In silico Analysis Predicts that Mir-6770-5p Can Target the X Gene of All Hepatitis B Virus Genotypes

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Received: December 08, 2022 **Accepted: May 05, 2023**

Published: September 30, 2023

Abstract: *To date, effective medication against hepatitis B virus (HBV) has not been developed. MicroRNAs (miRNAs) comprise a promising therapeutic approach to inhibit the virus. In this study, 1917 miRNAs in the miRBase database were screened using bioinformatics software to obtain candidates that can target HBV genotype B. Two parameters, namely pairing pattern and minimum free energy were used to select the qualifying miRNAs. Three miRNAs targeting the X gene and one miRNA targeting the C gene were identified out of 39 initial candidates. Uniquely, miR-6770-5p was the only candidate that could target the X gene of all HBV genotypes, with a higher potency of inhibition compared to other candidates. The three other candidates also showed good potency for some genotypes; thus, the identified candidates show promise as therapeutics for hepatitis infection.*

Keywords: *Human miRNAs, HBV, X gene, in silico analysis.*

Introduction

Hepatitis B virus (HBV) is a DNA virus around 3.2 kb in size [23] that infects the human liver and causes cirrhosis, liver failure, and hepatocellular carcinoma [33]. Of the 240 million people with chronic HBV infection, 75% live in Asia [24]. Approximately 600,000 people die every year due to HBV-related diseases [1]. To date, effective medication against HBV has not been developed.

MicroRNAs (miRNAs) have been explored as a potential therapeutic approach to inhibit the replication of viruses such as hepatitis C virus (HCV) [11], primate foamy virus type 1 (PFV-1) [19], and vesicular stomatitis virus (VSV) [28]. Some miRNAs related to HBV have also been discovered, such as miR-192-3p, which can promote HBV replication by inhibiting Akt/mTOR signalling [21].

MiRNAs comprise short, non-coding RNAs of 20-24 nucleotides that function as gene expression regulators [26]. They are derived from pri-miRNA, which is transcribed from promoter genes [18] and then undergoes an enzymatic reaction to become pre-miRNA. The pre-miRNA is then exported to the cytoplasm by exportin-5 and cleaved by dicer, forming miRNA duplexes that eventually separate into 5p and 3p single mature miRNAs. MiRNAs inhibit human or viral mRNA by repressing translation [4, 8, 13]. MiRNAs can target both the 3' untranslated region (3' UTR) and the coding region of mRNA [31]. DNA methyltransferase 3b (DNMT3b) expression is inhibited by miR-148 through the targeting of its coding sequences [9].

Mir-3145 is an example of a miRNA candidate targeting influenza A viruses identified through *in silico* analysis whose real effectiveness was confirmed in a laboratory in vitro test [14, 15]. In this study, *in silico* analysis was used to predict the potency of miRNA candidates in targeting HBV mRNA.

Materials and methods

The nucleotide sequence of HBV genotype B (accession number LC456112) was downloaded from the National Center for Biotechnology Information (NCBI). In this study, a modified *in silico* analysis protocol adapted from Khongnomnan [14] was used. The HBV sequence was divided into smaller segments of 50 nucleotides, with an overlap of 25 nucleotides between each segment.

Each of the small segments was inputted into the miRBase website, which contains 1917 datapoints of human miRNA (https://mirbase.org/search.shtml), using the SSEARCH tool instead of BLASTN. This tool identified miRNAs that were homologous to the DNA sequence and its complement. However, homology did not always indicate the actual candidate miRNA, which could be the 5p or 3p variant.

To be considered a candidate, the miRNA had to exhibit effective hybridization with the mRNA target. RNA Hybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) [30] was used to check this indicator. The first essential parameter was the pairing pattern between the miRNA and mRNA. For effective binding, one of three pairing patterns was required: 5' seed, 5' canonical, and 3' complementary. The 5' seed pattern required a perfect match between the 2^{nd} and 8^{th} nucleotides of the miRNA and the target, although another study suggested that a match in the 2^{nd}-7^{th} nucleotides was sufficient [20]. The 5' canonical pairing pattern required nucleotide matches in the 3' part in addition to a 5' seed. The 3' complementary pattern indicated no perfect match in the 5' seed part but required matches of more than half the miRNA at the end of the 3' part [7].

The second essential requirement was low pairing energy between the miRNA and the target to stabilize the binding. A minimum free energy (mfe) of less than –10 kcal/mol was required, along with one of the three pairing patterns mentioned above, to conclude that the miRNA candidate had good potency [11].

Results and discussion

Viral nucleotide sequences

The HBV genome is circular, has a length of 3215 bp, and contains four main genes, namely the surface (S), polymerase (P), core (C), and X genes. The location and length of the DNA sequences are shown in Table 1. Some genes have overlapping nucleotides, and these data vary between different genotypes of HBV.

Table 1. Location of genes in the HBV DNA sequence [27]

Genes	HBV genotype B (LC456112*): location of genes	Length of DNA sequences (nucleotides)
S	2848–835	1203
P	2307–1623	2532
X	1374–1838	465
C	1901–2452	552

*Accession number

The four genes have different lengths, with some overlapping nucleotides. These nucleotides were divided into short segments consisting of 50 nucleotides each. Between these short segments, 25 nucleotides overlapped. They were inputted into the miRBase website containing 1917 datapoints of human miRNA to identify miRNAs that are homologs of the short segments and their complements.

To identify homologs or candidates for human miRNAs, alternative software and databases can be used instead of miRBase, including DIANA-microT, PITA, TargetScan, and miRWalk [22]. DIANA-microT predicts miRNA targets based on sequence and thermodynamic features [29], while PITA uses thermodynamic stability to identify miRNA targets and their sites [11]. TargetScan predicts the target by examining sequence complementarity and conservation across species [2]. MiRWalk identifies potential miRNA targets using various databases and algorithms [10]. It is recommended to use multiple tools to obtain a comprehensive list of candidate miRNAs, as each database may have different lists of miRNAs.

Thirty-nine miRNAs were homologs of HBV DNA segments and their complements

A total of 39 miRNAs out of the 1917 miRNAs listed on the miRBase website were found to be homologs of HBV genotype B, with 21 homologs corresponding to the DNA sequence and 18 to the complementary sequence (as shown in Tables 2A and 2B). Tables 2A and 2B display data obtained from miRBase by inputting short segments of HBV DNA sequences containing 50 nucleotides each into the website, which then revealed the homology of these sequences with miRNAs. These miRNAs were classified based on the gene types, as outlined in Table 3.

The limited number of homologs may be attributed to the small genome size of HBV (only 3215 bp). As the genome size increases, the chances of identifying miRNA homologs also increase.

Table 2A. Homology of viral DNA sequences with miRNAs in miRBase [25]

No.	Human miRNAs	Target viral gene	Homology with DNA sequences
1	hsa-miR-12116	C (2274–2292)	UserSeq 1 AUAUUUGGUGUCUUUUGGAGUGUGGAUUCGCACUCCUCCUGCAUAUAGAC 50 hsa-miR-12116 1 UUAGGCUUCCCCCUCCUCCUGC 22
2	hsa-miR-3675-5p	C (1925–1944)	UserSeq 1 AUGGACAUUGACCCGUAUAAAGAAUUUGGAGCUUCUGUGGAGUUACUCUC 50 hsa-miR-3675-5p 1 UAUGGGGCUUCUGUAGAGAUUUC 23
3	hsa-miR-500b-3p	C (2060–2129)	UserSeq 1 CACCAUACGGCACUCAGGCAAGCUAUUCUGUGUUGGGGUGAGUUGAUGAA 50 hsa-miR-500b-3p 1 GCACCCAGGCAAGG-AUUCUG 20
4	hsa-miR-6793-5p	C (2076–2140)	UserSeq 1 CACCAUACGGCACUCAGGCAAGCUAUUCUGUGUUGGGGUGAGUUGAUGAA 50 hsa-miR-6793-5p 1 UGUGGGUUCUGGGUUGGGGUGA 22
5	hsa-miR-4758-5p	X (1727–1745)	UserSeq 1 AGGCAUACUUCAAAGACUGUGUAUUUACUGAGUGGGAGGAGUUGGGGGAG 50 hsa-miR-4758-5p 1 GUGAGUGGGAGCCGGUGGGGCUG 23
6	hsa-miR-370-3p	S (3065–3082)	UserSeq 1 CACCCCUCCCCAUGGGGGCCUGUUGGGGUGGAGCCCUCAGGCUCAGGGCA 50 hsa-miR-370-3p 1 GCCUGCUGGGGUGGAACCUGGU 22
7	hsa-miR-4528	S (735–751)	UserSeq 1 GGCUUUCCCCCACUGUCUGGCUUUCAGUUAUAUGGAUGAUGUGGUAUUGG 50 hsa-miR-4528 1 UCAUUAUAUGUAUGAUCUGGAC 22
8	hsa-miR-4746-5p	S (507–527)	UserSeq 1 UUCCAGGAUCAUCAACCACCAGCACCGGACCAUGCAGAACCUGCACGACU 50 hsa-miR-4746-5p 1 CCGGUCCCAGGAGAACCUGCAGA 23
9	hsa-miR-6846-5p	S (3060–3080)	UserSeq 1 CACCCCUCCCCAUGGGGGCCUGUUGGGGUGGAGCCCUCAGGCUCAGGGCA 50 hsa-miR-6846-5p 1 UGGGGGCUGGAUGGGGUAGAGU 22
10	hsa-miR-6851-5p	S (743–763)	UserSeq 1 AGUUAUAUGGAUGAUGUGGUAUUGGGGGCCAAGUCUGUACAACACCUUGA 50 hsa-miR-6851-5p 1 AGGAGGUGGUACUAGGGGCCAGC 23
11	hsa-miR-15b-3p	P (2737–2758)	UserSeq 1 CCAGACGAAACAUUAUUUACAUACUCUUUGGAAGGCGGGUAUCUUAUAUA 50 hsa-miR-15b-3p 1 CGAAUCAUUAUUUGC-UGCUCUA 22
12	hsa-miR-370-3p	P (3065–3082)	UserSeq 1 CCAUGGGGGCCUGUUGGGGUGGAGCCCUCAGGCUCAGGGCAUACUCACAA 50 hsa-miR-370-3p 1 GCCUGCUGGGGUGGAACCUGGU 22
13	hsa-miR-4495	P (960–978)	UserSeq 1 AUGUUUUAGGAAACUUCCUGUAAACAGGCCUAUUGAUUGGAAAGUAUGUC 50 hsa-miR-4495 1 AAUGUAAACAGGCUUUUUGCU 21
14	hsa-miR-4528	P (735–751)	UserSeq 1 CCACUGUCUGGCUUUCAGUUAUAUGGAUGAUGUGGUAUUGGGGGCCAAGU 50 hsa-miR-4528 1 UCAUUAUAUGUAUGAUCUGGAC 22
15	hsa-miR-4746-5p	P (507–527)	UserSeq 1 CAUCAACCACCAGCACCGGACCAUGCAGAACCUGCACGACUCCUGCUCAA 50 hsa-miR-4746-5p 1 CCGGUCCCAGGAGAACCUGCAGA 23

16	hsa-miR-670-3	P (2541–2563)	UserSeq 1 ACUCCUUCUUUUCCUGACAUUCAUUUGCAGGAAGACAUUGUUGAUAGGUG 50 hsa-miR-670-3p 1 UUUCCUCAUAUUCAUU--CAGGA 21
17	hsa-miR-6739-5p	P (2782–2799)	UserSeq 1 CUUUGGAAGGCGGGUAUCUUAUAUAAAAGAGAGACAACACGUAGCGCCUC 50 hsa-miR-6739-5p 1 UGGGAAAGAGAAAGAACAAGUA 22
18	hsa-miR-6846-5p	P (3060–3080)	UserSeq 1 CCAUGGGGGCCUGUUGGGGUGGAGCCCUCAGGCUCAGGGCAUACUCACAA 50 hsa-miR-6846-5p 1 UGGGGGCUGGAUGGGGUAGAGU 22
19	hsa-miR-6851-5p	P (743–763)	UserSeq 1 CCACUGUCUGGCUUUCAGUUAUAUGGAUGAUGUGGUAUUGGGGGCCAAGU 50 hsa-miR-6851-5p 1 AGGAGGUGGUACUAGGGGCCAGC 23
20	hsa-miR-6871-5p	P (891–910)	UserSeq 1 CCUUCACUUCAUGGGAUAUAUAAUUGGGAGUUGGGGUUCCUUGCCACAGG 50 hsa-miR-6871-5p 1 CAUGGGAGUUCGGGGUGGUUGC 22
21	hsa-miR-873-5p	P (1240–1257)	UserSeq 1 CCCUAGGCCAUCAGCGCAUGCGCGGAACCUUUGUGUCUCCUCUGCCGAUC 50 hsa-miR-873-5p 1 GCAGGAACUUGUGAGUCUCCU 21

Table 2B. Homology of the complements of viral DNA sequences with miRNAs [25]

No.	Human miRNAs	Target viral gene	Homology with complementary DNA sequences
1	hsa-miR-611	C (2334–2356)	UserSeq 1 UUCUUCUAGAGGACCUGCCUCGUGGUCUAACAACAGUAGUUUCCGGAAGU 50 hsa-miR-611 23 GCGAGGACC--CCUCGGGGUCUGAC 1
2	hsa-let-7a-2-3p	X (1762–1774)	UserSeq 1 ACCAAUUUAUGCCUACAGCCUCCUAGUACAAAGACCUUUAACCUCAUCUC 50 hsa-let-7a-2-3p 22 CUGUACAGCCUCCUAGCUUUCC 1
3	hsa-miR-214-3p	X (1534–1553)	UserSeq 1 CCGGCAGAUGAGAAGGCACAGACGGGGAGUCCGCGUAAAGAGAGGUGCGC 50 hsa-miR-214-3p 22 ACAGCAGGCACAGACAGGCAGU 1
4	hsa-miR-6770-5p	X (1532–1544)	UserSeq 1 CCGGCAGAUGAGAAGGCACAGACGGGGAGUCCGCGUAAAGAGAGGUGCGC 50 hsa-miR-6770-5p 24 UGAGAAGGCACAGCUUGCACGUGA 1
5	hsa-miR-6871-5p	X (1617–1636)	UserSeq 1 ACCUUGGGCAAGUUCCGGUGGGCGUUCACGGUGGUUUCCAUGCGACGUGC 50 hsa-miR-6871-5p 22 CAUGGGAGUUCGGGGUGGUUGC 1
6	hsa-miR-6880-3p	X (1740–1756)	UserSeq 1 GUACAAAGACCUUUAACCUCAUCUCCUCCCCCAACUCCUCCCACUCAGUA 50 hsa-miR-6880-3p 21 CCGCCUUCUCUCCUCCCCCAG 1
7	hsa-miR-6888-5p	X (1800–1817)	UserSeq 1 CAGGAGAUGAUUAGGCAGAGGUGAAAAAGUUGCAUGGUGCUGGUGAACAG 50 hsa-miR-6888-5p 20 AAGGAGAUGCUCAGGCAGAU 1
8	hsa-miR-4678	S (735–752)	UserSeq 1 UCAAGGUGUUGUACAGACUUGGCCCCCAAUACCACAUCAUCCAUAUAACU 50 hsa-miR-4678 22 AAGGUAUUGUUCAGACUUAUGA 1

9	hsa-miR-4713-3p	S (732–753)	UserSeq 1 CCAAUACCACAUCAUCCAUAUAACUGAAAGCCAGACAGUGGGGGAAAGCC 50 hsa-miR-4713-3p 22 UGGGAUCCAGACAGUGGGAGAA 1
10	hsa-miR-6834-5p	S (327–347)	UserSeq 1 AUUGGAGGACAACAAGUUGGUGAGUGACUGGAGAUUUGGGACUGCGAAUU 50 hsa-miR-6834-5p 21 GUGAGGGACUGG-GAUUUGUGG 1
11	hsa-miR-142-3p	P (2478–2497)	UserSeq 1 ACCGUAGAAGAAUAAAGCCCCGUAAAGUUUCCCACCUUAUGAGUCCAAGG 50 hsa-miR-142-3p 23 UGUAGUGUUUCCUACUUUAUGGA 1
12	hsa-miR-450b-5p	P (903–920)	UserSeq 1 AAUUUGAUUUUUUGUACAAUAUGUUCCUGUGGCAAGGAACCCCAACUCCC 50 hsa-miR-450b-5p 22 UUUUGCAAUAUGUUCCUGAAUA 1
13	hsa-miR-4678	P (753–770)	UserSeq 1 UAAAGGGACUCAAGGUGUUGUACAGACUUGGCCCCCAAUACCACAUCAUC 50 hsa-miR-4678 22 AAGGUAUUGUUCAGACUUAUGA 1
14	hsa-miR-4731-3p	P (763–782)	UserSeq 1 UAAAGGGACUCAAGGUGUUGUACAGACUUGGCCCCCAAUACCACAUCAUC 50 hsa-miR-4731-3p 22 CACACAAGUGGCCCCCAACACU 1
15	hsa-miR-568	P (796–812)	UserSeq 1 GAGGGUUUAAAUGUAUACCCAAAGACAAAAGAAAAUUGGUAACAGCGGCA 50 hsa-miR-568 20 AUGUAUAAAUGUAUACACAC 1
16	hsa-miR-611	P (2346–2368)	UserSeq 1 GAGUUCUUCUUCUAGAGGACCUGCCUCGUGGUCUAACAACAGUAGUUUCC 50 hsa-miR-611 23 GCGAGGACC--CCUCGGGGUCUGAC 1
17	hsa-miR-6828-3p	P (2822–2834)	UserSeq 1 ACCUCCCAUGCUGUAUCUCUUGUUCCCAAGAAUAUGGUGACCCGCAAAAU 50 hsa-miR-6828-3p 20 AUCUGCUCUCUUGUUCCCAG 1
18	hsa-miR-6834-5p	P (295–315)	UserSeq 1 UUGGUGAGUGACUGGAGAUUUGGGACUGCGAAUUUUGGCCAAGACACACG 50 hsa-miR-6834-5p 21 GUGAGGGACUGG-GAUUUGUGG 1

Of the 39 homologs, five, seven, eight, and 19 homologs were identified for the C, X, S, and P genes, respectively (as indicated in Table 3). HBV has some overlapping genes (as illustrated in Table 1), wherein the P gene completely overlaps the S gene and some parts of the C gene. Therefore, miRNAs that were homologs of S were automatically considered homologs of the P gene. Similarly, some miRNAs homologous to the P gene were also homologous to the C gene.

Not all miRNAs homologous to the DNA sequences were considered miRNA candidates. As mentioned earlier, miRNAs comprise two single mature miRNAs, 5p and 3p, which complement each other, although not entirely. If the miRNA is homologous to the viral DNA sequence (Table 2A), the most likely candidate would be the complementary variant (e.g., miR-3675-3p for hsa-miR-3675-5p) to ensure the targeting of the corresponding mRNA. However, if the miRNA is homologous to the complement of the viral DNA sequence (Table 2B), the most likely candidate would be the miRNA itself (e.g., miR-6871-5p for hsa-miR-6871-5p). It is, however, useful to test both the 5p and 3p versions of miRNAs as a next step using the RNA Hybrid software because both may have a good nucleotide match to the viral DNA sequences. In this case, both miR-6770-5p and -3p could be identified as candidates.

Table 3. Cellular miRNA homologs associated with HBV genes
(derived from Tables 2A and 2B and presented categorically based on gene type)

Genotype B			
C gene	**X gene**	**S gene**	**P gene**
hsa-miR-12116	hsa-let-7a-2-3p	hsa-miR-370-3p	hsa-miR-142-3p
hsa-miR-3675-5p	hsa-miR-214-3p	hsa-miR-4528	hsa-miR-15b-3p
hsa-miR-611	hsa-miR-4758-5p	hsa-miR-4678	hsa-miR-370-3p
hsa-miR-6793-5p	hsa-mir-6770-5p	hsa-miR-4713-3p	hsa-miR-4495
hsa-miR-500b-3p	hsa-miR-6871-5p	hsa-miR-4746-5p	hsa-miR-450b-5p
	hsa-miR-6880-3p	hsa-miR-6834-5p	hsa-miR-4528
	hsa-miR-6888-5p	hsa-miR-6846-5p	hsa-miR-4678
		hsa-miR-6851-5p	hsa-miR-4731-3p
			hsa-miR-4746-5p
			hsa-miR-568
			hsa-miR-611
			hsa-miR-670-3
			hsa-miR-6739-5p
			hsa-miR-6828-3p
			hsa-miR-6834-5p
			hsa-miR-6846-5p
			hsa-miR-6851-5p
			hsa-miR-6871-5p
			hsa-miR-873-5p

In certain cases, such as those of hsa-mir-12116 and hsa-miR-4528, the miRNA may not conform to the 5p or 3p designation as only a single mature miRNA sequence has been recorded in miRBase. This phenomenon may be attributed to various factors, such as the structural characteristics of the pre-miRNA, processing preferences, or post-transcriptional modification or degradation [16]. It is plausible that both 5p and 3p sequences may exist but have yet to be identified. However, the absence of a clear designation is not of significant concern as long as the miRNA sequence satisfies the pairing pattern and mfe requirements.

Four miRNAs qualified based on the requirements

The effective hybridization of the miRNAs with the mRNA targets was assessed using RNA Hybrid. To qualify as potential miRNA candidates, the pairing pattern had to be either 5' seed, 5' canonical, or 3' complementary, and the mfe had to be less than –10 kcal/mol.

Both the 3p and 5p versions of the 39 homologs were subjected to RNA Hybrid analysis to assess their pairing patterns and mfe values. Four miRNAs met the abovementioned criteria and were considered potential candidates (see Table 4). The data in Table 4 were based on the pairing pattern and the mfe values and generated using RNA Hybrid. Only four candidates were

selected after inputting all the miRNA candidates. Specifically, miR-6793-3p targeted the C gene (2057–2089), while miR-6770-5p, miR-6770-3p, and miR-6888-5p targeted the X gene at positions 1538–1566, 1552–1573, and 1829–1848, respectively.

The X and C genes are both critical for the HBV life cycle. The X gene is a regulatory gene that encodes the HBx protein, which is involved in regulating viral replication and transcription and host cell signalling pathways [17]. The HBx protein can also interact with host proteins to promote cell proliferation and inhibit apoptosis, which may contribute to the development of HBV-associated liver cancer [3].

The C gene, on the other hand, encodes the core protein, which forms the viral capsid and packages the viral genome during assembly. The core protein also plays a role in viral persistence by interacting with host proteins to evade immune detection and promote viral replication [32].

Notably, miR-6770-5p and miR-6770-3p were derived from the same precursor and could target the X gene at nearby locations. All the identified miRNAs had low mfe values, which suggested stable binding.

Among the miRNAs, miR-6793-3p, miR-6770-5p, and miR-6888-5p displayed strong pairing patterns, namely 5' canonical 7mer-m8, 8mer, and 7mer-m8, respectively. Although miR-6770-3p had a 5' canonical 6mer, it was still regarded as a potential candidate since it was found to target mRNA according to Lewis et al. [20].

Three types of miRNA recognition elements (MREs) were found in this study, namely 7mer-m8, 8mer, and 6mer. The MRE is the region of the mRNA that base-pairs with the miRNA. 7mer-m8 is the most effective MRE at downregulating gene expression as it allows for the most stable miRNA-mRNA interaction. It consists of seven nucleotide miRNA sequences that are complementary to the mRNA, followed by an "A" nucleotide and then any nucleotide. The "A" nucleotide is located at the 8th position from the 5' end of the miRNA, hence the name "7mer-m8" [20].

8mer is less common than 7mer-m8 but still effective at regulating gene expression. It consists of an eight-nucleotide miRNA sequence that is perfectly complementary to the mRNA. 6mer is the least effective at regulating gene expression and consists of six nucleotide miRNA sequences that are complementary to the mRNA [20].

Mfe was also a requirement for filtering the best candidates. Mfe is a widely used metric for assessing the stability of miRNA-mRNA interactions. A lower mfe value indicates that miRNA and mRNA sequences are more stably bound together. Mfe lower than –10 kcal/mol is needed for a strong bond [11].

MiR-6770-5p could target all HBV genotypes

The four miRNAs mentioned in Table 4 could target HBV genotype B. However, it is important to ascertain whether they can also target other genotypes of HBV to ultimately manage antiviral therapy more efficiently.

Thus, the DNA target of HBV genotype B was inputted into NCBI BLAST to determine whether other genotypes also had homologous DNA sequences. The other genotypes were found to have varying homologies from 0 to 100% compared to HBV genotype B (Table 5).

However, the differences in nucleotide sequence do not matter as long as they are not in the pairing region, especially in the 5' seed region.

Table 4. Qualifying cellular miRNA candidates that target HBV

No.	miRNA	DNA target	Scheme of pattern	Pairing pattern	mfe (kcal/mol)
1	**hsa-miR-6793-3p**	C (2057–2089)	target 5' A CACU CAAGCUAUUCU U U 3' CGG CAGG G GUUGGGG GCC GUCC C CAACCCC miRNA 3' GAC C U 5'	5' canonical, 7mer-m8	–21.7
2	**hsa-miR-6770-5p**	X (1538–1566)	target 5' A GACUCCCC U U 3' CGCG G CUGUGCCUUCUCA GUGC C GACACGGAAGAGU miRNA 3' A ACGUU 5'	5' canonical, 8mer	–32.7
3	**hsa-miR-6770-3p**	X (1552–1573)	target 5' U CCUU U U 3' CUGUG C CA CUGCCGG GACAC G GU GGCGGUC miRNA 3' UUCU U C 5'	5' canonical, 6mer	–21.3
4	**hsa-miR-6888-5p**	X (1829–1848)	target 5' C AAU G 3' UCUGCCU CAUCUCCU AGACGGA GUAGAGGA miRNA 3' U CUC A 5'	5' canonical, 7mer-m8	–29.9

Table 5. miRNAs targeting all HBV genotypes, compared to genotype B

miR-6770-5p	Location	Target sequence	Identity, %	Pairing pattern	mfe (kcal/mol)
A	1538–1566	ACGCGG**T**CTCCCCGTCT GTGCCTTCTCAT	96.55	5' canonical 8mer	–33.1
B	1538–1566	ACGCGGACTCCCCGTCT GTGCCTTCTCAT	100	5' canonical 8mer	–32.7
C	1538–1566	ACGCGG**T**CTCCCCGTCT GTGCCTTCTCAT	96.55	5' canonical 8mer	–33.1
D	1526–1554	ACGCGGACTCCCCGTCT GTGCCTTCTCAT	100	5' canonical 8mer	–32.7
E	1538–1566	ACGCGG**T**CTCCCCGTCT GTGCCTTCTC**GC**	89.60	5' canonical 8mer	–32.3
F	1538–1566	ACGCGG**T**CTC**G**CC**TG**CT GT**T**CCTTCTCAT	82.70	5' canonical 8mer	–33.6
G	1483–1511	ACGCGG**T**CTCCCCGTCT GT**T**CCTTCTCAT	93.10	5' canonical 8mer	–26.5
H	1538–1566	ACGCGGACTCCCCG**C**CT GTGCCTTCTCAT	96.55	5' canonical 8mer	–32.7

I	1538–1566	ACGCGG**T**CTCCCCGTCT GTGCCTTCTCAT	96.55	5’ canonical 8mer	–33.1
J	1538–1566	ACGCGG**T**CTCCCCGTCT GT**A**CCTTCTCAT	93.10	5’ canonical 8mer	–26.5
miR-6770-3p	**Location**	**Target sequence**	**Identity, %**	**Pairing pattern**	**mfe (kcal/mol)**
A	1552–1573	TCTGTGCCTTCTCATCTG CCGG	100	5’ canonical 6mer	–21.3
B	1552–1573	TCTGTGCCTTCTCATCTG CCGG	100	5’ canonical 6mer	–21.3
C	1552–1573	TCTGTGCCTTCTCATCTG CCGG	100	5’ canonical 6mer	–21.3
D	1540–1561	TCTGTGCCTTCTCATCTG CCGG	100	5’ canonical 6mer	–21.3
E	1552–1573	TCTGTGCCTTCTC**GC**CTG CCGG	90.90	NQ	–21.1
F	1552–1573	**G**CTGT**T**CCTTCTCAT**G**T GCCG**C**	81.80	NQ	–17.3
G	1497–1518	TCTGT**T**CCTTCTCATCTG CCGG	95.40	5’ canonical 6mer	–18.3
H	1552–1573	**C**CTGTGCCTTCTCATCTG CCGG	95.40	5’ canonical 6mer	–21.5
I	1552–1573	TCTGTGCCTTCTCATCTG CCGG	100	5’ canonical 6mer	–21.3
J	1552–1573	TCTGTA**C**CTTCTCATCTG CCGG	95.40	5’ canonical 6mer	–26.5
miR-6888-5p	**Location**	**Target sequence**	**Identity, %**	**Pairing pattern**	**mfe (kcal/mol)**
A	1829–1848	CTCTGCCTAATCATCTC**T** TG	78.90	5’ canonical 7mer m8	–27.2

B	1829–1848	CTCTGCCTAATCATCTCC TG	100	5’ canonical 7mer m8	–29.9
C	1829–1848	CTCTGCCTAATCATCTC**A** TG	94.73	NQ	–25.2
D	1817–1836	CTCTGCCTAATCATCTC**T** TG	58	5’ canonical 7mer m8	–27.2
E	1829–1848	CTCTGCCTAATCATCTC **G**TG	78.90	NQ	–25.5
F	1829–1848	CTCTGCCTAATCATCTT**T** TG	94.73	5’ canonical 7mer m8	–27.2
G	1774–1790	CTCTGCCTAATCATCTC**T** TG	58.00	5’ canonical 7mer m8	–27.2
H	1829–1848	CTCTGCCTAATCATCT**TT** TG	89.40	5’ canonical 7mer m8	–24.5
I	1829–1848	CTCTGCCTAATCATCT**C A**TG	89.40	NQ	–25.2
J	1829–1848	CTCTGCCTAATCATCTC**T** TG	47.30	5’ canonical 7mer m8	–27.2

miR-6793-3p	**Location**	**Target sequence**	**Identity, %**	**Pairing pattern**	**mfe (kcal/mol)**
A	2071–2089	-	0.00	-	-
B	1552–1573	ACGGCACTCAGGCAAGC TATTCTGTGTTGGGGT	100	5’ canonical 7mer	–21.7
C	2071–2089	-	0.00	-	-
D	1540–1561	-	0.00	-	-
E	2071–2089	-	0.00	-	-
F	2071–2089	-	0.00	-	-
G	1497–1518	-	0.00	-	-
H	2071–2089	-	0.00	-	-
I	2071–2089	-	0.00	-	-
J	1552–1573	-	0.00	-	-

NQ – not qualified

Furthermore, the homologous DNA from other HBV genotypes was tested by RNA Hybrid to check the hybridization pattern (Table 5). Interestingly, miR-6770-5p could target all genotypes

with remarkable potency based on the pairing pattern of miRNA-mRNA 5' canonical 8mer, which is a strong bond with the lowest mfe (Table 6).

Table 6. Targeting of all HBV genotypes by the qualifying miRNAs

miRNA	Genotype									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
miR-6770-5p	+	+	+	+	+	+	+	+	+	+
miR-6770-3p	+	+	+	+	-	-	+	+	+	+
miR-6793-3p	-	+	-	-	-	-	-	-	-	-
miR-6888-5p	+	+	-	+	-	+	+	+	-	+

MiR-6770-3p placed second and could target most genotypes except E and F. The pairing pattern was weaker than that of miR-6770-5p, being only 5' canonical 6mer. Interestingly, both candidates were derived from the same precursor. Hence, if their anti-HBV effect is confirmed via experimental testing, they will be more efficient in miRNA-based treatment.

MiR-6888-5p placed third and could target HBV genotypes A, B, D, F, G, H, and J. It had the effective pairing pattern 5' canonical 7mer-m8 and a low mfe. Interestingly, some genotypes showed a difference in the 5' seed region in the last five DNA nucleotides which could affect the binding. However, because instead of C, G can also match with U (wobble pair), no effect was observed (Table 5). Table 5 displays qualifying miRNAs targeting HBV genotypes A–J, with bold font indicating nucleotide differences from genotype B. It is important to note that no homologs of the DNA sequence of HBV genotype B with other genotypes were found for miR-6793-3p as the target sequence was too long. Data were obtained from RNA Hybrid.

The fourth candidate, miR-6793-3p, could only target genotype B due to its long sequence of 33 nucleotides, which caused difficulty in creating homology with the continuously mutating HBV.

Of the four candidates, miR-6770-5p is the best one due to its targeting of the X gene of all HBV genotypes with the pairing pattern 5' canonical 8mer. However, laboratory tests are required to confirm its activity, including the dual luciferase assay, northern blot analysis, qRT-PCR, and western blot analysis [5]. MiR-6770-3p, as the complementary miRNA, also has potential; however, in another study, the overexpression of miR-6770-3p in human endothelial cells induced significant angiogenesis and reduced cell proliferation [6]. MiR-6770-5p may be better, but no study has been conducted on this miRNA thus far.

In Table 6 all candidate miRNAs can target HBV genotype B, but only miR-6770-5p can target all HBV genotypes (A–J). Data were obtained from RNA Hybrid.

Conclusion

In conclusion, this study used bioinformatics to predict the potential for miRNAs to target HBV based on the miRBase database. The results revealed four qualifying miRNAs that can target the HBV gene. MiR-6793-3p can target the C gene, and the three other miRNAs, namely miR-6770-5p, miR-6770-3p, and miR-6888-5p, can target the X gene.

MiR-6770-5p is the best candidate because it can target all HBV genotypes at the X gene, which is essential for efficient virus replication. All candidates can target HBV genotype B, which is

common in the Asia-Pacific region. However, in vitro analysis is still required to prove the inhibitory effect of all candidates on HBV.

Acknowledgements

This work was supported by a grant from The Indonesia Endowment Funds for Education (LPDP) and the Faculty of Medicine, Universitas Muslim Indonesia. We would like to thank Dr. Ag Phyo Wai and Dr. Hasta Handayani M. Kes for reviewing the manuscript.

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