RESEARCH ARTICLE

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CRISPR Target-based Single-guide RNA (sgRNA) for Diagnostic Testing of Hepatitis B Virus

Jeanne Elvia Christian, Hartiyowidi Yuliawuri, Edvan Arifsaputra Suherman

Biology Study Program, Faculty of Science and Engineering, Calvin Institute of Technology, Jakarta, Indonesia

Background: Indonesia is the second-highest country with hepatitis B cases in the South East Asian region. Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 12 (Cas12) could be developed as a diagnostic tool to detect hepatitis B infection. This study was aimed to develop a diagnostic method for hepatitis B virus by designing CRISPR target-based single-guide RNA (sgRNA).
Materials and method: The preCore/Core-gene sequences of hepatitis B virus were collected from the National Center for Biotechnology Information (NCBI) website. The selected sequence was submitted to Cas Designer and CRISPOR tools to design sgRNA. The resulting sgRNA was cloned *in silico* into an expression vector using Benchling software.
Results: The 23-nucleotide sequence 5'- GTAGTCAGTTATGTCAATGTTAA-3' had 30% GC content, 68.3 out-of-frame and 76 predicted efficiencies. This sequence had no mismatch based on analysis.
Conclusion: This preliminary study will help design a CRISPR-based diagnostic kit for the detection of hepatitis B virus in Indonesia. However, further *in vitro* and *in vivo* studies are required to demonstrate its potential and efficiency.

Keywords: *CRISPR-Cas12b, diagnostic, HBV, sgRNA*

Introduction

Hepatitis B virus (HBV) is still a burden, especially in Asia, the Pacific Islands, and Africa. Indonesia is regarded as one of the highly endemic areas because of the HBV infection cases. This viral infection could cause acute and chronic diseases in the liver, such as liver cirrhosis and hepatocellular carcinoma (HCC).[1,2] HBV is classified into 10 genotypes (A to J) with 8% nucleotide differences. From 10 HBV genotypes, they are divided into several sub-genotypes with 4-8% nucleotide differences. HBV genotype B (66%) is the most dominant type in Indonesia, followed by genotype C (26%), D (7%), and A (7%).[3] The transmission mainly occurs by exposure to infected blood and body fluids, and during childbirth. HBV infection has an incubation period ranging from 1-6 months and it could be detected outside the body for at least 1 week. To reduce the infection of the virus worldwide, care is mainly aimed at prevention since there is no specific treatment for acute hepatitis B. For chronic infection, patient needs regular examination to monitor tumor markers. Vaccination program and diagnostic testing are essential for viral prevention. The diagnosis can be done

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Corresponding Author:
Jeanne Elvia Christian
Biology Study Program
Faculty of Science and Engineering, Calvin Institute of Technology
Jl. Industri Blok B14 Kav.1 Kemayoran, Jakarta 10610, Indonesia
e-mail: jeanne.elviac@gmail.com

Cell and Biopharmaceutical Institute

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within one to two months after infection using patient's blood.[2,4,5]

Current diagnostic testing usually uses hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs), and total hepatitis B core antibody (anti-HBc) for three different serologic tests. These tests can be used to determine the condition of the infected patient, such as acute or chronic HBV infection, or susceptibility to infection and is in need of vaccination, or has immunity to HBV prior to infection or vaccination. Detection of HBV DNA is usually used to measure the viral load using real-time polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, or genotyping.[6,7] These techniques have their own disadvantages, such as the sensitivity or specificity to detect infection, and some of them require specialized labs and skilled personnel.[8] Therefore, the development of diagnostic testing for HBV is needed.

Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) system is one of the promising systems to prevent the transmission of HBV by accurately diagnosing the infection that occurs. Guide RNA (gRNA) directs the Cas effector proteins to target and cleave invading nucleic acid. CRISPR-Cas for diagnostic systems usually uses Cas13, Cas12a, and Cas12b. These Cas types have the ability to nonspecifically cleave surrounding nontarget single-stranded (ss)RNA and ssDNA.[8,9] A previous study tried to develop the CRISPR-Cas12b-LFB using the S protein of HBV genotypes B and C.[8] Hepatitis B e antigen (HBeAg) is one of the first viral markers detected in serum after infection that indicates the level of HBV replication and infectivity. This protein lowers T lymphocyte function in cells by increasing programmed cell death protein (PD)-1. The presence of anti-HBeAg indicates initiation of recovery. Anti-HBcAg is usually used to assess the response to HBV vaccine.[1,6,10] The aim of this study was to develop the diagnostic method for HBV by using preCore/Core protein from HBV genotypes B, C, and D. PreCore/Core encodes HBeAg and core protein (HBcAg).

Materials and methods

Construction of Phylogenetic Tree

The preCore/Core-gene sequences of HBV genotype B, C, and D from Indonesia[3,11,12] were selected from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) and compared to other geographical locations from the Hepatitis B Virus Database (https://hbvdb.lyon.inserm.fr/HBVdb/).[13] A phylogenetic tree was built with MEGA software version 11.0.13 (Penn State University, University Park, PA, USA) using neighbor-joining method and the bootstrap resampling 1,000 times.

CRISPR-Cas Target Design

The sequence of the preCore/Core-gene (Gene ID: D00331.1) was selected and submitted to Cas-Designer (http://www.rgenome.net/cas-designer/).[14] Then, the sequence was submitted to CRISPOR (http://crispor.tefor.net/) for further comparison.[15] The sequence should have GC content in the range of 20-80%, an out-of-frame score above 66, and no mismatches. The genomic targets of 23 nucleotide gene sequences preceding 5'-ATTN-3' protospacer adjacent motif (PAM) were generated. To confirm that the sequence has no similarity with other viruses, the target sequence was submitted to the basic local alignment search tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The vector for single-guide (sgRNA) was chosen to be expressed in mammalian cells. The pUC19-U6 vector which possesses a U6 promoter, M13 forward and reverse primer binding, *lac* promoter, and ampicillin resistance (*AmpR*) promoter was downloaded. To construct the sgRNA into the expression vector adjacent to the U6 promoter site, the Benchling tool (Benchling, San Francisco, CA, USA) was used.[16]

Results

Phylogenetic Tree

The sequences of pre-Core/Core gene from Indonesia were compared to other HBV sequences from other regions. A neighbor-joining tree was built based on the sequence alignment (Figure 1). Based on the phylogenetic tree analyses of the 638 bp preCore/Core gene sequence, sequences that were used in this study had genotype B (D000331.1, M54923.1, AB033555.1, AB334302.1, AB355454.1, AB644284.1) and D (AB355456.1).

CRISPR-Cas Target Design

Cas-Designer software was used to identify the PAM sequence. It also generated 24 possible sgRNA sequences from the selected sequences of pre-Core/Core HBV gene. The Cas-Designer tool determined several in-built parameters of the sequences, including GC content, out-of-

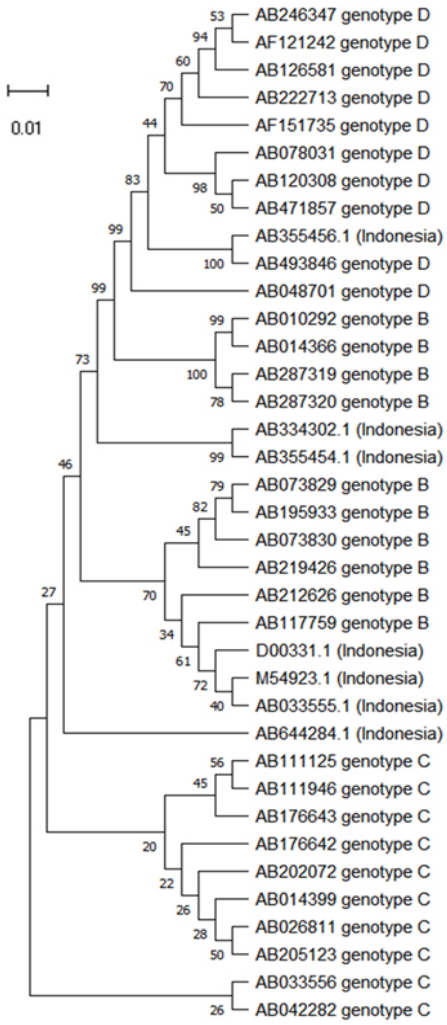

Figure 1. Phylogenetic tree of HBV preCore/Core gene from Indonesia compared to other regions using neighbor-joining method. The percentage next to the branching points indicates the relative support from bootstrap (1,000 times).

frame score, and mismatches (Table 1). From 24 possible sgRNA sequences, we selected 9 sequences that had GC content in range of 20-80%, out-of-frame score above 66 and no mismatch (Table 1, blue).

For comparison, we used CRISPOR tool to identify the off-target for mismatches and predict the efficiency. We excluded four sequences from the first analysis because they had at least one off-target for mismatches. The other five sequences from the second analysis did not have off-target for mismatches. One out of five sequences had the highest predicted efficiency, which was 76. Therefore, we chose one possible sgRNA sequence that had GC content of 30%, had out-of-frame score of 68.3, no mismatches in both tools and had predicted efficiency =76 in CRISPOR tool (Table 1, blue, italic). For visualization of cloned sgRNA into the expression vector, the selected 23 sgRNA sequences were attached to the U6 promoter site for transcription (Figure 2).

Discussion

The pre-Core/Core sequences of HBV that were used in this study had genotype B and D (Figure 1). According to the previous studies, AB334302.1, AB355454.1, and AB644284.1 formerly had genotype C.[3,11,12] However, phylogenetic tree analysis conducted in this study demonstrated that those sequences were closely related to HBV genotype B. This sequence similarity between HBV genotype B and C is also observed in another study.[17]

In this study, we designed sgRNA, especially the CRISPR RNA (crRNA) that is complementary to the target DNA using *in silico* method. The sgRNA region that we proposed was located in 336-359 bases. Using the Cas-Designer prediction, the sgRNA that we chose had an ideal GC content (30%), since it was still within the range of 20-80%. The GC content can affect the activity of sgRNA. If it is too low or high, it will lower the cleavage efficiency. The out-of-frame score was higher than the standard score (66), which was 68.3. This score is positively correlated with the frequency of frame-shift mutations. The higher the out-of-frame score, the higher the possibility of out-of-frame deletions caused by sgRNA.[18,19]

The sgRNA had no mismatches, which indicated that the target matched zero locations in the genome with no mismatch. Another comparison tool, CRISPOR, also confirmed the results of previous prediction. The sgRNA had no off-target for mismatches and the predicted efficiency was 76, which suggested that the target was likely to be cut by its gRNA sequence.[18] The sgRNA had 5'-ATTN-3' PAM that will be recognized by Cas12b. PAM is a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA that is specifically targeted by Cas nuclease.[20,21]

In this study, we tried to visualize the sgRNA with its expression vector that can be expressed in mammalian cells under the U6 promoter. When HBV is detected by sgRNA, the target region would be subjected to cleavage by Cas12b enzyme from another plasmid, pCAG-AaCas12b-2eGFP.[22] However, further study is needed to validate and test the accuracy of the sgRNA using *in vitro* and *in vivo* methods.

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Table 1. Cas-Designer results of HBV sgRNA.

#RGEN Target (5' to 3')	Position	Cleavage Position	Direction	GC Contents (w/o PAM; %)	Out-of-frame Score	Mismatch
ATTCTTTATACGGGTCAATGTCCATGC	86	13.8	-	43	66.4	Y
ATTGACCCGTATAAAGAATTTGGAGCT	94	18.5	+	39	68.6	N
ATTTGGAGCTTCTGTGGAGTTACTCTC	111	21.2	+	52	64.3	Y
ATTCGAGATCTTCTCGACACCGCCTCT	166	29.8	+	57	56.6	N
ATTGTTCACCTCACCATACGGCACTCA	227	39.3	+	52	63.9	Y
ATTCTGTGTTGGGGTGAGTTGATGAAT	262	44.8	+	43	69.5	N
ATTCATCAACTCACCCCAACACAGAAT	262	41.4	-	43	70.8	N
ATTACTTCCCACCCAGGTGGCTAGATT	286	45.1	-	57	67.4	N
ATTTGGAAGACCCAGCCTCCCGGGAAT	311	52.5	+	65	62.0	N
ATTCCCGGGAGGCTGGGTCTTCCAAAT	311	49.1	-	61	67.6	N
ATTGACATAACTGACTACTAATTCCCG	331	52.2	-	39	55.9	N
ATTAGTAGTCAGTTATGTCAATGTTAA	336	56.4	+	30	68.3	N
ATTAACATTGACATAACTGACTACTAA	337	53.1	-	30	73.1	N
ATTTTTAGGCCCATATTAACATTGACA	351	55.3	-	35	58.5	N
ATTGTGGTTTCACATTTCCTGTCTTAC	387	64.4	+	39	70.7	N
ATTTCCTGTCTTACGTTTGGAAGAGAA	400	66.5	+	43	64.1	N
ATTCAAGAACAGTTTCTCTTCCAAACG	413	65.0	-	39	68.6	N
ATTTGGTGTCTTTTGGAGTGTGGATTC	440	72.7	+	48	67.7	Y
ATTCGCACACCTCCAGCATATAGACCA	463	76.3	+	52	43.7	Y
ATTTGGTGGTCTATATGCTGGAGGTGT	469	73.8	-	52	58.0	Y
ATTGAGACCTTCGTCTGCGAGGCGAGG	569	89.5	-	65	41.9	Y
ATTGAGATCTTCTGCGACGCGGCGATT	593	93.3	-	57	46.0	Y
ATTCCCGAGATTGAGATCTTCTGCGAC	602	94.7	-	52	51.2	N
ATTGAGATTCCCGAGATTGAGATCTTC	608	95.6	-	43	N/A	Y

Y: one or more mismatch; N: no mismatch.

Conclusion

We proposed one possible sgRNA sequence for the development of the HBV diagnostic method. This sgRNA has good prediction scores based on two different sgRNA designing tools. *In vitro* and *in vivo* research should be conducted to test the efficiency of sgRNA in HBV diagnostic development.

Authors Contribution

JEC performed all roles from research design, data analysis and interpretation to manuscript writing with assistance from HY and EAS. HY took part in conceptualizing and planning the research, while EAS conducted the data analysis. In addition, HY and EAS helped interpret the research results. HY and EAS also contributed to the drafting of the manuscript by providing critical revisions to the manuscript.

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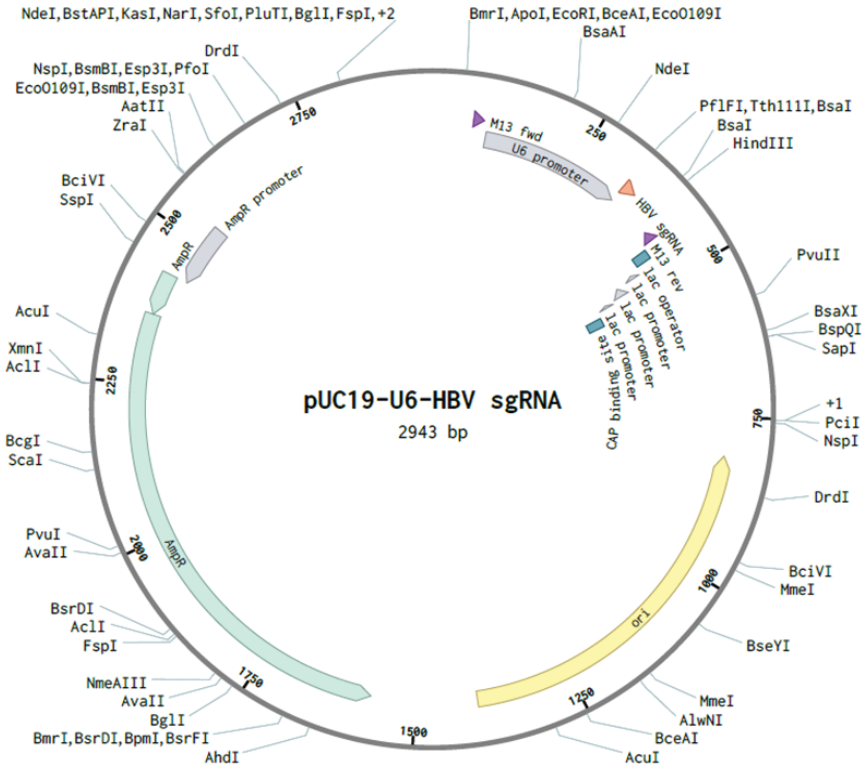

Figure 2. Visualization of pUC19-U6 expression vector with HBV sgRNA.

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