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Title	CRISPR-Cas13リボヌクレアーゼを使用したプログラム可能な特異的RNAノックダウン
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A Programmable RNA Knockdown Using CRISPR-Cas13 Ribonuclease Towards Gene Therapy

Abstract

Recent approvals in gene therapy have paved the road for an extensive second upsurge of therapies and forefront the groundwork for next-generation treatment strategies. CRISPR-Cas effectors have flourished as an overwhelming tool that can potentially endeavor future genetic medicine. In this doctoral thesis, I investigated the therapeutic potential of anaplastic lymphoma kinase (ALK) and developed optimal parameters for programmable RNA knockdown using CRISPR-Cas13a ribonucleoprotein. Furthermore, I applied the optimized protocol for echinoderm microtubule-associated protein-like 4 (EML4)-*ALK* transcript knockdown as a proof-of-principle for RNA-based cancer therapy. More explicitly, this dissertation consists of five chapters. Where, chapter one summarizes the recent development in RNA-targeted therapeutics including RNAi and CRISPR-Cas systems, and discusses the landscape of *EML4-ALK*-positive lung cancer treatment. This summarization of recent work will assist to understand the recent advancement of RNA-targeted CRISPR-Cas technologies and underpinning the contemporary *ALK*-positive cancer therapeutics.

In the second chapter, I explored the clinical outcome and associated genes of *ALK* expression using integrative bioinformatics. ALK is a tyrosine kinase receptor that is genetically altered in several cancers, such as non-small cell lung cancer (NSCLC), melanoma, lymphoma, and other tumors. Although *ALK* is associated with various cancers, the relationship between *ALK* expression and patient prognosis in different cancers is poorly understood. Here, I show a correlation between *ALK* expression and its clinical outcome in patients with lung adenocarcinoma (LUAD), melanoma, ovarian carcinoma (OV), diffuse large B-cell lymphoma (DLBC), acute myeloid leukemia (AML), and breast cancer (BC) using different computational assessments. I analyzed *ALK* transcriptional expression, patient survival rate, genetic alteration, protein network, and gene and microRNA (miRNA) co-expression. I found that deregulated expression of *ALK* is associated with a high mortality rate in *ALK*-positive cancers. I identified 214 missense mutations, 24 truncating mutations, seven fusions, and two in-frame mutations, with the highest alteration of *ALK* in melanoma. I further showed that 17 genes and 19 miRNAs were exclusively co-expressed and found that *EML4* was the most positively correlated gene. The gene ontology and signaling pathways of the genes co-expressed with *ALK* involved in these six cancers were also identified. My findings offer a basis for *ALK* as a prognostic biomarker and therapeutic target in cancers, which will potentially contribute to precision oncology.

In the third chapter, I developed optimal parameters for programmable and effective RNA knockdown using marker genes including firefly luciferase and mCherry transcripts. RNAi technology has noteworthy potential as a future medicine and could ideally be used to knock down disease-related RNAs. However, owing to frequent off-target effects, limited accessibility of nuclear transcripts, and low efficiency, the medical application of the technique remains challenging. Here, I first evaluated the stability of the Cas13a transcript and guide RNA. Next, I optimized the Cas13a and guide RNA expression vectors to achieve effective knockdown of firefly luciferase (FLuc) transcript, used as a target RNA. The knockdown specificity of Cas13a on target-search was next examined. I found that the 1:3 molar ratio concentration of Cas13a and guide RNA vector is preferable for effective knockdown than the vector amount. Based on

the Cas13a selectivity results, I observed restricted endonuclease activity in 3′ crRNA–gRNA orientation. I also found the highest activity between 24–30 bp long gRNAs with limited mismatch tolerance. Cas13a could effectively knock down FLuc luminescence (70–76%), and mCherry fluorescence (72%). Accordingly, Cas13a has strong potential for use in RNA knockdown and regulation.

Next, chapter fourth showed the feasibility of Cas13a ribonuclease in downregulation of oncogenic driver *EML4-ALK* expression in human disease model lung cancer cells. ALK tyrosine kinase inhibitors provoke a significant anti-tumor response; however, they inevitably succumb to the acquired resistance. Therefore, an alternative therapeutic strategy that limits *ALK* over-activation is necessary for the treatment of this lung cancer. Here, I show that the CRISPR-Cas13a effector possesses effective knockdown potency for oncogenic driver *EML4-ALK* transcript in lung cancer cells. I found the *EML4* transcript was not substantially expressed but *ALK* expressed 80–100 fold higher in *ALK*-positive lung cells compared to a non-fusion transcript in HEK293T cells. I also found that the EML4-ALK oncoproteins were robustly down-regulated (>80%) by employing Cas13a in those lung cancer cells based on western blot results. Consequently, the tyrosine kinase phosphorylation (50–70%) and cell growth (up to 40%) were inhibited. Overall the obtained data demonstrated that the CRISPR-Cas13a protein downregulated the ALK expression in the lung cancer cells. Thus, CRISPR-Cas13a mediated *EML4-ALK* RNA knockdown devises a potential therapeutic strategy for treating *ALK*-positive lung cancer.

Finally, chapter V recapitulates the total work and discusses the benefits, challenges, and future directions. In conclusion, Cas13a has strong potential for use in RNA regulation and could contribute to the development of next-generation genetic medicine.

Keywords: Prognosis of *ALK* expression; RNA knockdown; CRISPR-Cas13a; FLuc transcript; *EML4-ALK*-positive lung cancer.