VOLUME 11 NOMOR 2 DECEMBER 2024 ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

IN SILICO ANALYSIS OF SMALL INTERFERING RNA TARGETING THE NUCLEOPROTEIN GENE OF INFLUENZA VIRUSES

Analisa *Small Interfering RNA* secara *In Silico* terhadap Virus Influenza dengan Target Gen Nucleoprotein

Risza Hartawan[1]*, Atik Ratnawati[1], Indrawati Sendow[1], Ni Luh Putu Indi Dharmayanti[1]
[1]Research Center for Veterinary Science, Research Organization for Health,
National Research and Innovation Agency, Indonesia
*Email: risz001@brin.go.id

ABSTRACT

Small interfering RNA (siRNA) is a promising therapeutic against viral infection, including Influenza viruses. However, the Influenza viruses have massive variants with high mutation rates. Therefore, the siRNAs could be futile against newly emerging viruses. Thus, this study aimed to analyze siRNAs targeting the nucleoprotein gene of Influenza viruses. Using bioinformatic analyses, the siRNAs were simulated against 5 subtypes of Influenza viruses, including H1N1, H3N2, H5N1, H7N9, and H9N2. Bioinformatic tools for the folding structure of messenger RNA were utilized to select effective siRNA. As a result, 32 siRNA sequences targeting the nucleoprotein gene were identified. The precision medicine concept seems applied to the siRNA treatment for the Influenza virus since each siRNA is effective in its respective virus target. Based on the nucleotide mismatch parameter, most siRNA does not have coverage for the multiple infections of all five subtypes of Influenza viruses, except for NP1089 and NP1496. Later, the secondary and tertiary structure analysis of messenger RNA demonstrated that siRNA has different circumstances in its RNA target position. Therefore, siRNA mapping based on the RNA folding structure approach provides a tool for selecting more effective sequences against Influenza virus infection. Both siRNA NP1089 and NP1496 were predicted to have similar effectivity in knocking down Influenza virus infection. Moreover, the cocktail application of siRNA treatment may be effective as an alternative strategy in matching co-infection of multiple Influenza virus subtypes.

Keywords: *siRNA, Influenza, Nucleoprotein, Bioinformatic, RNA secondary and tertiary structure*

ABSTRAK

Small interfering RNA (siRNA) merupakan salah satu pendekatan terapi yang menjanjikan untuk melawan infeksi berbagai macam virus, termasuk virus Influenza. Namun, virus Influenza memiliki banyak varian dengan tingkat mutasi yang tinggi. Oleh karena itu, siRNA dapat menjadi sia-sia terhadap berbagai virus Influenza yang baru bermunculan. Oleh karena itu, penelitian ini bertujuan untuk menganalisis siRNA dengan target gen nukleoprotein virus Influenza. Dengan menggunakan analisa bioinformatika, siRNA disimulasikan terhadap 5 subtipe virus Influenza, termasuk H1N1, H3N2, H5N1, H7N9, dan H9N2. Selanjutnya, analsa bioinformatika untuk struktur pelipatan messenger RNA digunakan untuk pemilihan siRNA yang efektif. Sebagai hasilnya, 32 siRNA yang menargetkan gen nukleoprotein dapat diidentifikasi. Konsep pengobatan

Received: *28 October 2024* ***Accepted***: *10 December 2024* ***Published***: *20 December 2024*

presisi tampaknya berlaku pada pengobatan siRNA untuk virus Influenza karena setiap siRNA efektif pada target virusnya masing-masing. Berdasarkan parameter ketidakcocokan nukleotida, sebagian besar siRNA tidak memiliki cakupan untuk melawan infeksi kelima subtipe virus Influenza, kecuali NP1089 dan NP1496. Analisis struktur sekunder dan tersier messenger RNA menunjukkan bahwa siRNA memiliki kondisi yang berbeda pada posisi target RNA-nya. Oleh karena itu, pemetaan siRNA berdasarkan pendekatan struktur pelipatan RNA dapat digunakan untuk memilih siRNA yang lebih efektif terhadap infeksi virus Influenza. Baik siRNA NP1089 maupun NP1496 diprediksi memiliki efektivitas yang hampir sama dalam melawan infeksi virus Influenza. Selain itu, aplikasi koktail dalam pengobatan siRNA mungkin efektif sebagai strategi alternatif dalam mengantisipasi ko-infeksi beberapa subtipe virus Influenza.

Kata kunci: siRNA, Influenza, Nukleoprotein, Bioinformatika, Struktur sekunder dan tersier RNA

INTRODUCTION

Small interfering RNA (siRNA) is a post-translation gene silencing agent by RNA interference (RNAi) approach (Dong et al. 2019, Hu et al. 2020). As a short non-coding RNA sequence in about 21 nucleotides, the siRNA degrades a complementary messenger RNA (mRNA), so the encoded protein cannot be produced (Lam et al. 2015). Therefore, the siRNA approach has been developed for cancer, genetic disorders, and pathogen infections (Draz et al. 2014). To date, several medicines based on siRNA have been approved by the Food and Drug Administration as well as the European Union, such as patisiran, givosiran, lumasiran, inclisiran, vutrisiran, and nedosiran (Ahn et al. 2023; Guo et al. 2024a; Jadhav et al. 2024). Numerous siRNA therapies are also in the clinical trial stages waiting for approval. These legal approvals raise promising commercialization of siRNA as therapeutic agents in the future (Guo et al. 2024b).

Many siRNA studies have been dedicated to viral infection treatment such as respiratory syncytial virus, hepatitis C virus, human immunodeficiency virus, EBOLA virus, influenza virus, etc (Qureshi et al. 2018; Kang et al. 2023). Moreover, the siRNA technology provides an alternative for developing new viruses that may resist current antivirals (Earhart et al. 2009; Okomo-Adhiambo et al. 2015). Thus, siRNA development for influenza antiviral has been performed by targeting important genes of Influenza viruses (Qureshi et al. 2018; Kang et al. 2023).

Influenza A group is a single-stranded negative-sense segmented RNA virus belonging to the Orthomyxoviridae family (Bouvier and Palese 2008). Subsequently, Influenza A viruses are classified into subtypes based on the combination of surface glycoproteins, namely hemagglutinin (H1-18) and neuraminidase (N1-11). These viruses have a wide range of hosts, from avian to mammalian including humans. Several subtypes are endemic in humans, including H1N1 and H3N2 (Bui et al. 2017). Despite most viruses circulating in avian and swine, several subtypes can cross interspecies barriers with fatal causalities, such as H5N1, H7N9, H9N2, etc. The concern about Influenza viruses is related to the previous pandemics with massive fatalities, including Spain flu H1N1 in 1918, Asian flu H2N2 in 1957, Hong Kong flu H3N2 in 1968, and Swine flu H1N1pdm09 in 2009 (Harrington et al. 2021). Therefore, effective antivirals are essential for the next pandemic preparedness.

The genome of the Influenza A virus is separated into 8 segments, namely polymerase base 2 (PB1), polymerase base 1 (PB1), polymerase acidic (PA), hemagglutinin (H), nucleoprotein (NP), neuraminidase (N), Matrix (M), and a non-structural protein (NS) (Bouvier and Palese 2008). Excluding H and N, the siRNAs have been designed for all six segment genes for several subtypes, such as H1N1, H3N2, H5N1, etc (Szczesniak et al. 2023). The NP has

become the most favorable target for gene silencing because it has been considered a conserved gene with a crucial function in the virus life cycle, especially in the RNA packaging process (Soszynska-Jozwiak et al. 2015). Despite most studies showing promising outcomes, the unique characteristics of the viruses can cause siRNAs to become ineffective because of the incompatibility matters because of massive variants and high mutation rates (Huang et al. 2017; Tompkins et al. 2004).

The recent concerns related to the siRNA therapeutic are preoccupied with desirable delivery systems (Neary et al. 2024; Thangamani et al. 2021; Ranasinghe et al. 2022; Wang et al. 2022). However, key problems in the Influenza virus siRNA therapeutic also have arisen in the selection of effective designs due to massive virus variants in the field. Therefore, new approaches are indispensable for selecting effective siRNAs from both references and/or self-design trials. The main objective of this study was to conduct a systematic mapping of the siRNA designs targeting the NP gene of Influenza viruses. A meta-analysis and *in silico* bioinformatic simulation were applied to evaluate the effectiveness of these siRNA sequences against new Influenza viruses. This mapping and bioinformatic analyses should give different perspectives about the siRNA circumstances regarding the Influenza virus infection.

MATERIALS AND METHODS

Design siRNAs for Influenza viruses

siRNA sequences targeting the nucleoprotein genes of the nfluenza viruses were designed using free web-based software (Naito et al. 2009). Firstly, a consensus sequence of the NP gene was constructed using the BioEdit 7 software (https://thalljiscience.github.io/) from the NP sequences of the Indonesian Influenza viruses retrieved from the NCBI Genbank Influenza virus database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi) on September 1st, 2023. Then, the consensus sequence was used as a template for siRNA design to accommodate genetic variation between Influenza viruses, such as single nucleotide polymorphism (SNP). Subsequently, the siRNA design was performed using siDirect version 2.1β (http://si-direct2.rnai.jp) and GenScript siRNA target finder (https://www.genscript.com/tools/sirna-target-finder) as per the software instruction manual.

Mapping siRNAs for Influenza viruses

Identification of the siRNA sequences targeting the nucleoprotein gene of the Influenza virus was conducted from previous studies. The siRNAs were retrieved from these reference studies without any limitation of their virus subtype of interest as long as its target gene sequences are clearly stated. The nomenclature of these siRNAs follows their original name from the respective studies. Subsequently, the NP gene sequences of Influenza viruses of H1N1, H3N2, H5N1, H7N9, and H9N2 subtypes isolated from humans were retrieved from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi). Later, the systematic mapping of the siRNA sequences on its target recognition in the nucleoprotein sequences of several Influenza viruses was performed using Clustal W (BioEdit 7, https://thalljiscience.github.io/). The effectivity of the siRNA designs against these newest Influenza viruses was evaluated by nucleotide matching parameter. The siRNA design is considered still effective if it is displayed ≤1 mismatch with its target sequences.

Bioinformatic analysis for siRNA effectivity

Further analysis of the siRNA target position in the mRNA of the NP of Influenza viruses in the secondary RNA structure form was accomplished with the RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Subsequently, the tertiary structure of mRNA was developed by the RNAcomposer (http://rnacomposer.ibch.poznan.pl/;jsessionid=520617E206D60C426C4FE5A8C6704B2F) and visualized by PyMOL 3.1.0 (https://pymol.org/). The siRNA target position was pinpointed in the secondary and tertiary mRNA structures to highlight the pattern, free chain, and complexity.

RESULTS AND DISCUSSION

For decades, the siRNA technology has been explored as an alternative antiviral against Influenza virus infection with promising outcomes, either *in silico, in vitro*, or even *in vivo* studies (Ge et al. 2004; Huang et al. 2017; Piasecka et al. 2020). The viral infection rates are suppressed by knocking down the targeted viral genes (Lam et al., 2013). Moreover, the siRNA design for the Influenza virus is quite tricky since many virus variants are identified in the field with a high possibility of mutations. Therefore, specific software with several parameters and calculations that can accommodate consensus sequences with multiple single nucleotide polymorphisms (SNPs) is required to develop siRNA sequences for Influenza virus genes (Naito et al. 2009).

The nucleoprotein gene is believed to be a highly conserved gene among other influenza genes (Soszynska-Jozwiak et al. 2015). Our studies found that the NP gene of the Influenza viruses has a high genetic variant, especially in different subtypes. To develop siRNAs that are effective for several Influenza subtypes at the same time, the consensus sequence should be used as a template for the siRNA designing software. In this study, the siRNAs designed for the NP gene of Influenza viruses are presented in Table 1. Using siDirect version 2.1β, four siRNAs were designed, namely siRNA-NP674, siRNA-NP1085, sRNA-NP1089, and siRNA-NP1324. On the other hand, one siRNA has resulted from the GenScript siRNA Target Finder, which was siRNA-NP975. Actually, several web-based software programs also provide services for constructing siRNA; however, they do not accommodate sequences with SNP variance. Moreover, the price of typical siRNA for viruses is more expensive than normal siRNA for human genes since it needs to be customized (Hartawan et al. 2022).

Table 1. Mapping of siRNAs targeting the NP gene of the Influenza viruses

siRNA	Target Sequence (5'-3')	Size (nucleotide)	Target Position	Number of Nucleotide Mismatches					Reference
				H1N1	H3N2	H5N1	H7N9	H9N2	
NP-183	ACTCAAACTCAGTGATTAT	19	138-156	1	2	1	2	2	Piasecka et al. 2020
NP-231	TAGAGAGAATGGTGCTCTC	19	188-206	2	1	2	1	1	Ge et al. 2003
NP-390	TAAGGCGAATCTGGCGCCA	19	347-365	3	0	4	5	5	Ge et al. 2003
NP-412	GCAAACAATGGCGAAGATG	19	367-385	0	3	4	2	3	Piasecka et al. 2020
NP-448	CATATCATGATTTGGCATT	19	403-421	0	3	4	3	3	Piasecka et al. 2020
NP-471	CCTGAATGATGCCACATAT	19	426-444	1	4	2	4	1	Piasecka et al. 2020
NP-574	GAAAGGAGTTGGAACAATA	19	549-567	1	4	4	6	6	Zhiqiang et al. 2010
NP-613	GCAATGGAGTTAATCAGAA	19	568-586	2	5	7	8	7	Piasecka et al. 2020
NP-672	CATCCTCAAAGGGAAATTCCA	21	672-692	3	4	1	1	0	Hartawan et al. 2022
NP-674	TCCTCAAAGGGAAATTCCAAA	21	674-694	2	4	0	1	0	siDirect version 2.1β
NP-682	CGAAGGACAAGGGTTGCTT	19	637-655	1	6	4	5	5	Piasecka et al. 2020
NP-717	TTATGAGAGAATGTGCAACAT	21	654-674	2	0	2	1	3	Timin et al. 2012
NP-733	AACATTCTCAAAGGAAAATTT	21	670-690	2	1	4	2	3	Brodskaia et al. 2018
NP-749	AGAGCAATGATGCATCAAG	19	706-724	2	3	1	3	2	Zhou et al. 2007
NP-865	GTCCTGCTTGCCTGCTTGT	19	819-837	2	5	0	1	0	Behera et al. 2015
NP-901	GCAAGTGGGCATGACTTTG	19	856-874	1	5	4	3	2	Piasecka et al. 2020
NP-975	AAGAGTCAATTAGTGTGGATG	21	973-993	2	3	0	2	1	GenScript siRNA Target Finder
NP-1004	ATTCTGCTGCATTTGAAGATAA	21	1001-1022	3	2	8	7	6	Brodskaia et al. 2018
NP-1008	CCCAGCTCACAAGAGTCAA	19	963-981	1	3	4	3	2	Piasecka et al. 2020
NP-1085	GGGTTCAAATTGCTTCAAATG	21	1085-1105	1	2	0	0	0	siDirect version 2.1β
NP-1089	TCAAATTGCTTCAAATGAGAA	21	1089-1109	1	1	0	0	0	siDirect version 2.1β
NP-1155	AATTGCTTCAAATGAGAACAT	21	1092-1112	3	0	0	0	0	Timin et al. 2012
NP-1312	ATGCTGCCATAACGGTTGC	19	1265-1283	8	8	8	8	8	Piasecka et al. 2020
NP-1324	ACTGAAATCATAAGAATGATG	21	1324-1244	3	3	0	0	0	siDirect version 2.1β

siRNA	Target Sequence (5'-3')	Size (nucleotide)	Target Position	Number of Nucleotide Mismatches					Reference
				H1N1	H3N2	H5N1	H7N9	H9N2	
NP-1341	GAATGATGGAAAGTGCAAAAC	21	1337-1357	1	3	2	2	1	Brodskaia et al. 2018
NP-1342	AATGAAGGACGGACATCCG	19	1297-1315	0	5	5	6	8	Piasecka et al. 2020
NP-1383	TGATGGAAAGTGCCAGACC	19	1340-1358	4	4	0	0	1	Zhou et al. 2007
NP-1433	CCTTTGACATGAATAATGAAG	21	1433-1453	1	4	0	1	1	Hartawan et al. 2022
NP-1494	GAGACAATGCAGAGGAGTA	19	1469-1487	0	2	0	0	0	Zhiqiang et al. 2010
NP-1496	GGATCTTATTTCTTCGGAG	19	1453-1471	1	0	0	0	0	Ge et al. 2003
NP-7	CAGAGGAGTATGACAATTA	19	1478-1496	1	3	0	1	0	Villa et al. 2023
Oligo-NP	AATGGACTCCAACACTCTTCA	21	1119-1139	5	6	2	2	1	Zhou et al. 2008

On the other hand, a total of twenty-seven siRNAs targeting the NP gene of Influenza viruses have been identified from the ten references published from 2003-2023 targeting the proximal up to distal part of the NP gene (Behera et al. 2015; Brodskaia et al. 2018; Ge et al. 2003; Hartawan et al. 2022; Piasecka et al. 2020; Timin et al. 2012; Villa et al. 2023; Zhiqiang et al. 2010; Zhou et al. 2007; Zhou et al. 2008). These siRNAs are presented in Table 1. Most of the siRNA studies have been performed on the Influenza virus subtypes H1N1 and H5N1. The threat of the influenza virus is not solely limited to these two subtypes because many other subtypes have also been reported to cause infection in humans (Bui et al. 2017). However, no study of siRNA design for influenza virus claims to be effective in reducing infection of all influenza subtypes. The *in vivo* challenge test of siRNA NP-1496 and PA-2087 in mice against four different influenza subtypes demonstrated that these siRNAs provide different outcomes of virus titer reduction depending on the virus subtypes, including H1N1, H5N1, H7N7, and H9N2 (Tompkins et al. 2004).

Subsequently, thirty-two siRNA sequences from both self-design and referral sources were simulated against Influenza viruses, including A/Wisconsin/37/2023(H1N1), A/New Mexico/34/2023(H3N2), A/Nanjing/1/2015(H5N1), A/China/LN/2017(H7N9), and A/China/A_Szuhou_GIRD01_2019/2019(H9N2). Using complementary parameters between the siRNA sequence and its target position, these siRNAs were matched with all five Influenza isolates. The siRNA is still considered effective in silencing the NP gene expression when the dissimilarity with its target ≤ 1 nucleotide. As a result, most siRNAs fail to cover the divergence of the nucleoprotein gene from recent Influenza virus subtypes isolated from humans, including H1N1, H3N2, H5N1, H7N9, and H9N2. Five siRNAs have coverage for four Influenza virus subtypes, including siRNA-NP1085, siRNA-NP1155, siRNA-NP1433, siRNA-NP1494, and siRNA-NP7. Later, six siRNAs still have coverage for three Influenza virus subtypes, including siRNA-NP231, siRNA-NP672, siRNA-NP674, siRNA-NP685, siRNA-NP1324, and siRNA-NP1384. The remaining nine-teen siRNAs only cover two or fewer influenza virus subtypes. A plausible explanation for this phenomenon is massive variants of the nucleoprotein gene, especially across different Influenza subtypes. This situation is exacerbated by gene mutation over time (Bouvier and Palese 2008).

Interestingly, this study found two siRNAs have coverage for these Influenza virus subtypes, which were siRNA-NP1089 and siRNA-NP1496. This result agrees with the previous studies where siRNA-NP1496 was capable of reducing several influenza virus subtypes infection as *in vivo* challenge in mice, including H1N1, H5N1, H7N7, and H9N2, even though the effect on virus infectivity reduction was different on each virus subtype (Tompkins et al. 2004).

Subsequently, the following consideration of the siRNA should not be analyzed solely based on the primary or linear structure of messenger RNA. The secondary structure of mRNA was used to identify and visualize the attachment position of siRNAs on its mRNA target (Szabat et al. 2020). Table 2 displays the free chain in the mRNA-siRNA target position, whereas Figure 1,

Figure 2, and Figure 3 display circumstances in the mRNA target position of siRNA, including the free chain, pattern, and its complexity for five different I.

Table 2. Bioinformatic analysis of siRNAs based on the mRNA secondary structure

siRNA name	Size (nucleotide)	Free chain structure on siRNA target position				
		H1N1	H3N2	H5N1	H7N9	H9N2
NP-183	19	10		3		
NP-231	19		9		7	9
NP-390	19		9			
NP-412	19	8				
NP-448	19	3				
NP-471	19	10				9
NP-574	19	10				
NP-672	21			8	8	4
NP-674	21			8	5	7
NP-682	19	5				
NP-717	21		7		7	
NP-733	21		7			
NP-749	19			9		
NP-865	19			5	3	5
NP-901	19	6				
NP-975	21			7		10
NP-1008	19	4				
NP-1085	21	6		4	4	4
NP-1089	21	6	12	8	8	8
NP-1155	21		11	8	6	8
NP-1324	21			7	8	7
NP-1341	21	14				4
NP-1342	19	7				
NP-1383	19			2	12	2
NP-1433	21	8		5	5	5
NP-1494	19	7		6	4	4
NP-1496	19	8	6	7	8	9
NP-7	19	4		9	2	2
Oligo-NP	21					8

Note: the grey cell indicates for >1 nucleotide mismatches between siRNA sequence and its mRNA target

Influenza subtypes. For H1N1, 16 siRNAs are predicted still to be compatible with the nucleoprotein gene silencing (Figure 1A). Subsequently, four siRNAs have at least teen-free nucleotide parts, namely siRNA-NP183, siRNA-NP471, siRNA-NP574, and siRNA-1341. For H3N2, seven siRNAs are considered to be working, where only two siRNAs have at least teen-free nucleotide parts, namely siRNA-NP1089 and siRNA-NP1155 (Figure 1B). For H5N1, there are fifteen siRNAs considered to be still effective, whereas no siRNA has at least teen-free nucleotide parts (Figure 2A). For H7N9, there are fourteen siRNAs considered to be still effective and only one siRNA has at least teen-free nucleotide parts, namely siRNA-1383 (Figure 2B). For H9N2, there are seven teen siRNAs considered to be still effective, where only one siRNA has at least teen-free nucleotide parts, namely siRNA-975 (Figure 3).

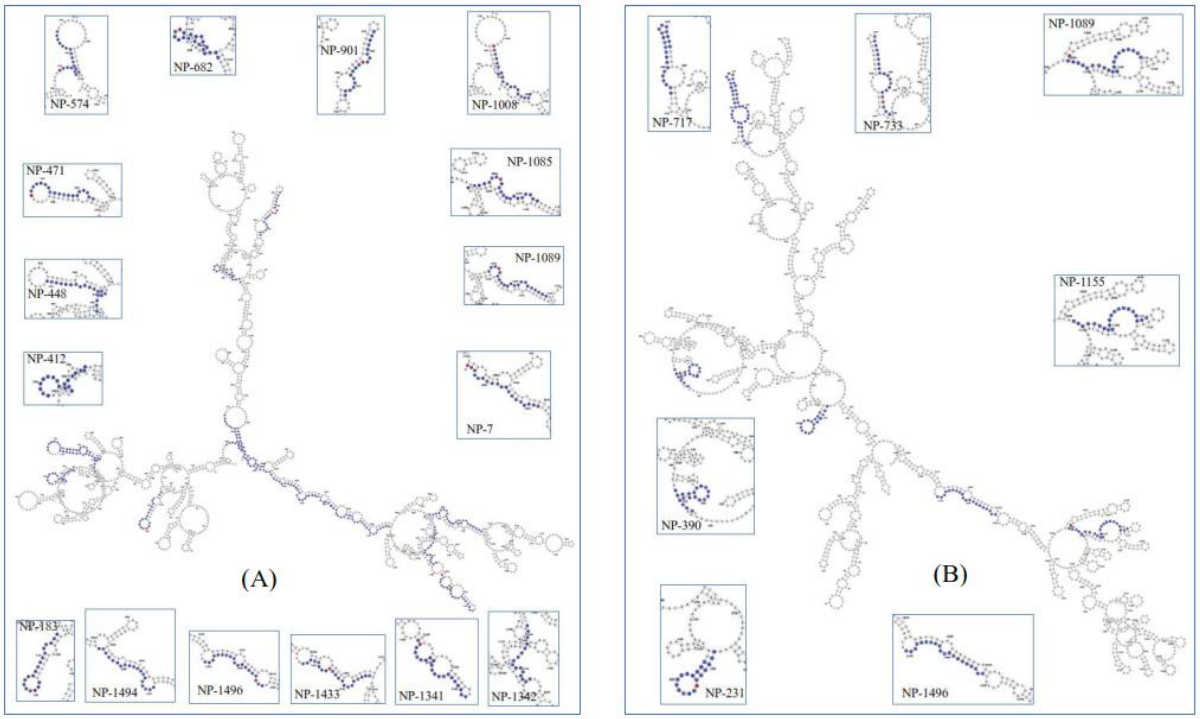

Figure 1. The sites of siRNA attachment in the mRNA secondary structure of the NP gene of the Influenza virus subtype H1N1 and H3N2. (A) A/Wisconsin/37/2023(H1N1) and (B) A/New Mexico/34/2023(H3N2)

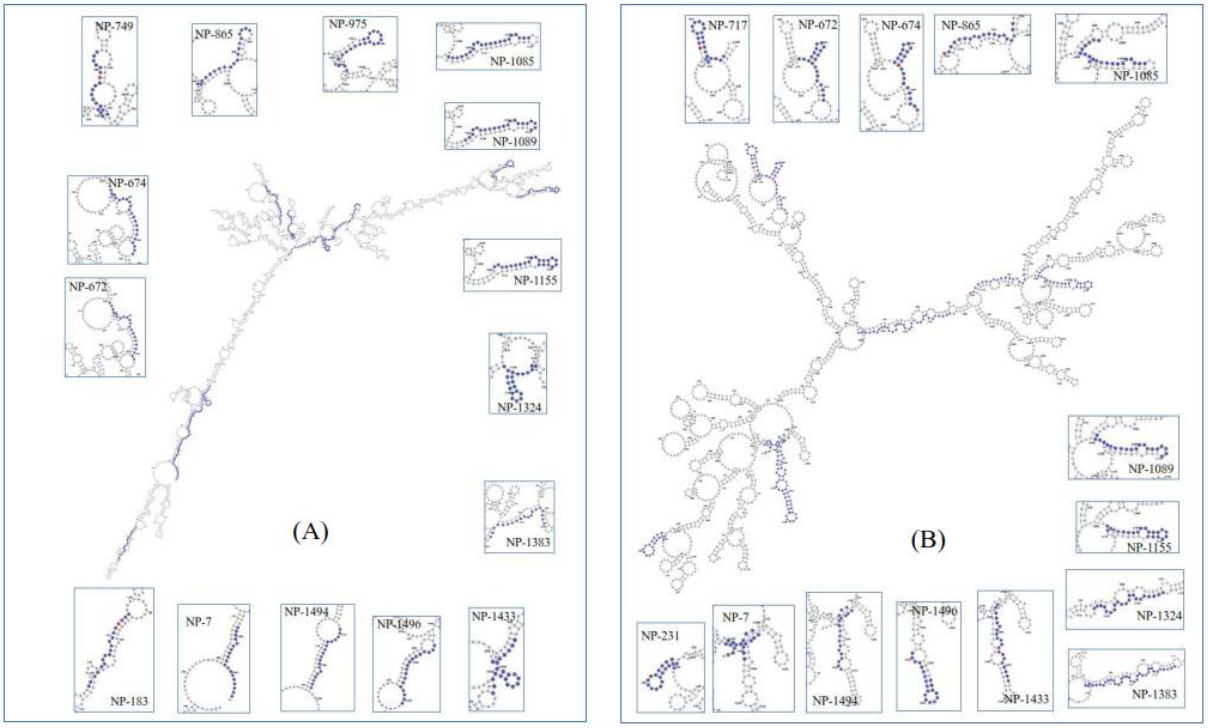

Figure 2. The sites of siRNA attachment in the mRNA secondary structure of the NP gene of the Influenza virus subtype H5N1 and H7N9. (A) A/Nanjing/1/2015(H5N1) and (B) A/China/LN/2017(H7N9)

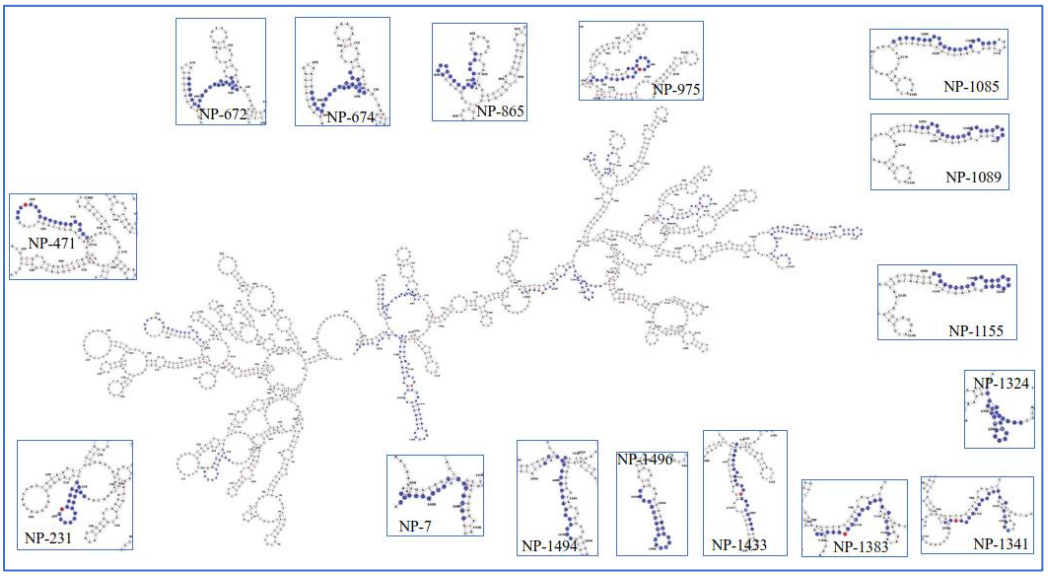

Figure 3. The sites of siRNA attachment in the mRNA secondary structure of the NP gene of the Influenza virus subtype H9N2 (A/China/A_Szuhou_GIRD01_2019/2019(H9N2))

Two kinds of siRNAs, siRNA-NP1089 and siRNA-NP1496, are interesting to compare since they are compatible with all five Influenza subtypes. Both siRNAs have target attachment in the distal part of the nucleoprotein gene. Therefore, these siRNAs are predicted to have similar effectivity since their target position in all five subtypes (H1N1, H3N2, H5N1, H7N9, and H9N2) in the simple structure with similar degree of exposed part. Thus, the effectivity of siRNA could also be estimated based on the accessibility of the target position in the mRNA structure, especially to avoid such complex structures. The siRNA is considered to be easier to attach with the target in the simple structure with free nucleotides, especially in the loop structure rather than the hairpin structure (Szczesniak et al. 2023, Chowdhury et al. 2021).

The three-dimensional structure of mRNA provides a better picture of the siRNA target attachment; however, the available software is limited. The RNA composer can predict the 3D structure of mRNA but only accommodates a maximum of 500 nucleotides (Sarzynska et al. 2023; , Chowdhury et al. 2021). This limitation may cause inappropriate prediction outcomes for the NP gene sequence with approximately a length of 1,500 nucleotides. Figure 4 demonstrates the attachment position of siRNA-NP1089 and siRNA-NP1496 on the tertiary structure of mRNA in all five Influenza subtypes. As a result, the target positions of both siRNA have quite similar structures and features. The mRNA will be folded as a consequence of the interaction between nucleotides into the secondary and tertiary structure to avoid degradation in the surrounding environment (Lorenz et al. 2011; Sarzynska et al. 2023). On the other hand, siRNA needs to attach with complementary mRNA through Watson-Crick base pairing to initiate the RNA-induced silencing complex (RISC) for mRNA degradation (Lam et al. 2013; Chan et al. 2009).

Based on the secondary and tertiary structure of mRNA our *in silico* analysis revealed that each siRNA has a different site, pattern, and folding recognition for its target position in the respective mRNA target for different Influenza subtypes. This evidence can be the explanation for why the same siRNA resulted in dissimilar effectivity for different subtypes. Other factors may influence the biological process of siRNA. For example, siRNAs with a target position at 600 reduced virus infection better than siRNAs in other positions (Hartawan et al. 2022; Szczesniak et al. 2023). Despite this fact needing further analyses and explanations, several parameters related to attachment position should also be considered for the siRNA selection. Finally, the *in silico* analysis only provides prediction so the siRNA

study for Influenza viruses should be confirmed with *in vitro* and/or *in vivo* studies because the outcome may be different.

CONCLUSION

The mapping of the siRNA targeting nucleoprotein gene of Influenza viruses showed that numerous siRNA sequences have been designed from the proximal up to the distal part of the gene segment. By complementary parameter, most siRNAs have no coverage to five Influenza virus subtypes, including H1N1, H3N2, H5N1, H7N9, and H9N2. This evidence may be caused by massive variants of the nucleoprotein gene, especially across different Influenza subtypes. This study found two siRNAs, siRNA-NP1089 and siRNA-NP1496, may have coverage for these five Influenza virus subtypes. The secondary and tertiary analysis of mRNA structure revealed that each siRNA has a different site, pattern, and folding recognition, especially on different Influenza subtypes. The siRNA is considered more effective if its mRNA target sequence demonstrates fewer miss-matches nucleotide, more free chain nucleotide, and simple structure. Furthermore, this *in silico* analysis should be confirmed with *in vitro* and/or *in vivo* studies. The recommendation related the siRNA utilization is a cocktail platform of several siRNAs at the same time to broaden sensitivity against massive variants and virus mutations in the fields.

ACKNOWLEDGMENT

This study was funded by the Research Grant (859.SDB.001: Vaccine and Drug) of The Organization Research of Health, National Research and Innovation Agency, Indonesia, 2023.

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