SYSTEMATIC REVIEW

Clustered regularly interspaced palindromic repeats-cas9-based strategies towards HIV eradication: A literature review

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Abstract

Objective: Despite Human Immunodeficiency Virus (HIV) being a major global health burden, no currently available therapy can eliminate it. One of the major challenges in developing treatment is the presence of latent HIV reservoirs. On the other hand, development of Clustered Regularly Interspaced Palindromic Repeats-Cas9 (CRISPR-Cas9) has made genome editing possible and thus can be used to address HIV latency and successfully treat HIV. This literature review aims to identify and appraise existing CRISPR-Cas9 strategies that address HIV treatment, particularly during latency.

Methods: The PubMed Database was used to retrieve relevant articles. This review included articles that mentioned the use of CRISPR-Cas9 as a treatment for HIV and are written in English and/or Indonesian language.

Results: The included studies (n = 17) showed that the CRISPR-Cas9 system can be utilized to disrupt the HIV-1 genome to inhibit viral reproduction and virulence. This system can be further optimized by combining several CRISPR-Cas9 systems. However, the use of CRISPR-Cas9 may cause HIV resistance, particularly to its guide RNA. This technique has also never been applied in vivo, thus more research is needed before wider implementation. A limitation of this review is the lack of data regarding CRISPR-Cas9 systems quality in some studies, thus limiting appraisal.

Conclusion: While the use of CRISPR-Cas9 to cure HIV seems promising, further studies regarding CRISPR-Cas9 quality, potential for development of gRNA-resistant HIV-1 strains and in vivo demonstration of the techniques are needed to progress this concept toward HIV eradication.

Keywords: HIV latency, CRISPR-Cas systems, CRISPR-associated protein 9, Guide RNA, RNA interference.

Introduction

HIV/AIDS has become a major global health issue since its emergence in 1981. In 2016, over 36.7 million people were living with HIV; 1.8 million of them were newly infected. The disease is also estimated to have claimed the lives of 35 million people.[1,2]

Despite such excessive morbidity and mortality, the focus of HIV therapy recommended by the World Health Organization (WHO) remains limited to controlling virus transmission and disease progression.[1] Although currently available antiretroviral therapy (ART) can suppress HIV to undetectable levels, interruption in its use rapidly leads to HIV rebound to pretreatment levels.[3] A cure, despite advancement in medical research, has yet to be found. Progress is impeded by the presence of reservoirs of memory CD4+ T cells, which harbour integrated latent HIV proviruses. These proviruses are transcriptionally silent and are thus unaffected by ART or immune responses. When memory cells become activated, latency may be reversed leading to transcription of provirus by T cells and resuming of virus production.[3,4] Because of this latency, it is as yet deemed impossible to reduce HIV from an infected person.[1,3] To address the issue, new strategies that can target HIV latency are urgently needed.

Consisting of Clustered Regularly Interspaced Palindromic Repeats (CRISPR) accompanied by the nuclease Cas9, CRISPR-Cas9 is known for its ability to cleave genetic material with high precision. CRISPR sequences are found in prokaryotic DNA and function to protect the organism from foreign genetic elements such as viruses or plasmids by targeting the associated Cas9 against CRISPR-complementary genomic sequences in such invading pathogens. In this way, CRISPR-Cas9 is known to play a major role in the acquired immune response in 40% of bacteria.[5] The CRISPR-Cas system can cleave foreign genetic materials by combining the nuclease activity of Cas with CRISPR-RNA (crRNA) and transactivating RNA (trRNA). Both crRNA and trRNA recognize complementary sequences in the target nucleic acid and thus guide the associated Cas9 to the correct site for cleavage. Many studies in diverse fields of research such as plant science, microbiology and animal studies use this technique to edit or cleave target genomes.[6,7]

Utilization of the CRISPR/Cas9 system has the potential to

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become a breakthrough to treat once-incurable viral infections, including HIV.[6,7] This literature review aims to identify and appraise existing CRISPR-Cas9 strategies related to HIV treatment. To meet this aim, we produced a database of HIV treatment strategies involving CRISPR-Cas9, as well as performing an appraisal of these strategies.

Methods

This literature review used the PubMed Database to retrieve relevant articles. The following search terms were used: "("HIV" OR "Human Immunodeficiency Virus") AND ("CRISPR Cas9" OR "CRISPR-Cas9") AND "treatment." The inclusion criteria were (a) articles mentioning the use of CRISPR-Cas9 as a treatment for HIV and (b) articles written in English and/or Indonesian language. Articles which only mentioned the treatment of HIV-2 were excluded from this study.

Three reviewers (AVM, LW, LAN) independently evaluated the articles based on the specified inclusion and exclusion criteria. We then extracted information as follows: type of HIV targeted in the study, viral target, nuclease, guide (g) RNA/sequence used, type of cell(s) used, and gene delivery technique. Data regarding sensitivity, specificity and efficiency were also extracted. The data were put into custom tables for easier organization as a database. Appraisal of the CRISPR-Cas9 strategies was based on data regarding their sensitivity, specificity and/or efficiency.

Results

Seventy-four articles in the PubMed database were initially selected. These were evaluated by the reviewers and fifty-seven articles were excluded that did not meet the specified inclusion criteria. Thus, only seventeen articles were used as the final research database (Table-1).

Discussion

CRISPR-Cas9 can be used to disrupt HIV-1 genes

We found several articles that demonstrated the ability of CRISPR-Cas9 to inhibit HIV production.[8-25] In treating HIV, CRISPR-Cas9 was used to target several genes in the HIV genome, including the structural (gag, env), enzymatic (pol), and accessory genes (vif, rev), as well as Long Terminal Repeat (LTR), which mediates integration of HIV DNA. By targeting the genes responsible for HIV structural building, the virus structures production can be hampered, thus limiting their assembling as a virus. On the other hand, targeting the LTR will inhibit HIV integration to human cell DNA, so that further HIV latency can be avoided. To target several gene loci that were considered to be conserved sites, all of the studies designed several gRNAs, an RNA type which can direct the CRISPR-Cas9 system to a particular site (Figure-1).[13] Conserved sites were chosen because of their relatively stable characteristics and low mutation rate. Avoiding potential for future mutation is important as deviation from the conserved sequences could reduce CRISPR-Cas9 system efficacy in the future.[8]

Most studies stated and explained the roles of each gRNA used, as well as their efficiency in inducing insertion and deletion (indel) in the HIV sequence. The efficiency ranged from 30% to 96.3%. Most gRNAs with efficiency above 80% targeted the LTR sequences,[8-10,12-16,21] although some targeted the structural genes.[8-10] However, there were some discrepancies regarding efficiency in targeting structural genes. Some studies showed that the efficiency of CRISPR-Cas9 in targeting structural genes was between 50-70%, while others found it to be more than 80%.[8-22] These variations could be caused by differences in gRNA design and the sequences they target. Some studies, unfortunately, did not indicate the efficiency of the gRNA used, and others only gave a graph without raw data to be reviewed.[8-10] Thus, it is difficult to conclude the efficiency of the technique.

While these results show promising evidence of CRISPR-

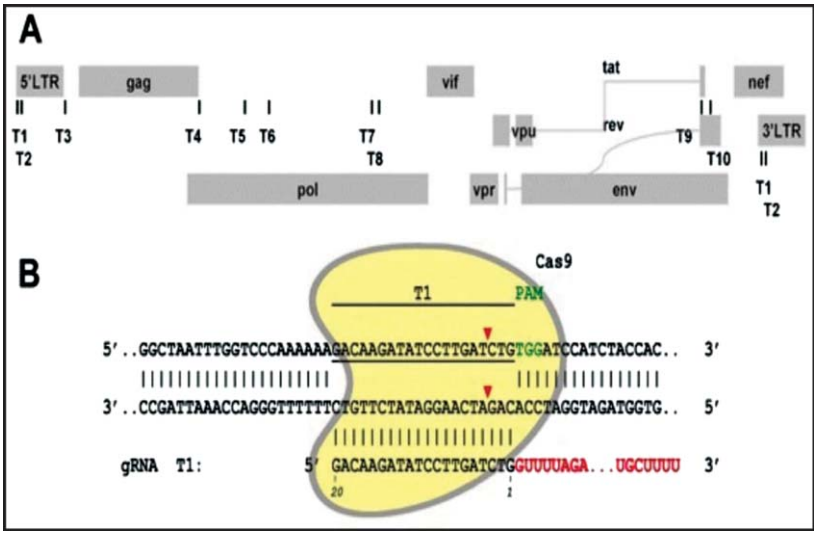

Figure-1: A) Locations of CRISPR-Cas9 targets in the HIV-1 genome. The figure also shows the location of gRNA T1-T10 utilized in trial by Zhu et al (2015). (B) Schematic depiction of T1 gRNA guide sequence binding (20 nucleotides, underlined) to the HIV-1 DNA with associated Cas9 (in yellow). The red arrows indicate the cleavage site by Cas9. Copyright © Zhu et al.; licensee BioMed Central. 2015.[13]

Table-1: Summary of CRISPR-Cas9 systems in included studies. Each CRISPR-Cas9 system described in the included studies targeted different viral target(s) and/or utilized distinct gRNA(s). The efficiency of the systems varies between 40%-96.3%. Note: Summary CRISPR-Cas9 systems combinations are not included in this table.

Viral targets	Utilized guide RNAs (gRNAs)	Efficiency	References
LTR	gLTR7	95.50%	Wang et al, 2010[8]
	gLTR8	94.70%	
Pol	gPol6	N.A	
	gPol7	N.A	
Env	gEnv5	N.A	
Vif	gVif2	N.A	
Rev	gRev3	96.30%	
LTR	gLTR1	In limiting of HIV DNA	Yin et al, 2018[9]
	gLTR2	Replication: 57 - 89%;	
	gLTR3	HIV virus production:	
Tat	gTat	44 - 76%	
Gag	gGag		
Pol	gPol		
Rev	gRev		
LTR	gRNA T1	85.40%	Zhu et al, 2015[10]
	gRNA T2	83.70%	
	gRNA T3	93.30%	
Pol	gRNA T4	59.60%	
	gRNA T5	87.60%	
	gRNA T6	82%	
	gRNA T7	87.70%	
	gRNA T8	86.50%	
Rev	gRNA T9	83.80%	
	gRNA T10	76.60%	
LTR + Pol + Rev	gRNA T3 + T6 + T9	N.A	
LTR + Pol + Rev	gRNA T1 + T6 + T9	N.A	
LTR + Pol + Rev	gRNA T2 + T4 + T10	N.A	
LTR	gLTR	N/A	Huang et al, 2017[11]
Nef	Gnef		
Pol	Gpol		
Tat	Gtat		
LTR U3	gRNA5	69%	Bialek et al, 2016[12]
		73%	
		89%	
LTR	gEmpty	89%	Liao et al, 2014[13]
	gMock	90.90%	
LTR	sg362F	95%	Saayman et al, 2016[14]
		16%	
		40%	
LTR U3/U5	sgRNA4	30%	Limsirichai et al, 2016[15]
	sgRNA6	85%	
LTR	gLTR1	78%	Wang et al, 2016[16]
	gLTR9	50%	
	gLTR7	60%	
	gGag1	64%	
	gGag3	67%	
	gGag4	44%	
	gGagPol	66%	
	gPol3	64%	
	gTatRev	54%	
	gEnv2	69%	
LTR	U3 sequence (gLTRa, gLTRb, gLTRc, gLTRd)	N/A	Hu et al, 2014[17]
LTR	T5 gRNA	62%	Ebina et al, 2013[18]
LTR	N/A	N/A	Kaminski et al, 2016[19]
LTR	gRNA LTR U3, R	N/A	Kaminski et al, Aug 2016[20]
LTR	gLTR4	40%	Lebbink et al, 2017[21]

Abbreviations: LTR = long terminal repeat; gag, env = HIV structural genes; pol = HIV enzymatic genes; vif, rev = HIV accessory genes; gRNA = guide RNA; sgRNA = single guide RNA.

Cas9 utilization in treating HIV, no included study involved in vivo experiments that directly used CRISPR/Cas9 in the clinical setting, therefore, as the data currently available are unlikely to accurately represent the in vivo scenario, further testing using applicable animal models is needed before this technique is used widely in humans.

CRISPR-Cas9 utilization to treat HIV infection involves several different approaches

There are three different strategies by which CRISPR-Cas9 targets HIV infection: (1) cleavage of HIV DNA that has not been inserted to the human DNA (pre-integration DNA); (2) direct editing of the genes in transcription-silent HIV genome, including pol, env, vif, rev and LTR; and (3) reactivation of the HIV provirus in combination with antiretroviral therapy (Figure-2). Targeting the pre-integration HIV DNA will prevent the insertion of it to human genes, thus preventing HIV latency.[13] On the other hand, direct editing of the pol, env, vif, rev, and LTR genes is also efficient in treating HIV as it does not require activation of viral gene transcription. Several studies showed that this characteristic has lead to efficient suppression of HIV-1 proviral reactivation.[8,10,13,17-19,21] The study by Liao et al. even showed that resistance to HIV-1 caused by this editing could be induced in human pluripotent stem cells and be maintained after cell differentiation.[13] This demonstrates the possibility of utilizing the direct editing strategy not only as a treatment option for HIV but also as a preventive measure against the disease.

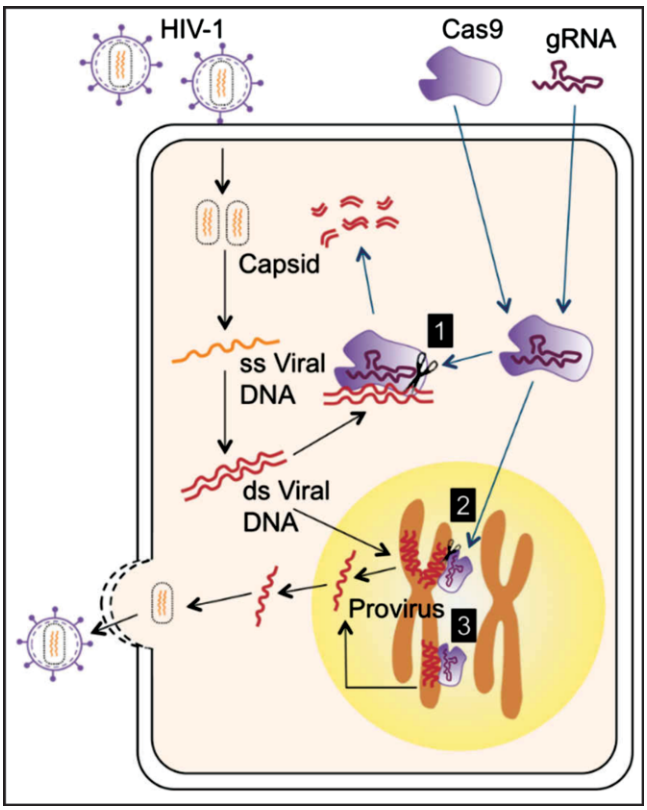

Figure-2: Model of CRISPR-Cas9 directed against HIV-1 infection. Treatment of HIV infection may be treated with CRISPR-Cas9 by utilizing one or more of the three targeting strategies: (1) cleavage of HIV DNA that has not yet been inserted into the human genome; (2) cleavage of HIV provirus; (3) reactivation of HIV provirus in conjunction with antiretroviral therapy (ART). With this strategy, ART is responsible for elimination of viruses produced after the reactivation process. Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature Nature Communications Liao HK, Gu Y, Diaz A, Marlett J, Takahashi Y, Li M, et al. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. Nat Commun 2015;6:6413. Copyright © 2015.[13]

In addition to disrupting silent HIV-1 provirus, HIV treatment can also be performed by reactivating inserted HIV genes in human cells, enabling its transcription. These occurrences lead to HIV production.[12,14,15] This approach utilizes the ability of latency reversing agents (LRA), a pharmacological agent that induces HIV proviral transcription in human genome.[14,15] When used alongside combined ART (cART), which destructs the viruses upon the end of their production, this approach may lead to complete removal of HIV from an individual.[15] However, there remains a drawback to this method; until now, no trials have shown significant reduction in latent reservoirs upon treatment using LRA. This is caused by the inefficient clearance of activated reservoir cells and insufficient latent proviruses activation by currently available LRAs. Bialek et al. addressed this problem by exploring CRISPR-Cas9-derived activator systems, which function by recruiting multiple transcriptional activation domains to the HIV 5' LTR. Induction of these activator systems was found to have similar or greater anti-HIV effect when compared with direct genome editing.[12]

Despite positive results of the approaches discussed above, to work optimally CRISPR-Cas9 requires certain conditions. A study by Yin et al. found while cytoplasmic Cas9 inhibited HIV-1 as strongly as nuclear Cas9, only nuclear cas9 could excise latent provirus.[9] These findings should be taken into consideration for designing further experiments regarding CRISPR-Cas9 system utilization for HIV treatment.

Combination of CRISPR-Cas9 systems may increase treatment efficiency

To improve the quality of HIV treatment CRISPR-Cas9 is used by increasing its sensitivity, specificity, and efficiency, two or more CRISPR-Cas9 systems have been combined in

Table-2: Summary of CRISPR-Cas9 systems combinations. Some of the systems listed in Table S2 can be combined in order to increase the CRISPR-Cas9 system efficiency in reducing HIV DNA replication and virus production. Note that the last two combinations studied by Zhao et al utilized CRISPR-Cas9, which uses gRNA, and RNA interference (RNAi), which uses shRNA.

Viral targets	Utilized RNA(s)	Efficiency	Reference
Gag + Pol	gGag8 + gPol7	96.3%	Wang et al, 2010[8]
LTR	gLTR7 + gLTR8	N/A	
LTR + Gag + Pol	gLTR7 + gGag8 + gPol 7		
LTR + Pol + Rev	gRNA T3 + T6 + T9	N/A	Zhu et al, 2015[10]
LTR + Pol + Rev	gRNA T1 + T6 + T9		
LTR + Pol + Rev	gRNA T2 + T4 + T10		
LTR + pol	gLTR + gpol	N/A	Huang et al, 2017[11]
LTR + tat	gLTR + gtat		
nef + pol	gnef + gpol		
nef + tat	gnef + gtat		
LTR	gRNA MA3+PR2	N/A	Lebbink et al, 2017[21]
	gRNA MA3+IN5		
	gRNA PR2+IN5		
Gag3 + Gag1	gGag3 + gGag1	N/A	Zhao et al, 2017[22]
	gGag3 + gGag5		

Abbreviations: LTR = long terminal repeat; gag, env = HIV structural genes; pol = HIV enzymatic genes; vif, rev = HIV accessory genes; gRNA = guide RNA.

recent studies (Table-2). Combinatorial attack between CRISPR-Cas9 and RNA Interference (RNAi), another system capable of treating HIV, has also been studied.

The most common CRISPR-Cas9 technique combination described in studies is a combination of two gRNAs, which has been found to increase the efficacy of CRISPR-Cas9 in combating HIV. However, these combinations require specific conditions to work effectively. A study conducted by Lebbink et al. demonstrated that gRNA combinations are effective only if the gRNAs used are both potent. A less potent gRNA, RT2, allowed viral breakthrough even when it was combined with a potent gRNA. It is also important to consider that potent gRNA combination may lead to minor cytopathogenic side effects at early time points, but not after prolonged treatment.[21]

Another study by Zhao et al. showed the possibility of combining two biomolecular approaches to cure HIV: CRISPR-Cas9 and RNA interference (RNAi). Defined as a cellular mechanism for gene expression regulation at the posttranscriptional level, RNAi is involved in the processing of small, noncoding microRNAs (miRNA), which control mRNA silencing. Small interfering RNAs (siRNAs) derived from RNAi machinery equipped with short hairpin RNA (shRNA) were used in the context of HIV treatment. These siRNAs can be used as miRNA mimics against the HIV-1 RNA genome.[22] An additive effect of combinatorial CRISPR-Cas9 and RNAi attack was found in different combinations, but this was not analyzed quantitatively as the overall effect was confounded by the potent antiviral effects of each individual inhibitor. It is also important to note that HIV-1 cross-resistance may occur when overlapping HIV sequences are targeted by both techniques as CRISPR-Cas9 attack triggers a unique mutagenic response, that is immediate non-homologous end joining (NHEJ) DNA repair. This will lead to generation of considerable genetic variation, mostly indels but also nucleotide substitutions, at the site of cleavage. Thus, raw material for the escape virus variants may be formed, enabling viral escape. Therefore, such overlap should be avoided.[22]

HIV gRNA-resistant strains

Despite the emergence of CRISPR-Cas9 utilization as an inhibitor of HIV, some studies have shown that the method is potentially susceptible to resistance. We found two studies that showed the possibility of HIV-1 provirus resistance development, which could be caused by either reverse transcriptase (RT) or insertion-deletion (indel) mutation.[23,24] A study by Wang et al. showed that the rate of sequence indel mutation in HIV genome increases by up to 35% when CRISPR-Cas9 was utilized to treat the virus.[23] On the other hand, a study by Yoder et al. showed contradictory results, with only a 3% increase of indel rate in HIV genome compared to non-treated HIV.[24] However, it is still vitally important to address this issue before wide implementation of CRISPR-Cas9-based HIV-1 treatment can be considered. Both studies recommended the use of simultaneously edited sequence to prevent the mutation; Wang et al. recommended utilization of more than one sgRNA (Streptococcus gRNA) to reduce indel more than three base pairs, while Yoder et al. recommended the use of more than one double strand breaks (DSB).[23,24]

Limitation of study

The limitation of our study is the lack of data regarding the sensitivity, specificity and/or accuracy of some CRISPR-Cas9 systems. Without these data, appraisal of the systems may not be done thoroughly for all included studies, especially for studies that lack the corresponding data.

Conclusion

In conclusion, seventeen studies included in this review showed promising data regarding the possibility of CRISPR-Cas9 utilization for HIV treatment. Furthermore, combination of techniques has been developed to further enhance treatment quality. However, this technique also has some issues that must be addressed before it could be implemented as a novel therapy, including lack of quality

data for some gRNAs, absence of in vivo experimental data and the potential emergence of HIV gRNA-resistant strains. Therefore, further research is needed. With accrual of more complete data for these techniques, in vivo experimentation and deeper knowledge of how to reduce the chances of resistance, HIV eradication with CRISPR-Cas9 may be possible in the future.

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