Targeted Cellular Delivery of CRISPR/Cas9 with RNA Aptamer-Cationic Liposome As A Potential Therapy For Metastatic Prostate Cancer

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Abstract

Prostate cancer is the sixth deadliest cancer worldwide due to its metastatic ability, rendering conventional treatment futile. Editing target oncogenes with the help of CRISPR/Cas9 is gaining recognition as promising treatment modality. However, the implementation of CRISPR/Cas9 in the clinical setting remains difficult due to challenges in delivery to target tissue. Therefore, this literature review focuses on exploring nanocarrier suitable for CRISPR/Cas9 delivery. The method used to assemble this literature review is by performing a comprehensive literature search with corresponding keywords. Results show that targeted delivery system of CRISPR/Cas9 using cationic liposome conjugated with RNA aptamer is an excellent candidate. Modification of cationic liposome, a well-established nanocarrier, with PEG and RNA aptamer as targeting ligand increases specificity, efficiency, stability, and circulation time in bloodstream. However, further research is still needed to confirm these findings for future implementation of this technology as prostate cancer therapy.

Keywords: *Aptamer, Cancer, CRISPR/Cas9, Liposome, Prostate*

Introduction

Prostate cancer affects approximately 899,000 men worldwide. In Asia, the incidence of prostate cancer ranges from 1 per 100,000 men until 53.3 per 100,000 men, making it the sixth leading cause of mortality by cancer worldwide.[1,2]

Prostate cancer is capable of metastasizing to distant regions of the body, such as the bone and the skin. While metastatic prostate cancer is traditionally treated with androgen deprivation therapy, the late-stage tumor usually contains androgen deprivation-resistant (castration-resistant) cells which will emerge after elimination of castration-sensitive cells through androgen deprivation therapy. This phenomenon is very problematic and often life threatening.[3]

The advancements in medical science have provided a better insight into the genetic basis of cancer. Genetic alterations involved in the cancer progression have been identified, with activation of oncogenes and inactivation of cell cycle regulators playing critical role. Accordingly, gene-based approach for the molecular therapy of cancer is emerging as a novel method of the treatment. Central to this approach is the modification of oncogenes in order to silence or obliterate them.[4]

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease Cas9 genome editing system has been gaining attention as a novel method for genome editing, owing to its specificity, simplicity, versatility, and affordability. With proper guide RNA (gRNA)—a complementary base-pairing nucleotide designed to locate specific genomic loci in the sea of chromatin—it confers extensive gene deletion with less off-target effects. Numerous studies have demonstrated CRISPR/Cas9's potential efficacy in the treatment of various cancers, prostate cancer included, through targeting of oncogenes *in vitro*.[5-11] Implementation of CRISPR/Cas9 in clinical setting, however, remains a major challenge due to difficult delivery of the nuclease and associated guide RNA *in vivo*.[12]

Targeted cellular delivery of CRISPR/Cas9 is therefore desired to obtain the genome-editing system's full potential. We sought to identify a highly-specific, efficient, and versatile method of delivery with adequate safety. We found that the transport of CRISPR/Cas9 with cationic liposome conjugated with RNA aptamer is a desirable delivery system for this. Cationic liposome acts as a carrier for the nuclease-gRNA complex with low toxicity. RNA aptamer acts as targeting ligand which can recognize cellular receptors with high affinity, therefore improving specificity and efficiency of delivery. The system is also highly flexible as RNA aptamers can be designed to target specific cell-type receptors.[11]

Material and Methods

We used PubMed, Google Scholar, ClinicalKey, and ScienceDirect to search for the research paper and articles used in this literature review with the following keywords "CRISPR/Cas9", "Prostate Cancer", "Prostate Carcinoma", "PLK1", "Gene silencing or gene knockout", "RNA aptamer", "Oncogene", "Cationic Liposome". We searched for appropriate articles which matched the topic of our literature review. From those articles, we excluded articles that were not yet published such as "accepted manuscript" and "author manuscript". Knowledge synthesis was done using those resources.

Result and Discussion

Pathogenesis of prostate cancer

Prostate cancers usually develop from excessive androgen production. Binding of androgen to androgen receptors (AR) in prostatic cells induces expression of pro-survival genes. Tumors that develop through this fashion exhibit androgen sensitivity, thus treatment using androgen receptor blocker effectively regresses the tumor size.[1]

Unfortunately, most tumors will become androgen-resistant. This progression is promoted by several mechanisms, such as: (1) AR gene amplification; (2) mutations in the AR gene resulting in improper activation of the receptor; and (3) mutations in the alternative signalling pathway which cause the expression of pro-survival genes despite the absence of androgen.[1]

One of the earliest events of genomic mutation in prostate cancer is the rearrangement of TMPRSS2-ERG gene. The excessive genomic rearrangement that occurs in prostate cancer cells may cause fusion of TMPRSS2 gene into ERG gene.

This event is highly observed at 16-20% in Prostate Intraepithelial Neoplasm (PIN), 40%-70% in prostate adenocarcinoma, and 45%-86% in prostatic small cell carcinoma.[2,3]

However, this fusion usually occurs in androgen-dependent prostate cancer. This is because TMPRSS2 is an androgen-regulated promoter gene, thus the absence of androgen represses this gene, which in turn will repress ETS gene family (one of them being ERG) expression. ETS itself is a gene that is responsible for the invasiveness of prostate epithelial cells although it does not directly transform the cells themselves.[1]

Despite the constant expression of TMPRSS2-ERG in later stages of carcinoma, it is highly doubted that the fusion gene has a major role in the disease progression since later stage cancers exhibit other androgen-independent pro-survival and anti-apoptotic genes, thus causing castration-resistant prostate cancer.[2]

Other gene that contributes to androgen-independent prostate carcinogenesis is Her-2/neu gene. This is done through the activation of AKT pathway which provides androgen-independent AR activation. The activation of MAP kinase, AKT, and P13 kinase pathways results in stabilization of AR which causes constant activation of AR without its ligand.[2,4] In addition, Her-2/neu gene activation can increase cell adhesion capability, thus elevating the chance of tumor metastasis.[2]

Another gene that is widely studied in the field of prostate cancer is MYC gene. This gene is thought to regulate 15% of all human genes. The expression of this gene results in cellular proliferation, thus overexpression of this gene due to mutation in the human prostate epithelial cell is linked to immortalization.[13]

Moreover, the expression of stem cell genes (including Oct-4, Klf4, Sox2, and c-Myc) is associated with poor outcome. However, the expressions of both Klf4 and c-Myc genes in the prostate cancer are significantly overexpressed compared to normal prostate epithelial cells.[14]

The exact role of Klf4 is still unknown. While it is observed to promote carcinogenesis in the breast and skin cancer, Klf4 acts as tumor suppressor in lung and skin cancer. However, it is certain that Klf4 prevents differentiation in embryonal stem cells. In the prostate cancer cells, the expression of Klf4 and c-Myc is higher compared to embryonic stem cells. Overexpression of Klf4 in the prostate carcinoma might be a step towards dedifferentiation of cancer cells, leading to a more malignant type of cancer.[14]

Another pro-survival gene that plays a role in prostate oncogenesis is polo-like kinase 1 gene (PLK1). PLK1 gene expression is highly enhanced in the tumor cells and is associated with poor outcome. The inhibition of PLK1 increases cell apoptosis and necrosis rate.[15]

Introducing CRISPR/Cas9

CRISPR (Clustered Regularly Interspaced Palindromic Repeats) is a prokaryotic-derived mechanism of adaptive immunity towards invasion of foreign nucleic acid such as viral nucleotides and plasmids.[16] Cas9, one of the CRISPR-associated proteins, is a nuclease capable of cutting both strands of DNA.[17] This system can recognize and cut non-self DNA, where small sequences of the cut DNA will later be incorporated into the CRISPR array in the host's genome, creating a memory for the adaptive immune system to protect the

host from further invasion.[18] Today, this system has been utilized in gene-editing field as it can correct errors in genome as well as turn on and off genes quickly and selectively. Moreover, this system is considered as cheap and easy to use.[19]

It is important to understand that there are 6 types of CRISPR-Cas system which are classified into 2 classes based on the diversity on the nature of the effector nuclease:[18] a multi-subunit protein complex of nuclease for class 1 (such as the CRISPR-associated complex for antiviral defense (CASCADE) complex assisted by the Cas3 helicase[20], the Csm complex[21], or the Cmr complex[22] within type I and type III CRISPR-Cas system); and a single large polypeptide of nuclease for class 2 (such as the Cas9 within type II and Cpfl within type V systems).[23] Hence, CRISPR/Cas9 is known as the CRISPR-Cas type II and is a part of class 2. In addition, Cas9 needs tracrRNA to activate the enzyme, as it is normally in a non-active form (Apo-Cas9).[24]

Compared to the other types, type II CRISPR mechanism is the most studied and is considered unique, as it needs only one Cas protein (Cas9) for gene editing.[25] The simplicity of the type II CRISPR-Cas system, requiring only two major components (Cas9 along with the gRNA), makes this system susceptible for genome editing.[26]

Comparison of CRISPR/Cas9 with Other Genome Editing Tools

Other genome editing tools, the Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), can both facilitate targeted Double Strand Breaks (DSBs) at specific loci like Cas9 nuclease system.[27] Until now, Cas9 has been considered as a major breakthrough in the field of genome editing,[28] as it offers several potential advantages when it is compared to ZFNs and TALENs. First, Cas9 has an advantage on target design simplicity as it relies on gRNA which can be easily retargeted new DNA through the customization of 20-nt sequence, while both ZFNs and TALENs use DNA-binding domain, an independently folded protein domain which recognizes double- or single-stranded DNA as targets, that are considered more expensive and time-consuming. Second, Cas9 system is super-efficient as it can target multiple genomic loci at once by injecting multiple gRNAs. Third, Cas9 offers a blunt cut in the target sequence, where in contrast TALENs cleave non-specifically between the pair of TALEN monomer-binding sites.[27,28,30]

CRISPR/Cas9: Insights into the Structure and Mechanism of Action

Overall, the mechanism of action of the CRISPR-Cas system can be divided into three stages: adaptation, expression and maturation, and interference.[18] The adaptation stage is where the unutilized-invading nucleic acids are turned into short fragments of DNA. These fragments will then be integrated into the CRISPR arrays within the host genome as new spacers, with the help of Cas1 and Cas2 proteins, thereby creating a genetic record of prior infection which provides protection to the host towards the same invader. Note that the sequences from which the spacers are derived from, prior to the merging into the CRISPR array, are called the protospacers.[18,31] The expression and maturation stages are the stages where the CRISPR array is transcribed and processed

into smaller units of spacer-contained CRISPR-RNAs (crRNA) then is combined with the Cas proteins to form an active Cas-crRNA complex.[18] The interference stage is where Cas-crRNA complex scans the cell to search for foreign nucleic acid targets that is complementary to the base-pairing of the crRNA sequence. Successful recognition will lead to the cleavage and degradation of the target nucleic acid. The Cas9 does not recognize spacers incorporated in the host genome as it does not possess the protospacer adjacent motifs (PAM), a short sequence of DNA 5′-NGG-3′.[24] Indeed, PAM plays a very important role in the recognition of self and non-self DNA as it is only present in the foreign DNA, sparing the CRISPR system to delete itself.[18,24]

CRISPR/Cas9 relies on the extensive complementary base-pairing via the 20-nt guide sequence on the single guide RNA (sgRNA) to locate specific genomic loci in the sea of chromatin. The presence of PAM directly downstream the 3' of the 20-bp target sequence is required for it to be recognized and adhered by the Cas9. Note that different Cas9 orthologs have different PAM sequence. For instance, wild-type *S. pyogenes* Cas9 (SpCas9) requires a 5'-NGG PAM. This requirement does not severely limit the targeting range of the Cas9, as some studies show that in the human genome, sites of 5'-NGG-3' can be found every 8-12 base pairs.[27,32,33]

CRISPR-Cas9 system is then adapted for uses in the genome editing tools. The guide RNA used in this system is a 18-20 nucleotides long of sgRNA which arises from merging crRNA with a part of the tracrRNA into a hybrid RNA.[34] Transport of cas9 nuclease and a sgRNA will generate double stranded-DNA break at the target chromosomal location in eukaryotic cells. The Cas9-induced Double Strand Breaks (DSBs) is re-repaired by two mechanisms: NHEJ (Non-homologous End Join) and HDR (Homologous Directed Repair). NHEJ is a cell's natural way to repair a cleaved DNA in which the ends of the DSB are rejoined through endogenous DNA repair system. This repair may cause random mutations that results in the frameshifts and creation of a premature stop codon, thus it is considered as error-prone. Moreover, HDR provides a single-stranded template of oligonucleotide in the form of oligodeoxynucleotide (ssODN) which has the ability to provide high fidelity and precise gene editing.[16,27]

Furthermore, the possible mechanism to deactivate the gene is by using the deactivated version of Cas9 (dCas9) which has the ability to recognize and bind to the target DNA, but loses its ability to cleave the sequence, hence its purpose is to block transcription and repress target gene expression.[35]

CRISPR/Cas9 Application: Inactivation of Mutations in Cancer

Genetic mutation is one of the hallmarks of cancer development. Until now, there has been more than 140 cancer driver genes described.[36,37] Therefore, identification of all genes in the actual tumor cells are emerging, yet the exact function and role of most mutations have not yet been resolved.[38] Moreover, despite its ability to reduce the development of cancerous cells and its capability to increase lifespan to a maximum of five years, the current cancer treatments (chemotherapy, radiotherapy and surgery) have harmful side effects and toxicity although it significantly reduces the quality of life. Using CRISPR/Cas9 technology, a powerful tool known to its

capability to correct many mutations at once in a cell's genome, researchers hope to resolve the functional role of the mutations in the cancer cells through the targeting of mutated genes, elucidating gene function involved in tumor progressions, correcting mutations, inactivating activated oncogenes or activating inactive cancer suppressor genes, which overall has shown promise towards reliable long term cancer therapy.[39]

CRISPR/Cas9-mediated gene silencing in prostate cancer models

Several studies have demonstrated the potential efficacy of CRISPR/Cas9 system in reducing the malignancy of prostate cancer models through silencing of genes which are believed to be involved in the tumors' pathogenesis and progression. These target genes include *NANOG* and *GPRC6A*.[9,10]

NANOG is an essential transcription factor in the embryonic stem cells, providing pluripotency and driving embryonic development.[40] However, it is also expressed in various cancers, including ovarian cancer, colorectal cancer, and prostate cancer.[41-45] Increased *NANOG* expression is observed in cancer stem-like cells (CSCs), whose presence in tumors is believed to increase lethality.[8,10,13] Consequently, increase of *NANOG* expression has been linked to poor prognosis in many cancers and increase in Gleason score, a poor indicator for prostate cancer.[40,44-46]

Kawamura et al (2015)[9] conducted CRISPR/Cas9-mediated gene knockout of *NANOG* and *NANOGP8*—a significant *NANOG* pseudogene—in DU145 prostate cancer cell line, which resulted in significant attenuation of malignant traits.[9] Sphere-forming capacity of knockout cells—a trait highly related to tumorigenicity—was decreased to 50% compared to parental (control) DU145 cells.[9,47] Migration of knockout cells was decreased by 40-60%.[41] Knockout of this gene also increased drug sensitivity, a finding consistent with other studies.[9,48] *In vivo* tumorigenicity was also decreased considerably following *NANOG* knockout; tumor formed within mice implanted with knockout cells had a much lower volume than control after 7-week-long observation.[9]

GPRC6A is an abundantly expressed G-protein-coupled receptor which regulates many biological processes, such as energy metabolism, sexual reproduction, and bone formation. Its ligands include amino acids, calcium, zinc, testosterone, and osteocalcin.[49,50] Implication of *GPRC6A*'s involvement in the prostate cancer is based on numerous evidences, including: (1) its expression is increased in prostate cancer cell lines; (2) its activation results in proliferation and migration of prostate cancer cells; (3) high serum level of its ligand namely osteocalcin, is used as a biomarker for prostate cancer.[49,51-53]

Ye et al (2017)[10] evaluated *GPRC6A*'s role in the prostate cancer's progression both *in vitro* and *in vivo*. CRISPR/Cas9 was used to target *GPRC6A* gene in PC-3 prostate cancer cell line. Modified and parental (control) cell lines were then treated with osteocalcin to assess the receptor activity. Treating modified cells with osteocalcin did not induce proliferation well and the expression of proliferation marker PCNA was decreased. Osteocalcin-induced cellular migration was also reduced considerably in modified cells. *In vivo* cancer progression was also inhibited;

osteocalcin treatment to mice implanted with modified PC-3 cells failed to increase tumor size and weight.[10]

Targeted cellular delivery of CRISPR/Cas9 system with RNA aptamer-cationic liposome chimera

To utilize CRISPR/Cas9-based therapy well, the transport of the system to the appropriate tissue or cell type is of paramount importance. The success of CRISPR/Cas9 treatment therefore relies on the development of carriers, whose delivery *in vivo* remains difficult. Thus, CRISPR/Cas9-based therapy is often considered non-applicable in the clinical setting.[54]

Conventional delivery methods of CRISPR/Cas9 involve physical introduction of the system into the nucleus and viral-vector-mediated insertion of the Cas9-gRNA complex.[54] Physical delivery is more suitable *in vitro* as it is simpler and highly reproducible, but less suitable *in vivo* as subject's physiology and pathological status vary.[55,56] Viral vectors are highly efficient, but bear safety concerns regarding potential immunogenicity and carcinogenicity. In addition, some viral vectors have limited capacity to carry large enzymes like Cas9 and limited number of cell types which can be infected.[57,58] Thus, the usage of non-viral vectors as delivery alternative is done in the consideration of their low immunogenicity, ability to carry larger cargo, and simpler large-scale production.

Ideal *in vivo* delivery system of CRISPR/Cas9 needs two components: (1) a carrier which can protect the cargo from degradation in the bloodstream; (2) a targeting ligand to recognize specific receptors on the appropriate target cell.[11,54]

An excellent carrier candidate is cationic liposome: a phospholipid-bilayer vesicle surrounding an aqueous core. The gRNA-nuclease complex can be incorporated inside, where it will be protected.[59] Cationic liposomes provide stable storage and low immunogenicity. They are well studied, being the first non-viral vectors to enter clinical trials.[59] However, cationic liposomes may induce inflammatory response and have low specificity, reducing transfection efficacy.[51,60] Thus, *in vivo* application of cationic liposome-based delivery is still restricted.

Modification of cationic liposome's surface with targeting ligand can help improve its efficacy. A promising targeting ligand is aptamer: small, single-stranded RNA or DNA oligonucleotide of 20-60 nucleotides. Aptamers bind target molecules with high specificity and affinity, making them comparable to antibodies. Furthermore, they have easier and cheaper production, lowly immunogenic, non-toxic, and bind to various molecules, making them an exceptional targeting ligand.[61,62] Of all aptamers, prostate-specific membrane antigen (PSMA) aptamers, such as RNA aptamer A9 and A10, are the most studied.[63,64]

Aptamers can be attached to the lipid heads of liposomes with the help of a hydrophilic polymer namely polyethylene glycol (PEG) as seen in Figure 1. In addition to mediate conjugation of aptamers, coating liposomes with PEG also improves stability. Mononuclear phagocyte system cannot recognize the 'stealth' hydrophilic surface of PEG, thus preventing removal of liposomes. PEG also prevents attachment of

proteins and opsonins in the blood. Therefore, blood clearance is avoided and circulation time is improved, allowing more efficacious targeting & delivery.[54,59,60]

Implementation of RNA aptamer in tumor treatment has been reported. A gp120 aptamer-silencing RNA (siRNA) chimera was able to deliver siRNA targeting HIV-1 *tat/rev* common exon.[62] The same chimera was also able to deliver siRNA into tumor cells.[62-64] RNA aptamer-conjugated cationic liposome has been used to deliver anticancer drug doxorubicin into prostate cancer cells *in vivo*.[68] However, the delivery of CRISPR/Cas9 using RNA aptamer has not been reported.

Zhen et al. undertook this challenge in their 2016 study, incorporating the RNA aptamer-cationic liposome to deliver CRISPR/Cas9 which targeted prostate cancer.[11] The RNA aptamer A10, which targets PSMA, was used, and LNCaP cell line was selected because of the presence of PSMA on their surface, as opposed to PC-3 cell line.[11,69]

Packaged CRISPR/Cas9-gRNA was targeted to the pro-survival gene polo-like kinase 1 (*PLK1)*. *PLK1* phosphorylates numerous proteins during mitosis. Its role in cellular proliferation is critical; *PLK1* is only expressed in dividing cells, with highest expression during G2/M phase.[70-72] Mutation and subsequent overexpression of *PLK1* result in chromosomal instability, aneuploidy, and improper cell division—all of which are the hallmarks of cancer cells.[70,71] Accordingly, *PLK1* overexpression is observed in many cancers, such as breast cancer, rectal cancer, and prostate cancer.[72-75]

PLK1 has been a target of cancer treatment, and numerous studies have demonstrated that inhibition of *PLK1* induces apoptosis, tumor growth reduction, and reduced proliferation.[70,76,77] Thus, it is expected that CRISPR/Cas9-mediated knockout of *PLK1* will reduce malignancy. Consequently, the success of aptamer-liposome chimera as a delivery system can be evaluated via attenuation of malignant traits. The results were favorable, as described below.[11]

(1) A10-liposome-CRISPR/Cas9 chimera was able to bind cell significantly with high specificity. Binding of A10-liposome-CRISPR/Cas9 chimera to PSMA showed significant competition with PSMA antibody. This indicates that the chimera's binding to prostate cells is comparable to an antibody. The chimera's binding strength was similar to A10 aptamer alone, showing that the aptamer retained its binding ability.[11]

(2) A10-liposome-CRISPR/Cas9 chimera had substantial *in* vitro gene silencing effect, down-regulating *PLK1* mRNA transcription significantly (63%). The down-regulation achieved was the highest of all CRISPR/Cas9 reagents formulations, showing the chimera's efficacy in transfecting CRISPR/Cas9. Without A10, the liposome carrying CRISPR/Cas9 had similar silencing effect as lipofectamine-2000 (a transfection reagent), demonstrating that on its own the liposome had considerable transfection ability, which was improved with A10 aptamer.[11]

(3) Cellular uptake assay showed that the A10-liposome-CRISPR/Cas9 chimera mediated the most efficient delivery of fluorescent-labeled CRISPR/Cas9. Fluorescent signal from cells treated with A10-liposome-CRISPR/Cas9 chimera was

the strongest, showing that uptake of CRISPR/Cas9 was best achieved through its delivery. This also means that the strong gene silencing effect from the A10-liposome-CRISR/Cas9 chimera resulted from the efficient delivery.[11]

(4) Cell viability was the lowest following A10-liposome-CRISPR/Cas9 chimera treatment. As established before, knockdown of *PLK1* is expected to reduce LNCaP cells' survival. Therefore, efficient delivery of *PLK1*-targeted CRISPR/Cas9 would reduce cell viability the most, as provided by A10-liposome-CRISPR/Cas9 chimera. Yen et al. also demonstrated that the aptamer-liposome chimera is inherently non-toxic.[11]

(5) A10-liposome-CRISPR/Cas9 chimera promoted regression of tumor *in vivo*. Mice with xenograft tumor (derived from LNCaP cells) were treated with different CRISPR/Cas9 formulations for 27 days. The A10-liposome-CRISPR/Cas9 chimera exhibited the most significant tumor regression (0.40 cm^3 to 0.15 cm^3). Free CRISPR/Cas9 and A10-liposome-scrambled CRISPR/Cas9 (no gRNA included) did not reduce tumor volume, showing importance of proper CRISPR/Cas9 delivery and the need for targeting *PLK1*. Importantly, neither mortality nor significant change of mouse body weight were observed after 27 days of treatment.[11]

(6) A10-liposome-CRISPR/Cas9 chimera had low immunogenicity. IFN-α and IL-12 levels of mice were assessed to determine immunogenicity. Compared to positive control, all formulations conferred low immunogenicity. However, adding A10 to the liposome chimera resulted in even lower cytokine levels, suggesting that A10 helps to prevent non-specific induction of immune response by increasing specificity.[11]

As shown by Ye et al.[11], *in vitro* observation of A10-liposome-CRISPR/Cas9 chimera's ability to silence gene and decrease cell viability was consistent with *in vivo* efficacy of the delivery system in promoting tumor regression. This may result from the enhanced binding of the chimera and the consequent improvement of Cas9-gRNA complex uptake. Moreover, the chimera confers no significant immunogenicity. Therefore, targeted cellular delivery of therapeutic CRISPR/Cas9 using RNA aptamer-cationic liposome chimera may help the implementation of CRISPR/Cas9 in clinical setting.

Limitations of this method

Despite its potential, CRISPR/Cas9 also have several limitations. First, similar to other nucleases, Cas9 has the potential to cleave off-target DNA targets, although at reduced frequencies.[33,78] A double nicking strategy can be used as a strategy to minimize off-target mutagenesis. However, it is important to understand the structure of the Cas9. The Cas9 nuclease is a bi-lobed structured protein consists of both alpha-helical and nuclease lobe, connected by a single bridge helix. The gRNA itself is located between those two lobes. The nuclease lobe has two other domains called the RuvC whose function is to cleave non-target DNA strand and the NHN nuclease whose function is to cleave target DNA strand.[78] Normally, the Cas9 will perform a double strand break on the foreign DNA site, yet mutation of the domains, either the RuvC or HNH will result into a single strand break. These mutants are known as Nickase, which can cut either complementary or on-

complementary DNA.[79] As single-stranded nicks will be repaired, DSB would only possible if both sgRNA are able to successfully cleave the defined targets and hence prevent un-wanted mutagenesis. These strategies are then considered promising in the attempt to increase the specificity in genome editing using CRISPR/Cas9.[27]

Second, not every cancer cell has the same mutation therefore this therapy is highly specific since the A10-Liposome-CRISPR/Cas9 without PLK1 gene exhibit lower tumor volume reduction. This means this method will only work if the gene mutation in the tumor matches with the gRNA of the A10-Liposome-CRISPR/Cas9 system.

Third, the aptamer-liposome chimera requires understanding regarding the target cell receptor because aptamer is very specific towards a certain receptor. Without the knowledge of the receptor and ligand, it is not possible to create the correct aptamer-liposome chimera.

Fourth, there are some limitations in this method due to the vulnerability towards opsonization of the liposome, accelerated blood clearance, immune response, and lack of research in this field. This problem can be solved by incorporating cholesterol into the phospholipid bilayer. Further research should be done to know the solution to these problems, its clinical efficacy, and side effects.

Last, this gene editing system is still a very new technology. There are not many researches about CRISPR/Cas9 with aptamer-liposome chimera. Another research should be done to reinforce and support the researches used in this literature review.

However, if this method is combined with other conventional therapy of prostate cancer, it might help in reducing metastatic prostate cancer. While conventional therapy can be used in androgen-sensitive prostate cancer, A10-Liposome-CRISPR/Cas9 system can be used as androgen-resistant metastatic prostate cancer to specifically target prostate cancer cells that have metastasized to another part of the body.

Conclusion

The therapy of prostate cancer with CRISPR/Cas9 as a cutting edge genome editing technology shows promising future thanks to its affordability and specificity. Both *in vivo* and *in vitro* studies have demonstrated its potential in reducing malignancy and promoting tumor regression. However, its clinical implementation remains difficult due to challenges in delivering the Cas9-nuclease complex to target tissue. Using targeted cellular delivery system with aptamer-conjugated liposome, a well-established carrier, can be a way to work around this problem. Modifying liposome surface with aptamer as targeting ligand can improve its efficacy through increased specificity towards target tissue. Attachment of RNA aptamer to liposome surface with PEG helps increase stability, reduce blood clearance, and increase circulation time. This approach can be used as the therapy of many diseases by targeting various cell types with the appropriate aptamer and target gene sequence.

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Conflict of Interest

The authors have no conflict of interest

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Appendices

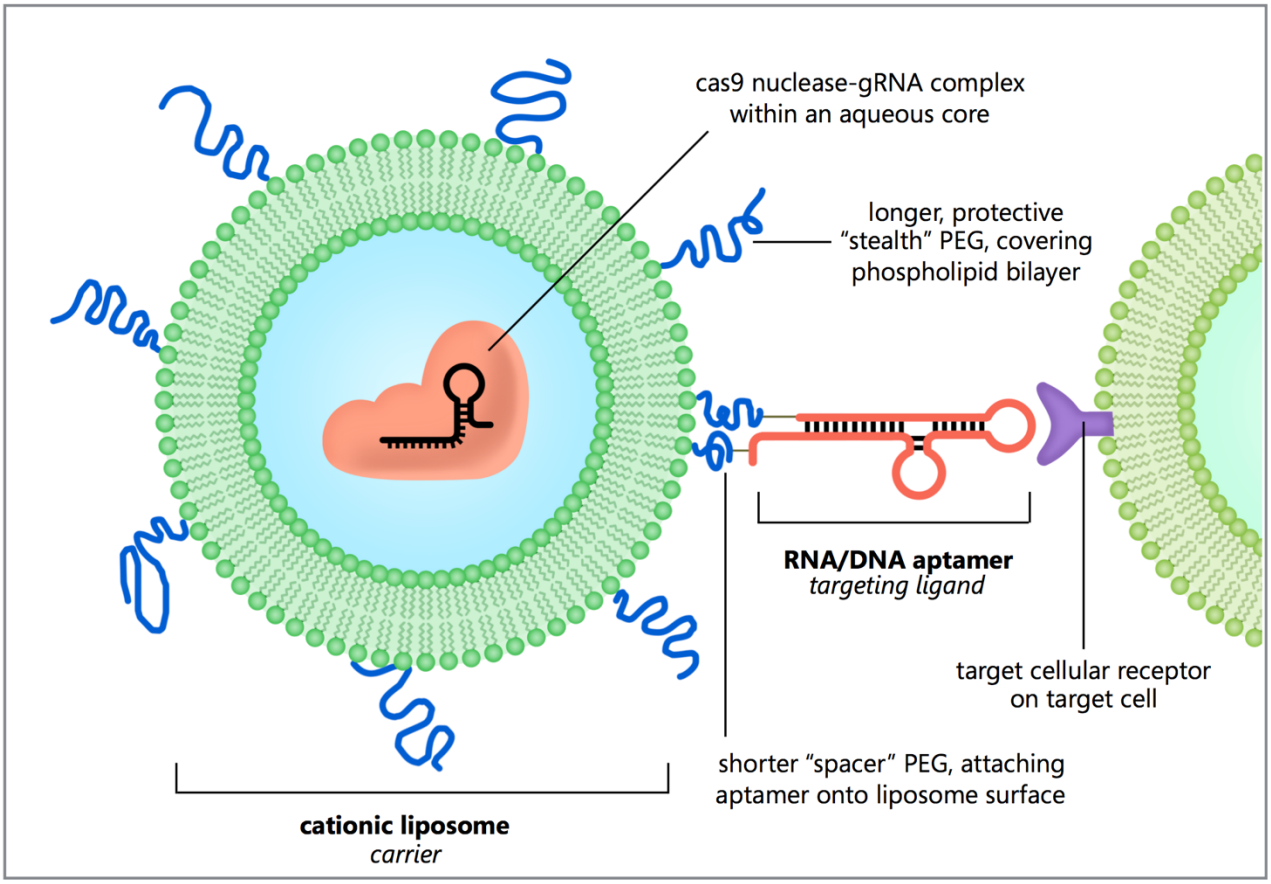

Figure 1. Schematic representation of RNA aptamer-cationic liposome chimera as a targeted delivery system of CRISPR/Cas9. The phospholipid bilayer, which encloses an aqueous core containing the gRNA-nuclease complex, has aptamers attached onto its surface with the help of polyethylene glycol (PEG), which also provides stability and increases circulation time.

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