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Review

Avian influenza and micro RNA: Role of bioinformatics

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Avian influenza virus is a major cause of influenza all over the world. Influenza virus being a RNA virus shows high mutation rates, antigenic shift and drift. These phenomena contribute to ineffective chemotherapy against influenza viruses. Recent advances in the current therapy, drugs and vaccines are restricted with many factors such as toxicity, complexity, cost and resistance. New technologies particularly RNA interference (RNAi) mediated by microRNA (miRNA) have become more and more interesting and effective therapeutic entities to silence pathogenic gene products associated with viral infections. Today, RNAi technology is a leading technology in sequence specific therapeutics. The flexibility of miRNAs in function makes them good candidates for use in sequence specific therapeutics. Although, miRNAs have been shown to be useful in combating against viral infections, there are problems associated with miRNA prediction, designing and function. Following review focuses on avian influenza virus (H5N1), the role of miRNAs in its pathophysiology and the computational prediction of miRNAs as antiviral therapeutics.

Key words: Avian influenza, miRNA, sequence specific therapeutics.

INTRODUCTION

Influenza is a highly infectious, acute respiratory disease which affects all age groups and possesses potential to occur repeatedly in any individual. Avian Influenza, popularly known as “Bird Flu” is one of the most severe respiratory viral infectious diseases of this decade. Highly pathogenic avian influenza (HPAI) H5N1 virus has created industrial and economical problems in the affected countries; the most affected industry being the poultry industry (Elci, 2006). This has lead to the need to find out effective measures which can be applied to combat with or to eliminate influenza threat. The host range of avian influenza virus includes birds and mammals, but its natural hosts are wetland birds such as wild ducks, gulls, and shore-birds (Zambon, 1999). Avian influenza is recurrently been found to be present in poultry birds, because of the transmissibility of the virus within bird species. The causative agent of avian influenza is Influenza A virus H5N1 strains. H5N1 is *Orthomyxoviridae* family member, with negative sense, single stranded RNA genome. The genome of H5N1 is segmented with 8 RNA segments, coding for eleven recognized proteins in all. These are PB1, PB1-F, PB2, and PA polymerases, HA, NP, NA, M1, M2, NS1, and NS2 proteins (Cheung and Poon, 2007). Two proteins, Hemagglutinin (HA) and Neuraminidase (NA) are the major antigens of H5N1. Being a RNA virus with segmented genome, H5N1 shows high mutation rates, especially in the antigenic regions. The phenomena of antigenic shift and antigenic drift make H5N1, highly unpredictable for chemotherapeutic interventions. The most widely used protective agents against AI viruses are vaccines. Either inactivated influenza viruses or antiviral agents are used as anti-influenza vaccines (Suarez and Schultz-Cherry, 2000; Palese and García-Sastre, 2002).

A variety of drugs are already available and some are in the process of formulation against H5N1. Five classes of drugs are available today; these include: neuraminidase inhibitors, M2 ion channel blockers, IMP dehydrogenase inhibitors, interferon and siRNAs, RNA polymerase inhibitors (De Clercq and Neyts, 2007; Ludwig et al., 2003) (Table 1). Most of the available drugs fail to provide effective protection against H5N1 virus, because of the highly variable nature of the antigenic part of the virus. Inactivated influenza virus in the form of a

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Table 1. Available drugs against H5N1 virus and their modes of action.

Drug	Example	Mode of function
Neuraminidase Inhibitors	Zanamivir, Oseltamivir, Peramivir	Blocks the release of viral particles, prevents spread of viral particles
M2 Ion channel blockers	Amantadine, Rimantadine	Interferes with the viral uncoating process
IMP dehydrogenase inhibitors	Ribavirin, Viramidine	Interferes with the function of IMP dehydrogenase
Interferon and siRNAs	α-interferon	RNA interference, host antiviral pathway
RNA polymerase inhibitors	T705, flutimide	T705 is recognized as a nucleobase, which in turn inhibits viral polymerase

vaccine may not always provide active immunity against new pandemic strains of influenza (García-Sastre, 2002). According to Clercq et al. (2007), double-, triple-, quadruple- combinations of currently available anti-influenza drugs can be used potentially against H5N1 (De Clercq and Neyts, 2007). Such combinations, may lead to possible emergence of new influenza strains resistant to multiple drugs. This kind of treatment may also produce toxic side effects in host systems, if not properly implemented.

Interfering RNAs are one of the most reliable drugs which candidates can be used against H5N1 to achieve sequence specific inhibition of viral genes. Interfering RNAs work on the lines of RNA interference (RNAi). RNAi refers to post-transcriptional gene silencing mediated by either degradation or translation arrest of target RNA (Ryther et al., 2005). Many reports and research articles cite use of interfering RNAs as a potential drug candidate use against H5N1 (Bennink and Palmore, 2004; Zhou et al., 2007; Ge et al., 2003; Brahmachari et al., 2008; Tompkins et al., 2004). With the advent of bioinformatics, it has become easy to predict and design interfering RNAs against specific genes or gene products thereof. No doubt interfering RNAs can be used as a therapeutic against RNA viruses, there are certain discrepancies and problems related to *in silico* prediction of interfering RNAs and *in vivo* use of these compounds.

THE INFLUENZA VIRUSES

The influenza virus family *Orthomyxoviridae* includes four types of influenza viruses (A, B, C) and thogotovirus (influenza D). Among Influenza viruses A, B and C viruses can be distinguished by the differences in their antigenic proteins namely NP and M proteins. Influenza A viruses are further divided according to the antigenic nature of their HA and NA glycoproteins. All influenza viruses are enveloped viruses having single stranded, linear, segmented, negative sense RNA genome. Influenza viruses typically contain 8 segments of RNA, except influenza C virus which contains 7 segments of RNA molecules (Lamb and Krug, 2001). All the 8 segments code for different proteins, encoding 11 proteins in all (Cheung and Poon, 2007). Thogotoviruses consists of 6 - 7 segments of RNA genome (Lamb and Krug, 2001; http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). All the four groups of influenza viruses differ in their host preferences, antigenic nature, morphological features, and in their respective mechanisms of encoding proteins. Influenza A viruses, which are the major contributors to the influenza spread worldwide, are categorized into different subtypes, based on the nature of HA and NA glycoproteins. There are total of 16 HA and 9 NA subtypes known (Osterhaus et al., 2008). These subtypes are perpetuated in aquatic birds. This gene pool of AI viruses seems to be limited but it can evolve rapidly once it enters domestic avian or mammalian host. Some of the strains which can evolve rapidly into highly pathogenic strains are H5 and H7. H1, H2, H3 strains have previously caused pandemics and epidemics in humans. H5, H7 and H9 strains are transmissible to human through avian hosts. H5N1 is the only strain of avian influenza which has caused mortality in humans, so far (Webster and Hulse, 2004).

H5N1

Among all the proteins, encoded by H5N1 genome, HA and NA proteins are the major determinants of biology of H5N1. HA glycoprotein is responsible for attachment of virus to the host cell, while NA glycoprotein is responsible for release of virus from infected cell and consequently spreading the virus throughout the respiratory tract. HA protein attaches to the cell membrane glycoproteins via sialic acid linkage, triggering viral fusion with host cell membrane and entry into the cell (Proença-Módena et al., 2007). Table 2 shows the H5N1 proteins and their functions. The host cell preference for any influenza A viral strain depends on binding to cell surface receptor that consists of terminal sialic acid residues with a 2-3

Table 2. Functions of proteins coded by H5N1 genome segments.

Segment	Protein product	Function
1	PB2	Polymerase proteins
2	PB1, PB1-F	Polymerase proteins
3	PA	Polymerase proteins
4	HA	Attachment of viral particle to host cell, major antigen
5	NP	Major structural protein
6	NA	Integral membrane protein, major antigen, release and spread of mature virions
7	M1, M2	Matrix protein
8	NS1, NS2	NS1 counteracts with type I interferon antiviral pathway of the host, NS2 is Involved in nuclear transport and viral assembly

linkage [Neur Ac(α2-3)Gal] to a penultimate galactose residue of glycoproteins or glycolipids. The tracheal epithelia of birds and mammals express influenza A receptors with a 2-3 and 2-6 linkage of sialic acid, respectively, whereas pig tracheal epithelia expresses both 2-3 and 2-6 linkages of sialic acid. This has lead to the hypothesis of pig as a “mixing bowl” for influenza A strains.

Another mechanism suggested by Matrosovisch et al. (2004), states that H5N1 viruses may infect humans, by infecting ciliated respiratory epithelial cells, as these cells preferentially express receptors with 2-3 linkages of sialic acid in culture. Although, ciliated cells are minor constituents of human respiratory epithelia, they are significant in number. The other part of respiratory epithelia consists of nonciliated epithelial cells, which preferentially express receptors with 2-6 linkages of sialic acid in culture (Matrosovich and Matrosovich, 2004). Hence, it poses a possibility that recurrent infection with H5N1 in humans might lead to highly pathogenic strain with a preference to 2-6 linkage of sialic acid (Lewis, 2006). According to a recent review by Zhang (2009); the generalized view that α2-3 and α2-linked sialic acid residues are the sole receptors determining tissue and host tropism is not true. Other factors such as glycan topology, lipid raft microdomains, local density of sialic acid receptors, concentration of invading virus, coreceptors and sialic acid independent receptors, may also be important in determining tissue and host tropism of human, avian and animal influenza viruses (Hong, 2009).

Epidemiology of influenza A and H5N1

The occurrence of influenza viruses is widespread throughout the world. Influenza viruses are responsible for symptomatic and asymptomatic infections in many vertebrate species, lower animals and aquatic birds (Webster et al., 1992). Many articles (Brown, 2000; Trampuz et al., 2004; Peiris et al., 2007), cite that aquatic birds are the primary source of influenza viruses in other species. Over past 150 years, at least four pandemics of influenza occurred at irregular intervals (Table 3).

According to recent World Health Organization (WHO) reports (http://www.who.int/csr/disease/avian_influenza/en/), 471 cases of avian influenza in humans have been reported, with 282 deaths world-wide. The maximum numbers of cases (161) and deaths (134) are from Indonesia, since 2003. The current threat of swine influenza, the causative agent of which is H1N1 viral strain, is nothing but a H1N1 human-swine-avian reassortant strain (Zhang and Chen, 2009; Smith et al., 2009). From the first report of swine influenza 2009 in humans in April 2009 (Zhang and Chen, 2009) at least 13,554 deaths have been reported to WHO regional offices from more than 208 countries and overseas territories or communities.

Avian influenza viruses can be categorized into two groups: low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI). Avian influenza (AI) viruses are generally LPAI, but upon introduction of H5 or H7 subtypes into domestic poultry these viruses may change their virulence level and may turn into HPAI strains (Webster and Hulse, 2004). The first report of AI in humans was reported from (Hong Kong, 1997; Chan, 2002). The H5N1 virus has spread worldwide from Southeast Asia to Europe and Africa, with occasional infections in humans, and continues to spread across the world (De Clercq and Neyts, 2007; Yen et al., 2008). Southern China is considered as the hypothetical pandemic epicenter of influenza (Horimoto and Kawaoka, 2001). Three earlier pandemics of influenza in Asia and recent threat of swine influenza H1N1 virus suggests that the next flu pandemic is highly possible, the causative agent of which could be avian influenza H5N1 virus.

Modes of mutations of H5N1

The variable nature of influenza viruses is contributed by some of the characteristic phenomena shown by them, such as, antigenic drift, antigenic shift, and recombination.

Table 3. Influenza pandemics and their impact in terms of human deaths.

Pandemic	Date	Deaths	Virus subtype involved
Asian (Russian) Flu	1889 - 90	1 million	H2N2
Spanish Flu	1918 - 20	50 million	H1N1
Asian Flu	1957 - 58	1.5 - 2.0 million	H2N2
Hong Kong Flu	1968 - 69	1 million	H3N2
Swine Flu	April 2009 till December 2009	~7.880 - ~16.460*	H1N1

*Data Source: (http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm).

These characteristics make them highly mutable. Antigenic drift occurs due to errors during replication, which are irreparable. These errors accumulate in the form of mutations in amino acid sequences within the viral genome, resulting in the existing strain being replaced by a new antigenic variant strain. The phenomenon of reassortment leads to exchange of RNA segments in two genotypically different influenza viruses infecting a single cell. This can result in the emergence of a novel strain and/or subtype. This phenomenon is known as antigenic shift; whereas recombination results in a single influenza RNA segment containing genetic material from two different sources. The current circulating swine influenza H1N1 virus is a reassortant of human, swine and avian influenza viruses, comprising of reassortant genomes of avian H1N1, H1N1 classical swine virus (Eurasian and North American) and H3N2 seasonal flu virus (Smith et al., 2009). The choice of mutations is most pronounced in HA and NA proteins, where selection is antibody mediated. Antigenic drift is common to all influenza viruses, but it is prominent in human influenza viruses as compared to AI viruses (Webster and Hulse, 2004) (Figure 1).

WHO FOOLED THE VACCINES?

A variety of vaccines and chemotherapeutic drugs are available which can act on H5N1 by inhibiting certain proteins or by interfering with viral metabolism. According to Clerq and Neyts (2007), five types of anti-influenza compounds are available and/or being evaluated for their anti-influenza properties. These include: neuraminidase inhibitors, M2 ion channel blockers, IMP dehydrogenase inhibitors, interferons and siRNAs, and RNA polymerase inhibitors (De Clercq and Neyts, 2007). Due to variable nature of viral antigens that is, HA and NA glycoproteins, neuraminidase inhibitors might not provide protection against all AI viruses (Bennink and Palmore, 2004; Fauci, 2005). Also, due to highly mutable nature of H5N1, other types of drugs might not provide protection at protein level. M2 ion channel inhibitors and neuraminidase inhibitors are also known to have side effects such as neurotoxicity (Brahmachari et al., 2008). According to Clerq and Neyts (2007), drug combinations can be used as a plausible anti-influenza treatment (De Clercq and Neyts, 2007). This may lead to emergence of highly drug resistant viral strains, at the same time; it might produce severe side effects in human hosts. Also, expense, potential side effects, and the timing of delivery have lead to the limited use of these drugs in high risk populations (Bennink and Palmore, 2004). Drugs which can specifically interfere with viral replication machinery may prove to be effective as it is more difficult for the virus to adapt to missing cellular functions (Ludwig et al., 2003).

Interferons play an important role as antivirals in immune system in humans. The Mx protein of human innate defense mechanism inhibits virus replication at various levels; while influenza NS1 protein has been reported to inhibit IFN pathway at various levels (Katze et al., 2002). Studies by Seo et al. (2002) show that H5N1 virus escapes host anti-viral responses. The exact mechanism of NS1 action is not yet clear, which may mark the use of interferons as controversial. Sequence specific inhibition that is, use of molecules which can inhibit viral genome at mRNA level, is one of the most promising anti-influenza therapy. Recent advances in RNA interference (RNAi) therapy, has lead to the discovery of small interfering RNAs (siRNAs) as antiviral agents. RNAi is the phenol-menon of sequence specific gene regulation in which a double stranded (ds) RNA mediates sequence specific degradation of target mRNA. Small regulatory RNAs include siRNAs, micro RNAs (miRNAs), and Piwi-interacting RNAs (piRNAs). Many publications (Ryther et al., 2005; Bennink and Palmore, 2004; Zhou et al., 2007; Ge et al., 2003; Rayburn and Zhang, 2008; Cejka et al., 2006; Colbère-Garapin et al., 2005; Novina et al., 2002; Dorsett, 2004; Aagaard and Rossi, 2007), cite use small regulatory RNAs as antiviral and anti-influenza com-pounds. The potential advantage in the use of sequence specific therapeutics (SSTs) is that these agents might not require an intact, functional immune system (Bennink and Palmore, 2004).

SMALL REGULATORY RNAs

Small noncoding RNAs (ncRNAs) have vital role in regulating a wide range of cellular pathways, develop-mental processes and the protection of genome

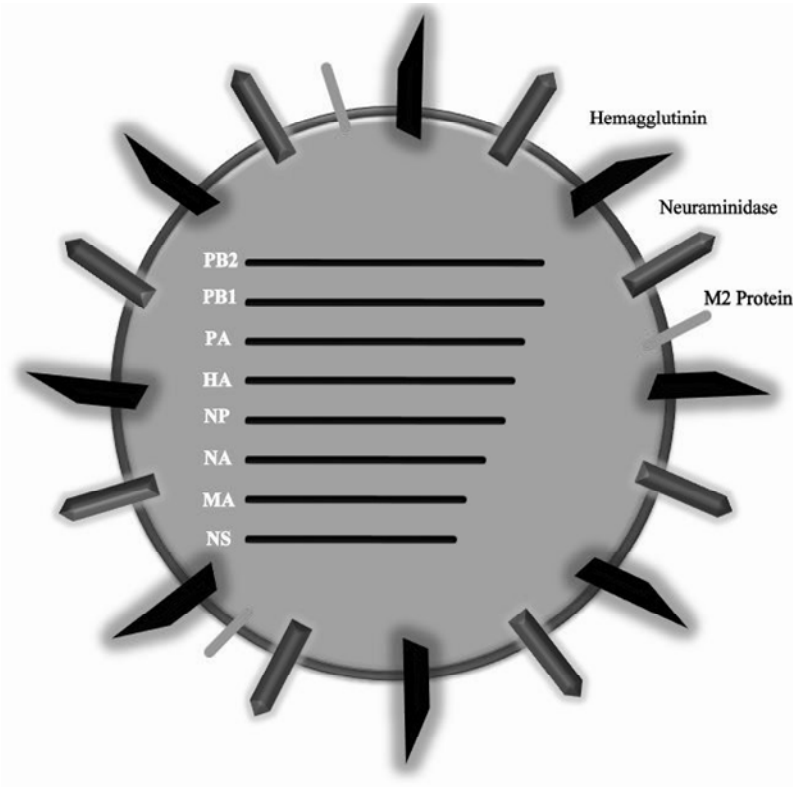

Figure 1. Diagram representing influenza virus. The eight segments of RNA code for eleven viral proteins.

against mobile genetic elements (Colbère-Garapin et al., 2005), such as viral genomes. In plants, siRNAs act as a conserved mechanism of antiviral immunity (Colbère-Garapin et al., 2005). Depending upon the type of ncRNAs, the mechanism of their biogenesis and action varies. The origin of miRNAs is endogenous while that of siRNAs is either endogenous or exogenous (Bushati and Cohen, 2007; Sontheimer and Carthew, 2005). The choice of type of ncRNA to be used in anti-influenza treatment is essential for achieving full-proof protection against the virus. There are conflicting views on efficacy of siRNA and miRNA as anti-viral therapeutics. Although, it is difficult to comment on the type of ncRNA to be used, as the treatment differs from virus to virus; miRNAs can be seen as promising ncRNA candidates; because of their flexibility in action.

miRNAs

miRNAs are generated by endogenous sources of nucleic acids. Most of the miRNAs are transcribed by RNA polymerase II, to generate pri-miRNAs, which are stem loop structures. Pri-miRNAs are processed by microprocessor, a multiprotein complex, consisting of RNase III enzyme Drosha and double stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha. This complex cleaves pri-miRNA converting it into hairpin precursor pre-miRNA. The 2 nucleotide (nt) 3' overhangs produced by RNase III is recognized by Exportin-5, which transports pre-miRNA to cytoplasm via Ran-GTP-dependent mechanism. In the cytoplasm, pre-miRNA is cleaved to produce mature miRNA: miRNA* duplex by Dicer protein. Argonaute (Ago) proteins, together with Dicer form a trimeric complex, known as the RNA-induced silencing complex (RISC). The miRNA strand with lower stability is incorporated into RISC, whereas the other RNA molecule namely miRNA* is degraded. Once incorporated into RISC, the miRNA guides the complex to its target mRNA. Depending on the complementarities the target mRNA is either degraded, cleaved or the translation of the target is repressed (Bushati and Cohen, 2007; Gregory and Shiekhattar, 2005; Chang and Mendell, 2007; Esquela-Kerscher and Slack, 2006; Bartel, 2004). miRNAs can also be produced by drosha independent pathway (Ruby, 2007).

The size of miRNAs varies depending on the species and miRNA genes, but is found to be approximately 22 nt. The seed region (2-7 or 2-8 nt) of miRNA consists of 7-8 nucleotides at the 5' end of the miRNA. Seed region is the primary determinant of miRNA target specificity

(Bushati and Cohen, 2007; Chang and Mendell, 2007). Depending on the coding sequence miRNAs are categorized as exonic and intronic miRNAs. The intronic miRNA is similar structurally and functionally to the exonic miRNAs, only difference is in the requirement for RNA polymerase II and other RNA splicing components for biogenesis (Lin et al., 2006). miRNAs show phenolmena of multiplicity or redundancy and cooperativity. Multiplicity refers to the ability of a single miRNA to regulate many targets, whereas cooperativity refers to the ability of many miRNAs to possess a common target (Xiao and Rajewsky, 2009).

According to Bushati and Cohen (2007), miRNAs function as regulatory molecules using five different modes of functioning, acting as: developmental switches, fine tuners of developmental programs, a part of feedback loop, inducers of proliferation and/or apoptosis, and molecules which establish threshold levels (Bushati and Cohen, 2007). miRNAs possess roles in human diseases such as neurodegenerative diseases for example, Tourette's syndrome (Abelson et al., 2005). miRNAs also possess roles as oncogenes and tumor supressors. Some of the human miRNAs such as miR-15a, miR-16-1 and let-7 family possess tumor suppressor activity, whereas miR-155, miR-17 cluster, miR-21 possess oncogenic activity Esquela-Kerscher and Slack, 2006. According to Chang and Mendell (2007), miRNAs have role in fragile X syndrome and synaptic function (Chang and Mendell, 2007).

Viral miRNAs

Several viral groups especially DNA viruses encode miRNAs to regulate the viral and host cellular transcripts. These miRNAs play an important role in viral infection, latency and metabolism. The functions of viral miRNAs include: regulation of viral gene expression, induction of degradation of viral transcripts, regulation of viral transcripts with partial homology, and regulation of host gene expression (Scaria et al., 2007). It seems possible that virus encoded miRNAs possess role in the process of inhibiting antiviral responses of host cell (Cullen, 2006). Jopling (2008) cites that viruses can also use host cellular miRNAs for their own benefit (Jopling, 2008). This phenomenon suggests that viruses might also be able to interfere with host cellular miRNA biogenesis and functions (Sullivan and Ganem, 2005). It seems possible that viral miRNAs play a role in either cancer causing or tumor suppressing activity in host cell (Fujii, 2009). The use of miRNAs by viruses can be attributed to certain properties of miRNAs; such as their non-immunogenicity, hence they do not require much of genomic space, and that, they are powerful regulators of gene expression (Sullivan, 2008; Qi et al., 2006).

Most of the identified viral miRNAs show poor conservation. Poor conservation of viral miRNAs does not necessarily indicate that the identified miRNAs do not have similar expression patterns and/or functions. This diversity suggests that viral miRNAs and their viral targets may have coevolved. The other facet is that, viral miRNAs may lose the functions due to point mutations in the seed region; this might result due to either loss or gain of a large number of cellular or viral miRNA targets (Gottwein and Cullen, 2008). The other interesting characteristic of viral miRNAs is their lack of homology with cellular miRNAs. This makes sure that viral miRNAs are only involved in virus specific regulatory relations. Some viral miRNAs show seed homology with some of the cellular miRNAs; for example, Kaposi sarcoma associated herpesvirus (KSHV) miR-K12-11 is an ortholog of cellular miR-155, they show strong seed homology (Skalsky et al., 2007). Such orthologous miRNAs may share functions resulting into regulation of major metabolic pathways of host by viral miRNAs.

Why miRNAs?

There are numerous articles present on use of sequence specific inhibition as an antiviral means. Although, most of the researchers go for the use of siRNAs, recent researches show that miRNAs can be effectively used as antivirals. Certain pros and cons are related to use of any kind of sequence specific therapeutic agents; the differences between siRNA and miRNA might provide insights into it. Although, both miRNAs and siRNAs depend on similar kind of biogenesis machinery and its components, the most important distinction can be the flexibility in regulation provided by miRNAs. The mode of action of miRNAs depends on the extent of complementarities in miRNA and its target. Also, miRNA show redundancy and cooperativity in function (He and Hannon, 2004). siRNAs work in a highly sequence specific manner and even a single point mutation in siRNA sequence may abrogate siRNA mediated silencing. This leads to viral RNAi escape mutants (Das et al., 2004; Westerhout et al., 2005; Giltin and Andino, 2003), which might prove to be highly virulent than their parent strains. To avoid this, multiple sequences per target and multiple targets per viral genome are necessary to effectively inhibit the virus (Ryther et al., 2005), this might clog miRNA pathway of host as siRNA and miRNA use same pathway for biogenesis (Aagaard and Rossi, 2007). Also, not all viral sequences are accessible as targets, may be due to RNA binding proteins or because of their complex secondary structure (Ryther et al., 2005). Most of the miRNAs are constitutively expressed, while some are restricted in expression which is temporal in nature.

On the other hand, siRNAs are mostly inducted due to exogenous sources, which are not constitutive, and temporal (Hutavágner and Zamore, 2002). siRNAs are typically less conserved as compared to miRNAs and

recognize targets with perfect complementarity, this is in accord with siRNAs being derived from the loci that they regulate or from the very closely related loci (Bartel, 2005). Endogenous siRNAs typically target same or similar loci, a phenomenon known as “auto-silencing”, whereas miRNAs show “hetero-silencing” (Bartel, 2004). It is known that long dsRNA triggers interferon-I (IFN-I) response; siRNAs when introduced into the cell, might trigger IFN response. This is due to recognization of “pathogen associated molecular patterns” by innate immune system of host, which results due to exogenous nature of siRNAs (Sledz et al., 2003; Behlke, 2006; Tosi, 2005). Computational prediction of miRNAs is relatively easier as it has been observed that miRNAs possess higher adjusted minimum folding energy (adjusted MFE to avoid potential effect of nucleotide sequence length on MFE) and a higher base pair rate in their predicted secondary structures as compared to other coding and non-coding RNAs (Zhang, 2006; Loong and Mishra, 2007).

Although, theoretically siRNAs may not seem to be a good choice for sequence specific therapeutics, many articles report usefulness of siRNAs as antivirals (Novina et al., 2002; McCaffrey et al., 2003; Jacque et al., 2002). A review by Bennink and Palmer, (2004), suggests promising use of siRNAs against influenza viruses (Bennink and Palmore 2004). Researchers such as Zhou et al. (2007); Ge et al. (2003); and Tompkins et al. (2004) have shown the potential of siRNAs in the treatment of influenza. Brahmachari et al. (2008) have filed a patent on targets for human miRNAs in H5N1 genome.

WHEN PREDATOR BECOMES PREY

The innate immunity antiviral pathway that is, IFN pathway is the prime pathway which can detect viral genomes and/or fragments thereof and can efficiently inhibit them. Many viruses, especially plant viruses, code for viral suppressors of IFN pathway. In case of influenza A virus, NS1 protein coded by segment 8 of viral genome, seems to interfere with IFN pathway of host. The NS1 protein seems to be involved in many essential functions such as mRNA transport, splicing, polyadenylation and translation Katze et al., 2002. Studies by Geiss et al. (2002); Garcia-Sastre et al. (1998), have shown that influenza viruses lacking functional NS1 protein are susceptible to antiviral mechanism of host cell (Geiss et al., 2002; García-Sastre et al., 1998). Many articles cite ability of NS1 to inhibit host antiviral responses either by interfering with JNK pathway or by inhibiting synthesis of interferons (Bergmann et al., 2000; Wang et al., 2000; García-Sastre, 2001; Ludwig et al., 2002). This poses possibility of NS1 being the primary culprit in the Hong Kong H5N1 infection which lead to human deaths (Palese et al., 2002). Also, experiments in plants have proved that NS1 acts as viral suppressor of RNAi (Delgadillo et al., 2004).

The reports on NS1 attracted attention of our lab as a target for SSTs. We have predicted viral miRNAs which show homology with 3 of human miRNAs. Also, prediction of metazoan miRNAs for NS1 homologous proteins from humans was also carried out. This was done using Softberry findmiRNA, miReval, TargetScanS and DIANA microT web tools. The average length of the predicted miRNAs is 22nt and they show dominance of guanine (G) in the seed region. The predicted miRNAs show role in MAPK, insulin signaling and pathways leading to renal cell carcinoma, pancreatic cancer, melanoma and chronic myeloid leukemia. This suggests possible roles of predicted miRNAs in host pathways related to cancer. The interaction map of miRNAs and their target genes, constructed using Osprey network visualization platform (Breitkreutz et al., 2003), is shown in Figure 2. Analysis of pre-miRNA structures of predicted miRNA shows that guanine (G) and uracil (U) are dominating nucleotides in pre-miRNAs; out of which G dominates in metazoan pre-miRNAs whereas U dominates in viral pre-miRNAs. Analysis of binding site of target mRNA reveals that hsa-miR-138, hsa-mir-525-3p and hsa-miR-124 show maximum value of MFE inferring that these reactions are most stable (Unpublished results). The role of NS1 as an inhibitor of host antiviral pathways is not fully elucidated yet. NS1 might possess other roles in relation with RNAi silencing and viral replication. Also, it can be inferred that RNAi against influenza virus proteins may not be effective due to interference created by NS1 protein (Figure 3).

LIMITS OF SEQUENCE SPECIFIC THERAPEUTICS

Apart from advantages provided by sequence specific therapeutic agents, these agents might also pose some problems. RNA viruses show high rate of mutations, hence they show a high degree of sequence diversity between different genotypes. RNA viruses are rapidly evolving viruses particularly those with segmented genomes. This is problematic in case of sequence specific therapeutics, particularly siRNAs, which are highly, sequence specific in action (Colbère-Garapin et al., 2005). To avoid this problem, if a variety of siRNAs are incorporated into the host cell, the implication might be clogging of endogenous miRNA pathway, as shRNA and siRNA resemble miRNA precursors (Aagaard and Rossi, 2007). Viral suppressors of host RNAi pathway pose another problem (Li and Ding, 2001). This may be overcome by inhibiting viral suppressor proteins. *In vivo* use of SSTs is difficult due to problems such as, blood stability, delivery to infected tissue, poor intracellular uptake, and nonspecific immune stimulation (Leung and Whittakar, 2005).

Serum nucleases can degrade siRNAs (Dykxhoorn and Lieberman, 2005; Paroo and Corey, 2004); but this can

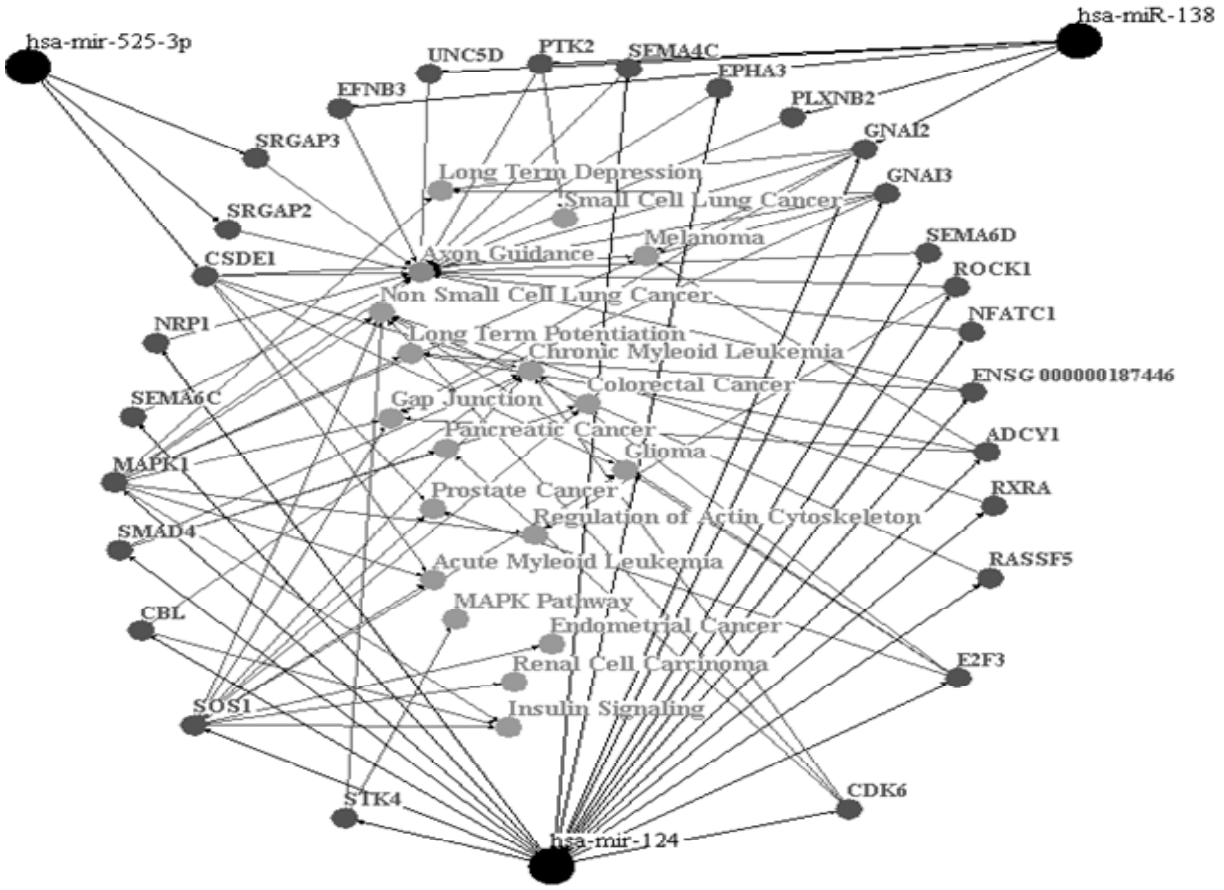

Figure 2. Interaction map of predicted miRNAs showing interrelationship between miRNA and their target genes. Predicted miRNAs show role in essential biochemical as well as cancer causing pathways of host cell.

be avoided to a certain extent by chemically modifying SSTs (Watts et al., 2008). Problematic delivery of SSTs to specific tissue types reduces efficacy of SSTs. Cationic polymers are usually applied to mediate delivery of SSTs into the host cell (Ge et al., 2003; Tompkins et al., 2004). SSTs should remain stable in the circulatory system, and should bind to blood proteins to a degree which should not be toxic (Dorsett, 2004). It has been suggested that the transfection agent may also contribute to the influence of expression independent of siRNA (Federov et al., 2005; Akhtar and Benter, 2007). Suppression off target presents another problem for SSTs. It has been observed that siRNA treated cells show off target gene silencing (Jackson et al., 2003; Jackson and Linsley, 2004; Couzin, 2004; Scacheri et al., 2004). Hence, it is of utmost importance to avoid off target gene silencing.

miRNA PREDICTION

Computational prediction of miRNAs is today's need for carrying out experiments related to or concerned with miRNAs. A variety of methods, protocols and online or offline web sources can be used and are in use for miRNA prediction. Table 4 shows the most popular web sources which are cited by most of the research articles. The computational prediction of miRNAs is based on the confirmed and known rules of miRNA and mRNA interactions (Wei et al., 2009). Several methods use evolutionary principles of miRNA sequence conservation, whereas recent methods have focused more on *ab initio* miRNA and target finding methods. Many methods use machine learning approaches (Legendre et al., 2005; Wang and Naqa, 2008). Although, machine learning approaches are efficient in prediction, the availability of data is less; this may affect efficiency of these methods (Lindow and Gorodkin, 2007). Biological properties of miRNA sequences are of great importance in computational prediction of miRNA and their respective targets. According to a review by Lindow and Gorodkin (2007), a variety of approaches can be applied for miRNA gene finding and for miRNA target prediction *in silico* (Lindow and Gorodkin, 2007). The miRNA gene finding takes into account approaches such as removal of exons and repeats, intergenomic matches, intragenomic matches, hairpin classification based on MFE values, rule

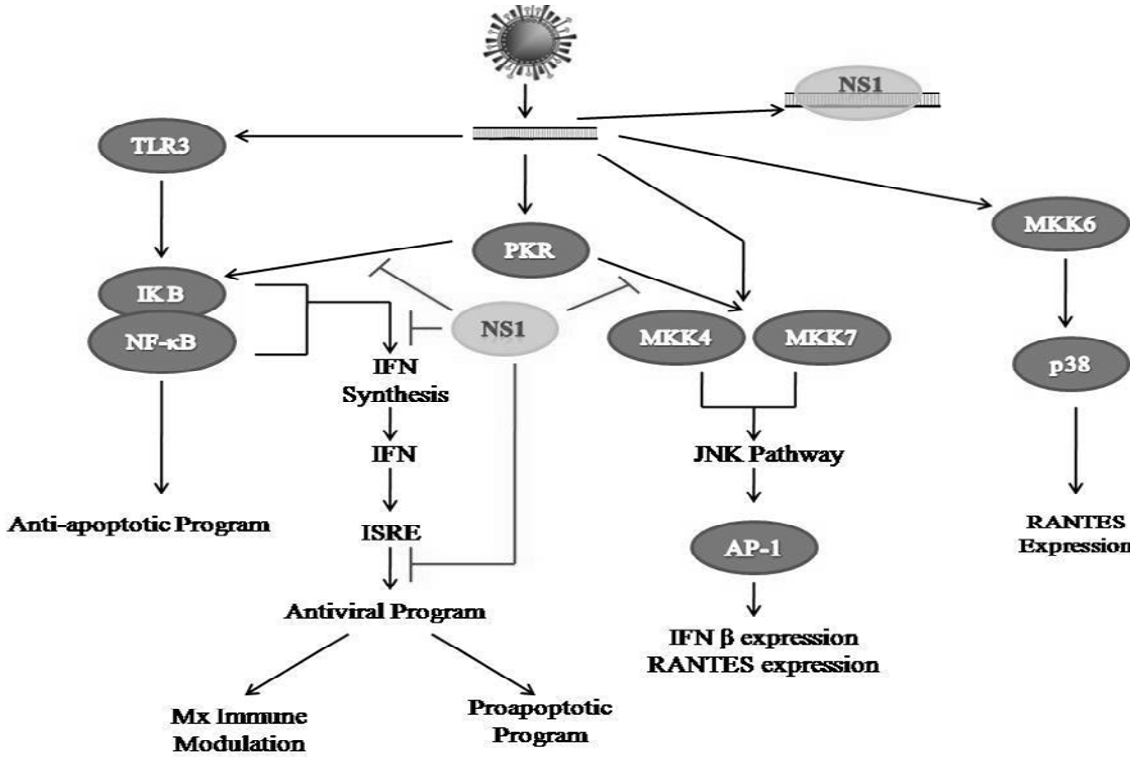

Figure 3. Involvement of NS1 protein of H5N1 in various cellular pathways leading to inhibition of IFN synthesis. Influenza virus infection activates a variety of chemokine and cytokine genes. H5N1 NS1 protein is found to be responsible for inhibition of antiviral mechanism of the host. This is mediated by inhibition of host proteins at different levels by NS1 as shown in the figure.

based classification of hairpins (Lindow and Gorodkin, 2007), and machine learning approach to classify hairpins (Lindow and Gorodkin, 2007; Xue et al., 2005). The miRNA target finding takes into account approaches such as conservation, use of target islands, seed matching. Target gene regulation by miRNAs and interrelationship between transcription factors and miRNAs can also be elucidated with the help of computational biology (Li et al., 2010).

Large scale genome analysis considering over 3853 reported miRNAs by Zhang et al. (2009) reveals that Uracil is the dominant nucleotide in the edges of seed region that is, at first and ninth position. This study also deduces the relationship between divergence in miRNA features and taxonomic level, inferring that as the taxonomic level increases diversity in miRNA features increases (Zhang et al., 2009). miRNA binding site location (3'UTR, coding sequence, 5'UTR), number of G: U wobble pairs in the seed region, and the kinetics and thermodynamics of miRNA and its target mRNA are some of the essential factors to be considered for computational miRNA prediction (Ghosh et al., 2007). The secondary structure prediction of miRNAs can be refined by eliminating pseudo knots, comparing with consensus structure, motif finding, statistical structure prediction and 2D structure prediction and comparison (Hamilton et al., 2007).

Avian influenza genome was computationally screened for human miRNA targets by Brahmachari et al. (2008). Two miRNAs namely hsa-miR-136 and hsa-miR-507 show targets in HA and PB2 protein, respectively. This suggests need of integrated *in silico* and *in vitro* approaches (Brahmachari et al., 2008). A research article by Hevik et al. (2007) emphasizes on the role of Drosha processing sites in miRNA precursors, citing that hairpins should not be annotated as miRNAs unless they are verified by Drosha and Dicer substrates (Helvik et al., 200). Whereas, Grimson et al. (2007) cite the effect of factors other than seed pairing in the miRNA target specificity in mammals (Grimson et al., 2007). Hence, it seems plausible that computational miRNA prediction may involve several parameters and factors, than in use. Also, sensitivity and specificity of prediction differs according to the algorithm used (Martin et al., 2007). Computational miRNA prediction may fail due to availability of less data, diverse nature of miRNA sequences, and flexibility in functioning of miRNAs interms of multiplicity and cooperativity. This creates a need to experimentally validate the predicted miRNAs and their target study *in vivo* (Barbato et al., 2009). This can be achieved by experimentally studying miRNA effects on target mRNA co-expression, target protein and

Table 4. Available web-sources for prediction of miRNAs and their targets.

Web tool	Link	Functions/uses
miRBase	http://www.mirbase.org/index.shtml	Database of predicted and known miRNAs
TargetScanS	http://www.targetscan.org/	miRNA gene finding, conservation analysis
RNA22	http://cbcsrv.watson.ibm.com/rna22.html	miRNA target and precursor miRNA finding
mir viRDB	http://140.109.42.4/cgiandbin/miRNA/miRNA.cgi	Sequence match, secondary structure prediction
miRanda	http://www.microrna.org/microrna/home.do	Target finding based on sequence match and MFE values
RNAHybrid	http://bibiserv.techfak.uniandbielefeld.de/rnahybrid/	Target finding based on sequence match and MFEs
DIANA microT	http://diana.cslab.ece.ntua.gr/pathways/	Target finding, Pathway annotations
Softberry findmiRNA	http://www.softberry.com/	miRNA finding, Target finding, folding studies
miRRim	http://www.ncrna.org/software/miRRim	HMM, conservation and thermal stability
Mfold	http://www.bioinfo.rpi.edu/applications/mfold	Nucleic acid folding and hybridization prediction
Pita	http://genie.weizmann.ac.il/pubs/mir07	Incorporates the role of target-site accessibility within traditional seed finding procedures
ViTA	http://vita.mbc.nctu.edu.tw/	Prediction of host miRNAs targets on viruses

target biological function (Kuhn et al., 2008).

Many of the mechanistic factors of miRNA biogenesis, sequence functionality and mode of action are still unexplored. Hence, it may reflect in the computational prediction of miRNA as most of the tools are based on machine learning approaches which use experimentally available data. Similar to siRNAs, there are chances of emergence of escape mutants in the treatment of miRNAs. On the other hand, due to flexibility in function, miRNAs might target host proteins leading to nonspecific reactions. This process of nonspecific interactions and viral protein inhibition, if visualized, considering the rapid evolution rate of viruses, suggests that use of miRNAs may lead to stronger viral suppressors and/or emergence of novel mechanisms of host RNAi inhibition.

CONCLUSION

The need of SSTs as antivirals is increasing due to emergence of drug resistant strains of influenza viruses. Recent advances in computational biology and SST research has opened up new avenues for *in silico* prediction of SSTs and *in vitro* validation of SSTs. Although, SSTs can be applied as antivirals, there are some innate problems related with their use. Designing, delivery and stability in the host system are some of the major hurdles in developing SSTs against influenza viruses. miRNAs seem to be more effective as anti-influenza therapeutics compared to siRNAs, as they are flexible in action. This flexibility in function might prove advantageous to treat continuously varying sequences of AI viruses. On the other hand, SSTs may also function in favour of viruses by nonspecifically inhibiting host metabolic pathways and/or proteins involved in essential metabolic pathways of the host. Computational prediction methods of miRNA prediction and miRNA target recognition have lead to easy and quick designing of SSTs. Although, there are certain discrepancies in different prediction methods applied by various algorithms; computational methods can be effectively used as a potential tool for further experimental validation of miRNAs.

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