p** is involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, glycosaminoglycan biosynthesis-chondroitin sulfate / dermatan sulfate, lysine degradation, morphine addiction, prion disease, and proteoglycans in cancer.
- **Hsa-miR-3145-3p** is involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, morphine addiction, prion disease, and proteoglycans in cancer.
- **Hsa-miR-3200-5p** is involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, and proteoglycans in cancer.
- **Hsa-miR-6841-3p** is involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, lysine degradation, morphine addiction, and proteoglycans in cancer.
- **Hsa-miR-6768-3p** is involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, glycosaminoglycan biosynthesis-chondroitin sulfate / dermatan sulfate, lysine degradation, morphine addiction, and prion disease.
- **Hsa-miR-518c-3p** and **Hsa-miR-6780b-3p** were not seen to be associated with any such pathways or conditions in particular.

The 3 leading microRNAs (hsa-miR-5009-5p, hsa-miR-3200-5p, hsa-miR-3145-3p) were found within the genome of 5 different Influenza A virus subtypes were found to be co-involved in TGF- beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, and proteoglycans in cancer.

A part from the above, many other microRNAs identified were also seen to have significant associations, mentioned as under-

- **Hsa-miR-6734-3p and hsa-miR-4778-3p** were observed to be involved simultaneously in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, glycosaminoglycan biosynthesis-chondroitin sulfate / dermatan sulfate, lysine degradation, morphine addiction, prion disease, and proteoglycans in cancer.
- **Hsa-miR-216a-3p and hsa-miR-3116** were involved in almost all the conditions mentioned above except, hsa-miR-216a-3p was not related in glycosaminoglycan biosynthesis and hsa-miR-3116 had no role in prion disease.
- **Hsa-6886-3p and hsa-miR-6841-3p** were co-involved in the TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, lysine degradation, morphine addiction, and proteoglycans in cancer, but both were not involved in glycosaminoglycan biosynthesis and prion disease

On looking into the microRNAs found within the whole genome sequence of A/Puerto Rico/8/1934 (H1N1) Influenza A virus subtype, Hsa-mir-5009-5p was observed twice within the genome sequence. Various

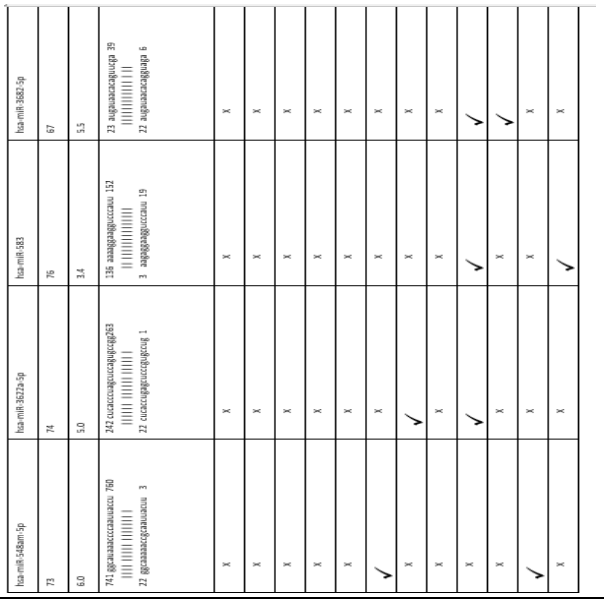
human microRNAs found within the whole genome sequence of A/Puerto Rico/8/1934 (H1N1) Influenza A virus subtype along with their scores, e-values, and alignments are depicted in the below-given table 1. (NB: e-value <10 and score >70 were considered as significant)

Table 1: MicroRNA (miR) Sequences Found in Influenza A virus (A/Puerto Rico/8/1934(H1N1) genome

miRnas	Score	E-value	Alignment
hsa-miR-5009-5p	72	7.3	360 cuuuuucaaaauugggga 377 6 cuuuuucagauuugggga 23
hsa-miR-634	73	6.0	118 uccaaagcuggacugcuggu 137 21 uccaaaguuggguugcuggu 2
hsa-miR-3145-3p	87	0.41	765 aauuccaaagacuaaaaaauu 785 23 aauuccaaacacucaaaauu 3
hsa-miR-567	74	5.0	710 uucugucauggaagcaaguacu 731 22 uucugucccggaagcaauacu 1
hsa-miR-4690-3p	72	7.3	262 ggccagcagaggccucug 269 4 gcccagcugaggccucug 21
hsa-miR-4646-5p	71	8.8	291 augaggagcugaggga 306 7 aagaggagccugaggga 22
hsa-miR-5009-5p	78	2.3	469 auacccaaagcugaaaaauuc 489 24 aucccaaauucugaaaaguc 4
hsa-miR-3686	86	0.50	329 aucuguaugagaaaguaaa 347 1 aucuguaagagaaaguaaa 19
hsa-miR-6873-5p	73	6.0	25 cacugggaauacagagggga 44 1 cagagggaauacagaggca 20
hsa-miR-6841-3p	77	2.8	427 ugggggugcagaugcaacg 445 20 uggggaugcagaugcaagg 2
hsa-miR-518c-3p	70	6.2	311 aaacucuacagagauucgcuugg 333 23 acacucuaaagagaagcgcuuug 1

From the above table 1, we can also observe the hsa-miR-3686 (329-347), hsa-miR-4646-5p (291- 306) and hsa-miR-5009-5p (360-377) are conserved regions. That means they are associated with structural and functional roles. The remaining miRs were less conserved in the evolutionary process.Again when the similar human miRNA's found within the whole genome sequence of 12 selected Influenza A v irus subtypes reported infecting humans (H1N1 H1N2, H1N5, H2N2, H3N2, H5N1, H6N1, H7N2, H7N7, H7N9, H9N2, H10N7) were taken into consideration, the most significant miRNAs seen were hsa-miR-5009, hsa-miR-3145-3p, hsa-miR-3200-5p, identified within the genome sequence of 5 different analyzed influenza A virus subtype (Table 2; NB: e-value <10 and score >70 were considered as significant).Finding similar miRs across different viral subtypes suggests having the same kind of etiology, pathogenesis, and symptoms during infection. This is because similar miRs will affect the same common pathways in each of the viral subtypes wherein they were identified. During evolution, the viral subtypes having similar miRs are suggested being more closely placed and the eventual clinical presentations as well remain somewhat the same

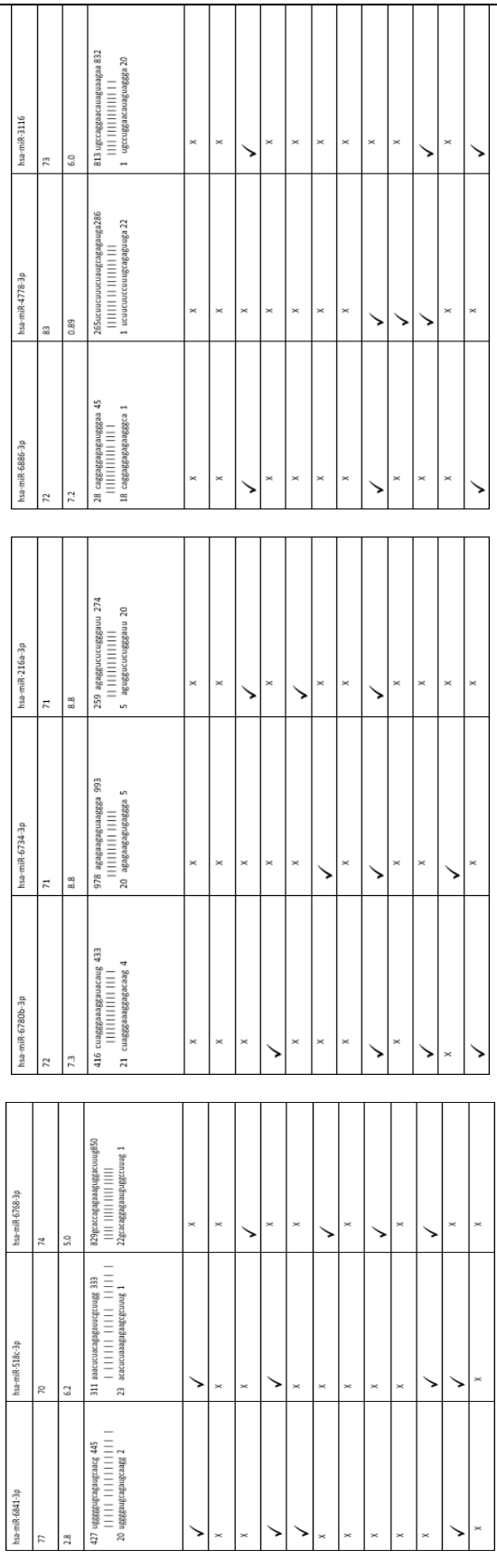
Table 2: Similar human microRNA (miR) sequences found within the genome of 12 Influenza A virus subtypes

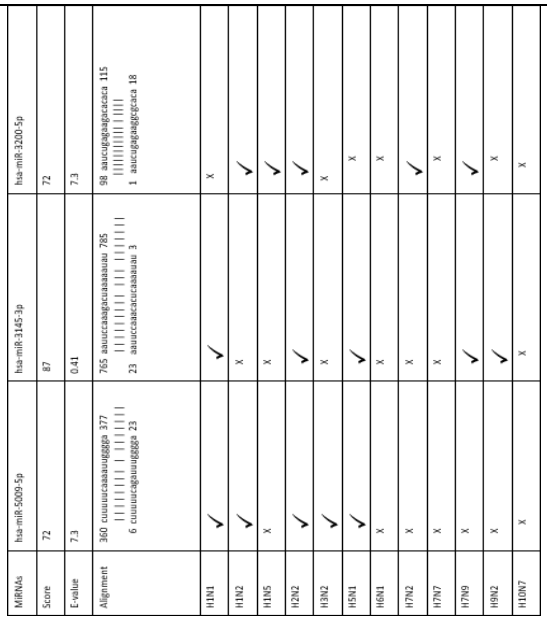


hsa-miR-514b-3p	hsa-miR-3680-5p	hsa-miR-548ag	hsa-miR-548c-5p	hsa-miR-548d-5p	hsa-miR-548o-5p
78	74	77	73	73	73
2.3	5.0	2.8	6.0	6.0	6.0
431 uccaagucacagagauguucaau 451 |||| ||||||||||| 21 uccaucucacagaggguucaau 1	285 gacuaaucucacaggauuacugaa3o6 |||| |||||||||| ||| 1 gacucucucucacaggauuugugca 22	742 gcauaaaccccaauuaccu 760 |||| ||||||||||| 21 gcagaaacccacaauuaccu 3	741 ggcauaaaccccaauuaccu 760 |||| ||||||||||| 22 ggcaaaaccccgcaauuacuu 3	741 ggcauaaaccccaauuaccu 760 |||| ||||||||||| 22 ggcaaaaccccacaauuacuu 3	741 ggcauaaaccccaauuaccu 760 |||| ||||||||||| 22 ggcaaaaccccgcaauuacuu 3
X	X	X	X	X	X
X	X	X	X	X	X
X	X	X	X	X	X
✓	X	X	X	X	X
✓	X	X	X	X	X
X	✓	✓	✓	✓	✓
X	X	X	X	X	X
X	✓	X	X	X	X
X	X	X	X	X	X
X	X	X	X	X	X
X	X	✓	✓	✓	✓
X	X	X	X	X	X

hsa-miR-4524b-3p	hsa-miR-3160-3p	hsa-miR-1178-3p
71	68	71
8.8	7.5	8.8
921 gagacuggauucaugcu 936 ||||| ||||||||| 1 gagacuggauucaugcu 16	435 ggacuuuucuguuucagcu 453 ||||||| || |||||| 21 ggccuuucuagucucagcu 3	361 ggaagaacagguuagca 376 |||||||||| |||| 17 ggaagaacagugagca 2
x	x	x
✓	✓	x
x	x	✓
x	x	✓
x	x	x
x	x	x
x	x	x
x	x	x
x	x	x
✓	✓	x
x	x	x
x	x	x

hsa-miR 6505-5p	hsa-miR-3686	hsa-miR-4524a-5p
71	86	71
8.8	0.50	8.8
334 uugaaauagggauau 349 |||||||||||||| 1 uugaaauagggauau 16	329 aucuguaugaaaguaaa 347 ||||||||||||||||| 1 aucuguaugaaaguaaa 19	921 gagacuggauucaugcu 936 ||||||||||||| 21 gagacuggauucaugcu 6
x	✓	x
✓	✓	✓
✓	x	x
x	x	x
x	x	x
x	x	x
x	x	x
x	x	x
x	x	x
x	x	✓
✓	x	x
x	x	x





MiRNAs	hsa-miR-5009-5p	hsa-miR-3145-3p	hsa-miR-3200-5p
Score	72	87	72
E-value	7.3	0.41	7.3
Alignment	360 cuuuuucaaaauuggga 377 |||||||||||||||| 6 cuuuuucagauuuggga 23	765 aauuccaaagacuaaaaauau 785 |||||||||||||||||||||| 23 aauuccaaacaccaaaaauau 3	98 aauccugagagacacaca 115 ||||||||||||||||| 1 aauccugagaggcgcaca 18
H1N1	✓	✓	X
H1N2	✓	X	✓
H1N5	X	X	✓
H2N2	✓	✓	✓
H3N2	✓	X	X
H5N1	✓	✓	X
H6N1	X	X	X
H7N2	X	X	✓
H7N7	X	X	X
H7N9	X	✓	✓
H9N2	X	✓	X
H10N7	X	X	X

The table above shows similar human miRNA's found within the whole genome sequence of 12 selected Influenza A virus subtypes reported to infect humans (H1N1 H1N2, H1N5, H2N2, H3N2, H5N1, H6N1, H7N2, H7N7, H7N9, H9N2, H10N7) along with their scores, e-values, and alignments. All similar miRNAs found were considered significant (NB: e-value <10 and score >70 were considered as significant). The most significant miRNAs are hsa-miR-5009, hsa-miR-3145- 3p, hsa-miR-3200-5p found within the genome sequence of 5 different analyzed influenza A virus subtype each.

4.2 Results of Heat maps created for MicroRNA pathway and target cell prediction

The Heat maps created to determine pathways and target cells altered in A/Puerto Rico/8/1934 (H1N1) influenza A virus subtype are described in the following table (3- A and B). While table 3A depicts the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, table 3B shows the Gene Ontology (GO) enrichment analysis result of MiRNA's found. The below tables presenting results also show the function affected, p-values, the number of genes affected, and miRNAs involved KEGG pathway analysis done adopting MirPath (Table 3A) suggests that many human microRNAs identified in the subtypes largely influenced multiple signaling pathways. To name a few, multiple (9) miRs were identified to have effects on signaling pathways regulating pluripotency of stem cells, Hippo signaling pathway, proteoglycans in cancer, pathways in cancer, Oocyte meiosis, Adrenergic signaling in cardiomyocytes and Ubiquitin mediated proteolysis. When a large number of miRs are seen to be involved in a particular pathway, the likelihood of seeing the pathway alterations and its consequential expression change across viral subtypes increases,

On the other hand, GO enrichment analysis results using MiRPath (Table 3B) has revealed that the microRNAs identified target genes involved in many crucial functions. Multiple microRNAs (10 in each) were seen to affect a large number of genes involved in vital physiological and biological functions associated with the cellular component (2305 genes), molecular function (2297 genes), biological process (2191 genes), and organelle (1471 genes). The more the number of miR target genes, the greater is the possibility of seeing the resultant function alteration among the viral subtypes.

Table 3: The KEGG pathway (A) and GO (B) enrichment analysis results for MiRNA's found in H1N1 Influenza A subtype.

3A

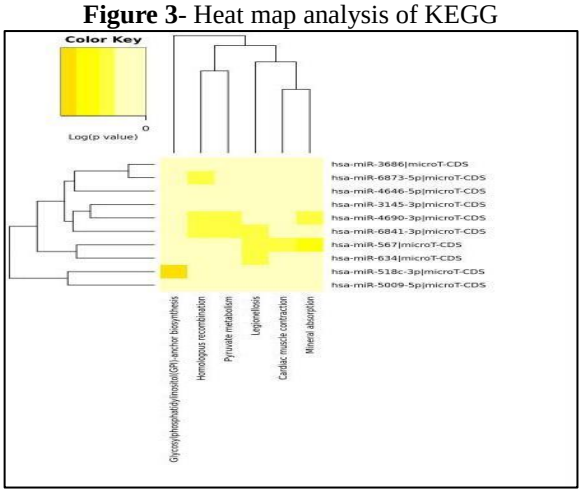
KEGG pathway	p-value	#genes	#miRNAs
Prion diseases	1.22E-14	4	4
TGF-beta signaling pathway	3.05E-06	23	8
Other types of O-glycan biosynthesis	0.0030272	8	5
Morphine addiction	0.0044641	21	8
Colorectal cancer	0.0157217	19	8
Hippo signaling pathway	0.0157217	37	9
Signaling pathways regulating pluripotency of stem cells	0.0157217	29	9
Wnt signaling pathway	0.0181841	31	8
Ubiquitin mediated proteolysis	0.0238014	34	9
Glycosphingolipid biosynthesis - lacto and neolacto series	0.0287027	6	4
Axon guidance	0.0287027	26	8
Oocyte meiosis	0.0287027	25	9
Adrenergic signaling in cardiomyocytes	0.0287027	32	9
Proteoglycans in cancer	0.0287027	44	9
Pathways in cancer	0.0380798	79	9
Long-term depression	0.0488129	16	7

3B

GO Category	p-value	#genes	#miRNAs
organelle	1.28E-82	1471	10
ion binding	1.11E-43	907	10
cellular nitrogen compound metabolic process	6.39E-41	719	10
biosynthetic process	1.08E-26	598	10
cellular protein modification process	1.78E-26	386	10
molecular_function	3.18E-15	2297	10
cytosol	6.16E-13	416	9
gene expression	1.08E-12	97	9
protein complex	4.03E-12	544	10
cellular_component	1.40E-11	2305	10
protein binding transcription factor activity	4.03E-11	92	9
small molecule metabolic process	4.03E-11	326	10
nucleic acid binding transcription factor activity	9.05E-11	162	9
neurotrophin TRK receptor signaling pathway	2.30E-10	49	8
post-translational protein modification	2.30E-10	39	9
cellular component assembly	5.47E-10	200	10
cellular protein metabolic process	3.16E-09	76	10
catabolic process	6.91E-09	272	10
synaptic transmission	1.20E-08	79	9
blood coagulation	2.17E-08	76	9
macromolecular complex assembly	3.18E-08	138	10
enzyme binding	8.14E-08	190	9
protein complex assembly	1.20E-07	124	10
Fc-epsilon receptor signaling pathway	1.88E-07	31	8
cytoskeletal protein binding	2.55E-07	124	9
symbiosis, encompassing mutualism through parasitism	4.38E-07	78	9
viral process	1.12E-06	69	9
platelet activation	1.46E-06	40	9
nucleoplasm	1.46E-06	173	9
nucleobase-containing compound catabolic process	1.67E-06	132	10
mitotic cell cycle	3.75E-06	60	9
cell death	5.24E-06	137	9
energy reserve metabolic process	7.34E-06	24	8
epidermal growth factor receptor signaling pathway	1.89E-05	39	8
cellular component disassembly involved in execution phase of apoptosis	4.18E-05	13	6
microtubule organizing center	6.91E-05	78	9
membrane organization	6.98E-05	85	9
enzyme regulator activity	7.01E-05	122	9
biological_process	7.82E-05	2191	10
chromatin organization	7.93E-05	28	9
cell-cell signaling	0.0001023	99	9
phosphatidylinositol-mediated signaling	0.0002361	28	9
cell junction organization	0.000318	30	9
protein N-linked glycosylation via asparagine	0.0003514	21	8
Fc-gamma receptor signaling pathway involved in phagocytosis	0.0004328	15	8
transmembrane receptor protein tyrosine phosphatase signaling pathway	0.0006076	6	6
response to stress	0.0006203	289	9
extracellular matrix disassembly	0.0033533	20	7
intrinsic apoptotic signaling pathway	0.0034771	16	8
regulation of insulin secretion	0.0037177	23	9
toll-like receptor 10 signaling pathway	0.004683	12	6
fibroblast growth factor receptor signaling pathway	0.004683	32	8
transcription, DNA-templated	0.004683	340	10
cellular lipid metabolic process	0.0047551	23	9
anatomical structure morphogenesis	0.0069947	21	6
platelet degranulation	0.0076412	14	9
toll-like receptor TLR1:TLR2 signaling pathway	0.0082763	12	6
toll-like receptor TLR6:TLR2 signaling pathway	0.0082763	12	6
glycosaminoglycan metabolic process	0.0082763	18	6
transmembrane transporter activity	0.0082763	143	9
toll-like receptor 9 signaling pathway	0.0109591	13	6
cytoskeleton-dependent intracellular transport	0.0109591	21	8
cytoskeleton organization	0.0136345	96	9
generation of precursor metabolites and energy	0.0140393	48	9
toll-like receptor 5 signaling pathway	0.0146954	12	6
apoptotic signaling pathway	0.0152736	24	8
RNA binding	0.0200101	238	10
platelet alpha granule lumen	0.0239969	9	7
phosphatidylinositol biosynthetic process	0.0269148	13	5
SMAD binding	0.0309385	19	8
inositol phosphate metabolic process	0.0311064	9	5
toll-like receptor 2 signaling pathway	0.0384991	13	6
cellular component movement	0.048146	19	8

On evaluating significant pathway union alteration for all human MicroRNAs found in A/Puerto Rico/8/1934(H1N1) influenza A virus subtype by looking into the heat map analysis results of KEGG pathway for the same many pathways were altered notably (Figure 3). MicroT-CDS database was referred to obtain the microRNA results and the miRPATH (version 3) database was used to draw heat maps. The neighborhood lines represent linked target mRNAs found in a specific pathway.

Figure 3- Heat map analysis of KEGG



From figure 3 it can be observed that hsa-mir-567 is associated with multiple pathways including mineral absorption, cardiac muscle contraction, Legionellosis. Hsa-mir-4690 is involved in mineral metabolism, pyruvate metabolism, and homologous recombination. Hsa-mir-567 is found to play roles in Legionellosis, pyruvate metabolism, and homologous recombination. In contrast to this, hsa-mir-518c (glycosylphosphatidylinositol-anchor biosynthesis) hsa-mir-634 (Legionellosis), and hsa-mir-6873 (homologous recombination) are seen to be associated in one pathway each. In Legionellosis co-involvement of hsa-mir-567, hsa-mir-567 and hsa-mir-634 were noticed. The darker the color, the farthest it is placed in evolution, and more will be the probability of finding it across different subtypes. Here, it can be said that glycosylphosphatidylinositol- anchor biosynthesis will be targeted by more commonly. Using the microT-CDS database pathway cluster dendrogram of MiRNA or pathway analysis of KEGG pathway (Figure 4E) and miRNA cluster dendrogram (Figure 4F) of A/Puerto Rico/8/1934 (H1N1) influenza A virus subtype human miRNAs were obtained. The cluster dendrogram indicates microRNA expression profile determining alterations in specific pathways.

5. Discussion

ThepresentstudyadoptedmethodstoidentifyhumanmicroRNAspresentin12differentInfluenza A virus subtypes, looked into the conserved regions within them, and compared their degree of closeness. Along with this the targeted genes and pathways by these identified microRNAs were studied. Not limiting to this, the presence of secondary protein structures was also checked.

5.1 Similar Human MicroRNAs Identified and Their Targeted Pathways

The study identifies about eighty-three similar human microRNAs across 12 different subtypes of Influenza A virus. Because one MicroRNA can target 100s of mRNA, these results raise interest to see the functional role they play and alterations they can cause. Although, so many miRs were found three (Hsa-miR-5009-5p, Hsa-miR-3145-3p, Hsa-miR-3200-5p) were seen within the genome of 5 different Influenza A virus subtypes and four (Hsa-miR-518c-3p, Hsa-miR-6841-3p, Hsa-miR-6768-3p, Hsa-miR-6780b-3p) were within the genome of 4 different Influenza A virus subtypes; suggesting more significant functional involvement. The below table presents the important MiRs found in the study

Table 4 Major microRNAs identified, pathways affected, and their significance.

MicroRNA	Functions seen (Current study)	Similar Findings	Scope
Has-mir-5009 -presence in 5 different subtypes (H1N1, H1N2, H2N2, H3N2, H5N1)	Involved in the TGF-beta signalling pathway, estrogen signalling pathway, signalling pathways regulating pluripotent of stem cells, glycosaminoglycan biosynthesis-chondroitin sulfate / dermatan sulfate, lysine degradation, morphine addiction, prion disease and proteoglycans in cancer.	Has-mir-5009 was proposed to be a potential biomarker for colon cancer (Xia et al. 2014).	These findings open the scope of considering it as a diagnostic marker for Influenza A virus infections. It also to encourage further studies to see if there are any association between Influenza virus A infection and cancer.
Hsa-miR-3145-3p - found in 5 different viral subtypes (H1N1, H2N2, H5N1, H7N9, H9N2)	Associated in functions involving TGF-beta signalling pathway, estrogen signalling pathway, signalling pathways regulating pluripotent of stem cells, morphine addiction, prion disease and proteoglycans in cancer.	Khongnomnan et al. 2015, also reported to find it in 3 influenza A virus subtypes; namely, pH1N1, H5N1 and H3N2. Their study states that this miR interferes with viral transcription and replication by inhibiting expression of one of the viral polymerase complexes PB1.	From this, it can be inferred that this miR can be used in stopping infection progression of the mentioned viral subtypes via gene silencing.
Hsa-miR-3200-5p -identified among 5 different viral subtypes (H1N2, H1N5, H2N2, H7N2, H7N9)	Influence TGF-beta signalling pathway, estrogen signalling pathway, signalling pathways regulating pluripotent of stem cells and proteoglycans in cancer.	Riazalhosseini et al. 2017, in their study, suggested using hsa-miR-3200-5p as a marker to monitor the prognosis of disease hepatitis patients.	Likewise, it can also be used to look more into the effect of Influenzavirus A on the liver and if it causes hepatic conditions.

Onobservingamoreconservedgenesequencewasseeninhsa-miR-5009-5p,indicatingittoplay On observing a more On observing a more conserved gene sequence was seen in hsa-miR-5009-5p, indicating it to play pivotal roles in crucial biological functions. Apart from the above major microRNAs identified in this work, there were four more microRNAs that were seen across four different Influenza A virus subtypes. Of these, two of them hsa-miR- 6841-3p and hsa-miR-6768-3p were co-involved functions related to TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, lysine degradation, morphine addiction. Along with the above, hsa-miR-6841-3p was also associated with proteoglycans in cancer and hsa-miR-6768-3p played roles in glycosaminoglycan biosynthesis-chondroitin sulfate / dermatan sulfate, and prion disease. In contrast to this, hsa- miR-518c-3p and hsa-miR-6780b-3p were not seen to be associated with any such functions particularly. There were some more microRNAs identified and were found to have significant involvement in many functions. Hsa-miR- 6734-3p, hsa-miR-4778-3p, hsa-miR-216a-3p, hsa-miR-3116, hsa-miR-216a-3p and hsa-miR-3116 were all involved in TGF-beta signaling pathway, estrogen signaling pathway, signaling pathways regulating pluripotent of stem cells, lysine degradation, morphine addiction, and proteoglycans in cancer.

However, in pathways signaling glycosaminoglycan biosynthesis and functions related to prion disease these microRNAs showed differences. While Hsa-miR-6734-3p, hsa-miR-4778-3p, hsa- miR-3116 were involved in pathways signaling glycosaminoglycan biosynthesis, hsa-miR-6734- 3p, hsa-miR-4778-3p, and hsa-miR-216a-3p were associated with prion disease. Hsa-6886-3p and hsa-miR-6841-3p both did not show involvements in glycosaminoglycan biosynthesis and prion disease. Similar microRNAs will have the same target genes and functional roles even across different subtypes. Viral subtypes having the same miRs can be said to have somewhat the same aetiology, pathogenesis, clinical presentation, and disease progression. However, the host's body immunity also has a pivotal role in the onset of a health condition and its progress into a disease. The same set of pathways and functions, but in different combinations were seen to be affected by the significant miRs identified across 12 different Influenza A virus subtypes.

KEGG pathway analysis done in this study suggested that most of the human microRNAs identified in the subtypes influenced multiple signaling pathways. Signaling pathways regulating pluripotency of stem cells, Hippo signaling pathway, proteoglycans in cancer, pathways in cancer, Oocyte meiosis, Adrenergic signaling in cardiomyocytes and Ubiquitin mediated proteolysis showed the maximum number of miRs (9) linked in H1N1. In the case of the 12 subtypes, miRNAs (11) were involved in bringing alterations in the calcium signaling pathway, MAPK signaling pathway, AMPK signaling pathway, and pathways in cancer. In the same manner GO enrichment analysis results of H1N1 found in this study revealed that the microRNAs identified target thousands

of genes involved in functions associated with the cellular component, molecular function, biological process, and organelle. For all 12 subtypes, microRNAs targeted a large number of genes linked with the cellular component, organelle, molecular functions, biological process, ion binding, cellular nitrogen compound metabolic process, and biosynthetic process. It was also seen that multiple miRs were targeting these genes simultaneously.

When the miRs target multiple genes and when multiple miRs target a gene, the chances of finding the associated function alteration largely increases. Furthermore, when such miRs are identified in many viral subtypes the likelihood of observing similar clinical pictures in an infection raises as the same linked pathways are targeted. Viral subtypes having similar miRs are suggested to be more closely placed during evolution and hence have resemblance in their aetiology and pathogenesis. Additionally, if miRs targeting multiple genes are silenced then multiple pathway alterations can be inhibited, thereby many symptoms can be resolved if used for therapeutic purposes. Heat map analysis of H1N1 done in this study showed many miRs associated with multiple pathways including mineral absorption, cardiac muscle contraction, Legionellosis, mineral metabolism, pyruvate metabolism, and homologous recombination. Glycosylphosphatidylinositol- anchor biosynthesis was observed to be more distant in evolution indicating miRs targeting it to be found more across different subtypes. The finding can be correlated with Aquino et al. that also suggested that a large number of pathogens depend on this pathway to achieve their fundamental activities. In heat maps of 12 subtypes, miRs involved in prion disease was seen more, indicating frequent chances of being present among the viral subtypes. Although no direct correlation was found, Koeller et al. also suggested a probable similarity in neurological symptoms presented in Influenza virus infection and prion disease

5.2 Degree of Homology Observed Between Influenza Virus A Subtypes

In this study, we observed that some subtypes of Influenza virus A are more closely placed than the others. The subtype is distinguished based on hemagglutinin and neuraminidase, which are surface glycoproteins of the virus. There are many genetically different subtypes identified, 18 subtypes of HA (H1 to H18), and 11 subtypes of NA (N1 to N11). However, H1, H2, and H3, and N1 and N2 are mostly seen to cause epidemics in humans (Zhuang et al. 2019). Viral subtypes that are more closely placed are more identical in their genetic constitution. They will have a similar mode of action survival and host invasion. As a result, the aetiology, pathogenesis, and clinical manifestations in the course of infection will also have a large degree of resemblance. An advantage to this is that the diagnostic tools and therapeutic approaches that need to be adopted will also remain much similar.

5.3 RNA secondary structure Predicted

RNA secondary structures were identified as well as hairpin loop formation was seen in this study. This can be advantageous as microRNA mimics (oligonucleotides) complementary to the loop region can be designed and used for gene silencing. Thus, inhibiting the viral particle form performing functional roles and giving therapeutic advances against the infection. Simon, L et al. also suggested the use of secondary protein structures in developing antivirals against Influenza virus.

Conclusion

The study efficiently found numerous similar human microRNAs across 12 different Influenza virus A subtypes. Among these few were frequently found across multiple subtypes. These were seen to target many genes and were involved in multiple functional roles and significant pathways. Thus, making them significant tools for diagnosis, monitoring pathogenesis, and developing therapeutic strategies. The degree of homology gave a picture of the related viral subtypes of the virus. This broadened the horizons for looking into the roles of similar microRNAs specifically in the related subtypes, and if one approach can be used to target close subtypes. RNA secondary structures observed again sows the seeds for further investigation of the scope of designing antivirals. To sum up, this piece of research paves the path for further work on the details of each frequently found similar human microRNAs identified, to formulate diagnostic and therapeutic approaches.

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