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Evaluation of double heptamer-type sgRNA as a potential therapeutic agent against multiple myeloma

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ABSTRACT

Emergence of drug-resistant mutations in the course of myeloma cell evolution and subsequent relapse of myeloma appears to be currently inevitable in most patients. To remedy this situation, we are trying to develop therapeutic small guide RNAs (sgRNAs) based on tRNase Z^L-utilizing efficacious gene silencing (TRUE gene silencing), an RNA-mediated gene expression control technology. We designed two sets of double heptamer-type sgRNA, which target the human BCL2 mRNA. Both sets of double heptamer-type sgRNA reduced viability of human myeloma cell lines, RPMI-8226 and KMM-1. We also performed a mouse xenograft experiment to examine how the double heptamer-type sgRNA DHa1(BCL2)/DHa2(BCL2) can reduce the growth of KMM-1 cells in vivo. Median survival periods of the sgRNA cohorts were greater than that of the control cohort by 11–43 days. Furthermore, we designed two sets of double heptamer-type sgRNA, which target the human CCND1 mRNA, and both sets synergistically reduced RPMI-8226 cell viability.

1. Introduction

The American Cancer Society estimates that 32,110 people will be newly diagnosed with multiple myeloma and that 12,960 deaths will take place from the disease in the United States for 2019 (https://www.cancer.org/cancer/multiple-myeloma/about/key-statistics.html; 2019/03/23). The advent of new therapeutic agents such as carfilzomib, pomalidomide, daratumumab, and venetoclax is expected to remedy the high fatality of multiple myeloma [1]. In addition, CAR-T cells targeting the B-cell maturation antigen appear to be a promising treatment modality. However, emergence of drug-resistant mutations in the course of myeloma cell evolution and subsequent relapse of myeloma appears to be currently inevitable in most patients.

We have shown that tRNase Z^L together with cellular small guide RNAs (sgRNAs) appears to form intra- and inter-cellular gene regulatory network [2–4]. In addition to the role in pre-tRNA processing, tRNase Z^L interacts with mRNAs to modulate gene expression by cleaving them under the direction of cellular small noncoding RNAs such as 5′-half-tRNA and miRNA as sgRNAs. Based on this network, we have been developing an RNA-mediated gene expression control technology called tRNase Z^L-utilizing efficacious gene silencing (TRUE gene silencing) [5–10]. This technology makes good use of the property that tRNase Z^L can cleave any target RNA at any desired site under the direction of an artificial sgRNA by recognizing a pre-tRNA-like or micro-pre-tRNA-like complex formed between the target RNA and the sgRNA [11–15]. We have demonstrated that sgRNA can be taken up by cells without any transfection reagents and that naked heptamer-type sgRNAs targeting BCL2 and WT1 mRNAs can efficiently induce apoptosis in human leukemia cells [16,17]. From the sgRNA library screening, we have also obtained 20 heptamer-type sgRNAs that can efficiently induce apoptosis in myeloma and/or leukemia cells [18,19].

To expand the utility of heptamer-type sgRNA, we have developed the double heptamer-type sgRNA method and have shown that two consecutively aligned heptamer-type sgRNAs targeting the BCL2 mRNA can downregulate its level in HEK293 cells more efficiently than a corresponding 14-nt sgRNA [20]. In this paper, we investigate whether the double heptamer-type sgRNAs targeting the BCL2 mRNA and the CCND1 mRNA can efficiently reduce myeloma cell viability and evaluate them as potential therapeutic agents against multiple myeloma.

2. Materials and methods

2.1. RNA preparation

Fully 2′-*O*-methylated, 5′- and 3′-phosphorylated heptamer-type sgRNAs were synthesized with a DNA/RNA synthesizer and subsequently purified through high-performance liquid chromatography with a buffer containing acetonitrile/n-hexylammonium acetate by

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Nippon Bioservice (Asaka, Saitama, Japan).

2.2. Cell culture

Human cell lines, RPMI-8226 and KMM-1, were cultured in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals Japan, Tokyo, Japan) and 1% penicillin-streptomycin (Invitrogen Japan, Tokyo, Japan) at 37 °C in 5% CO_2 humidified incubator. HEK293 cells were cultured in DME media (Wako, Osaka, Japan) likewise.

2.3. Cell viability assay

The human cells were plated at 500, 1000 or 3000 cells/100 μl/well on a 96-well dish in media containing 1 or 2 μM of naked heptamer-type sgRNAs. After 3 days, the viable cell counts were quantitated with Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) [18,19].

2.4. Mouse xenograft experiment

KMM-1 cells (~10^7 cells) were implanted subcutaneously into 24 female SCID/NOD mice (Charles River Laboratories, Yokohama, Japan). And 5 nmol of DHa1(BCL2) or DHa2(BCL2), or 2.5 nmol each of DHa1(BCL2) and DHa2(BCL2) dissolved in 50-μl saline without any carrier reagents were subcutaneously administered directly into the implanted place of each of 6 mice once a day for 4 consecutive days starting 12 day after the implantation. Each cohort consisted of three or four mice due to a low rate of the KMM-1 cell engraftment. They were sacrificed on the day when the tumor volume (length × width^2) / 2 reached to 1500 mm^3, and the data were subjected to the Kaplan-Meier survival analysis. The mouse xenograft experiments were performed in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by Niigata University of Pharmacy and Applied Life Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Niigata University of Pharmacy and Applied Life Sciences (Permit Number: NEW17-33). All efforts were made to minimize suffering.

2.5. Statistical analysis

In the cell viability assay, differences between control (mock) and experimental groups were evaluated by the Student's *t*-test. A difference between control and experimental cohorts in Kaplan-Meier curves was evaluated by the logrank test.

3. Results and discussion

3.1. Double heptamer-type sgRNA targeting the BCL2 mRNA

We designed two sets of double heptamer-type sgRNA, DHa1(BCL2)/DHa2(BCL2) and DHb1(BCL2)/DHb2(BCL2), which target the human BCL2 mRNA (Fig. 1A). And we examined these sets for the ability to reduce cell viability using two human myeloma cell lines, RPMI-8226 and KMM-1. DHa1(BCL2) and DHa2(BCL2) reduced viability of RPMI-8226 cells by 20% and 85%, respectively, under the conditions of a high cell density and a high sgRNA concentration, while DHa1(BCL2)/DHa2(BCL2) reduced the viability by 86% (Fig. 2A). A similar reduction pattern, albeit less efficient, was observed under the conditions of a low cell density and a low sgRNA concentration. The viability of KMM-1 cells was reduced with DHa1(BCL2) and DHa2(BCL2) by 42% and 43%, respectively, and with DHa1(BCL2)/DHa2(BCL2) by 81% (Fig. 2A). Although both DHa1(BCL2) and DHa2(BCL2) did not show the ability to reduce viability of the myeloma cells in the previous experiments [18], this would be due to the differences in experimental set-ups and cell conditions.

DHb1(BCL2) and DHb2(BCL2) decreased a viable RPMI-8226 cell number by 97% and 17%, respectively, under the conditions of a high cell density and a high sgRNA concentration, while DHb1(BCL2)/DHb2(BCL2) decreased the number by 91% (Fig. 2A). Similarly, DHb1(BCL2), DHb2(BCL2), and DHb1(BCL2)/DHb2(BCL2) decreased the living cell number by 57%, 13%, and 74%, respectively, under the conditions of a low cell density and a low sgRNA concentration. The viable KMM-1 cell count was reduced with DHb1(BCL2), DHb2(BCL2), and DHb1(BCL2)/DHb2(BCL2) by 49%, 38%, and 45%, respectively (Fig. 2A).

The observation that both sets of double heptamer-type sgRNA can work only additively at most suggests that both sets do not target the BCL2 mRNA but target other mRNAs/ncRNAs to affect myeloma cell viability. In a consistent manner, stable downregulation of the cellular BCL2 mRNA was not observed (data not shown). It should be noted that the effect of the two sets of double heptamer-type sgRNA on cell viability differs largely depending on myeloma cell lines.

As a control experiment, we examined how the two sets of double heptamer-type sgRNA affect viability of the non-cancerous cell line HEK293. None of single and double heptamer-type sgRNAs reduced the viability, and they rather augmented it (Fig. 3A).

We also performed a mouse xenograft experiment to examine how the double heptamer-type sgRNA DHa1(BCL2)/DHa2(BCL2) can reduce the growth of human myeloma cells in vivo. KMM-1 cells were implanted subcutaneously into SCID/NOD mice, and DHa1(BCL2) and/or DHa2(BCL2) was subsequently administered locally. Median survival periods of the sgRNA cohorts in the Kaplan-Meier curves were greater than that of the control cohort by 11–43 days, although the data were not statistically significant (p-values > 0.05) due to a low rate of KMM-1 cell engraftment (Fig. 4).

3.2. Double heptamer-type sgRNA targeting the CCND1 mRNA

We also designed two sets of double heptamer-type sgRNA, DHa1(CCND1)/DHa2(CCND1) and DHb1(CCND1)/DHb2(CCND1), which target the human CCND1 mRNA (NCBI Reference Sequence: NM_053056), and examined these for the ability to reduce cell viability as above (Fig. 1B). DHa1(CCND1) and DHa2(CCND1) reduced the RPMI-8226 cell viability by 38% and 31%, respectively, while DHa1(CCND1)/DHa2(CCND1) reduced the viability by 97% under the conditions of a high cell density and a high sgRNA concentration (Fig. 2B). A similar but less efficient reduction pattern was observed under the conditions of a low cell density and a low sgRNA concentration. The KMM-1 cell viability was reduced with DHa1(CCND1) and DHa2(CCND1) by 42% and 38%, respectively, and with DHa1(CCND1)/DHa2(CCND1) by 45% (Fig. 2B).

DHb1(CCND1) and DHb2(CCND1) decreased a viable RPMI-8226 cell number by 60% and 24%, respectively, while DHb1(CCND1)/DHb2(CCND1) decreased the number by 97% under the conditions of a high cell density and a high sgRNA concentration (Fig. 2B). In a similar fashion, DHb1(CCND1), DHb2(CCND1), and DHb1(CCND1)/DHb2(CCND1) reduced the cell viability by 40%, 17%, and 77%, respectively, under the conditions of a low cell density and a low sgRNA concentration. The viability of KMM-1 cells was reduced with DHb1(CCND1), DHb2(CCND1), and DHb1(CCND1)/DHb2(CCND1) by 37%, 42%, and 43%, respectively (Fig. 2B).

The results with respect to the RPMI-8226 cells indicate that both sets of double heptamer-type sgRNA work synergistically as expected, although we were not able to confirm stable reduction in the CCND1 mRNA level (data not shown). In contrast, both sets did not show synergistic effect on viability of the KMM-1 cells, suggesting that those sgRNAs target other mRNAs/ncRNAs. This is consistent with the observation that the CCND1 expression level is very low in the KMM-1 cells (data not shown).

The two sets of double heptamer-type sgRNA were examined for an effect on the HEK293 cell viability as a control experiment. Any of single and double heptamer-type sgRNAs did not reduce the viability,

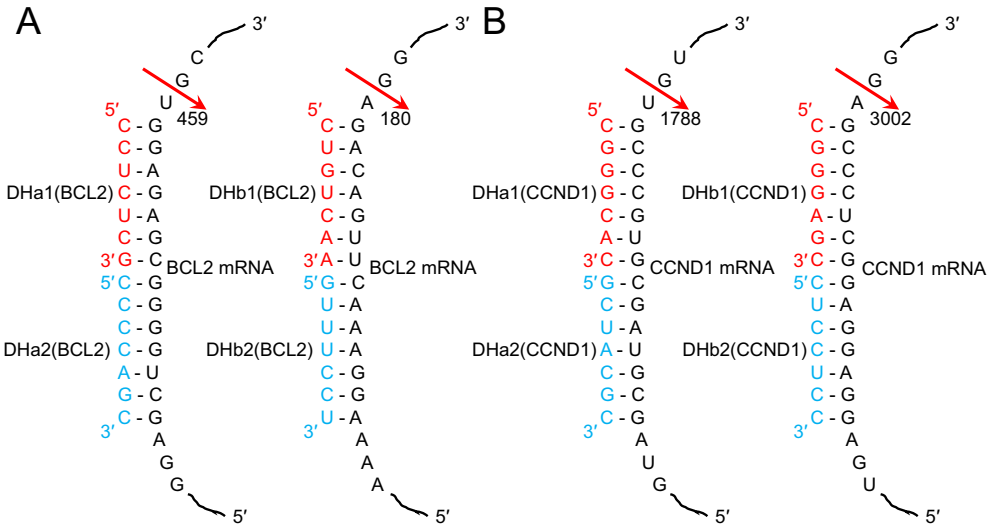

Fig. 1. Double heptamer-type sgRNA. (A) The secondary structures of the complexes between the human BCL2 mRNA (NCBI Reference Sequence: NM_000633) and DHa1(BCL2)/DHa2(BCL2) and between the human BCL2 mRNA and DHb1(BCL2)/DHb2(BCL2). (B) The secondary structures of the complexes between the human CCND1 mRNA (NCBI Reference Sequence: NM_053056) and DHa1(CCND1)/DHa2(CCND1) and between the human BCL2 mRNA and DHb1(CCND1)/DHb2(CCND1). Arrows denote expected cleavage sites by tRNase Z^L.

and rather increased it (Fig. 3B).

3.3. Conclusions

The observation that the two sets of double heptamer-type sgRNA targeting the CCND1 mRNA works synergistically to reduce RPMI-8226 cell viability suggests that these are promising potential therapeutic agents against multiple myeloma. In addition, the single heptamer-type sgRNA DHa2(BCL2) that efficiently suppressed the tumor growth in the mouse xenograft experiment can be one of the best candidate therapeutic sgRNAs. The current study encourages us to achieve personalized medicine for multiple myeloma patients using single or double heptamer-type sgRNA that consists of 16,384 species.

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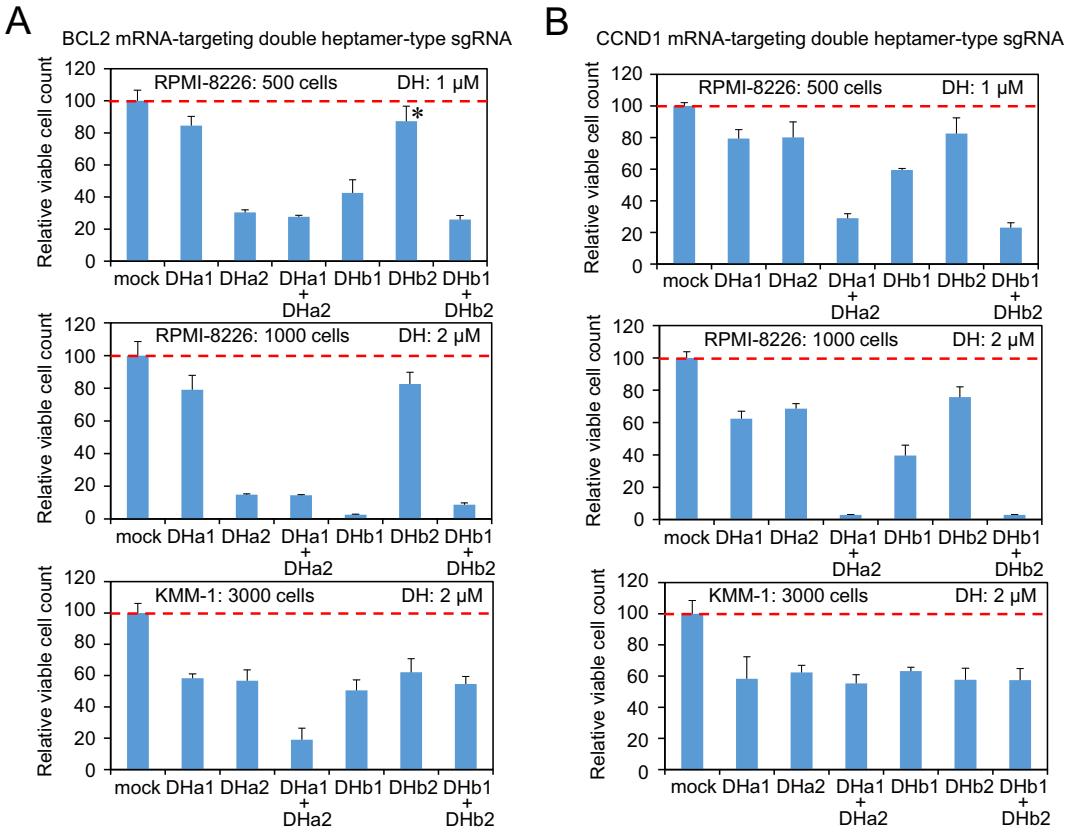

Fig. 2. Double heptamer-type sgRNA reduces myeloma cell viability. Relative viable cell counts were measured 72 h after RPMI-8226 cells or KMM-1 cells were cultured in the presence of 1 or 2 μM of the naked heptamer-type sgRNA targeting the human BCL2 mRNA (A) or CCND1 mRNA (B). The relative viable cell counts in the absence of sgRNAs (mock) are adjusted to 100. The p-values (n = 3) are < 0.05 with one exception denoted by *.

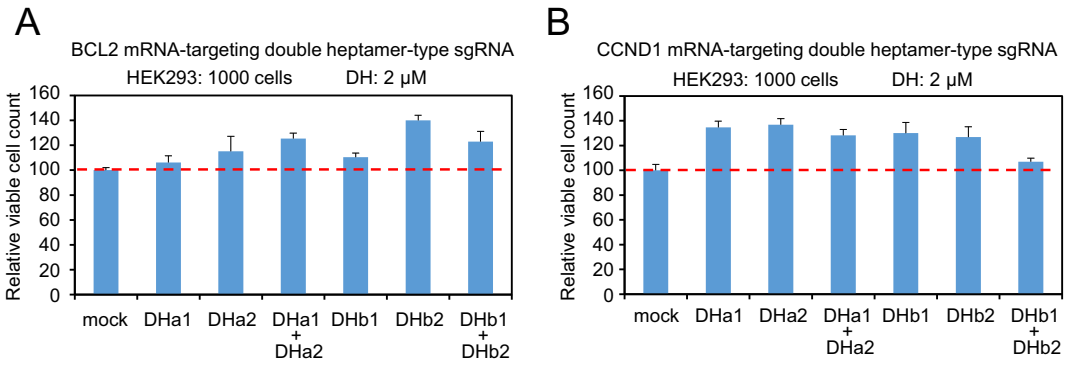

Fig. 3. Cell viability assay. Relative viable cell counts were measured 72 h after HEK293 were cultured in the presence of 2 μM of the naked heptamer-type sgRNA targeting the human BCL2 mRNA (A) or CCND1 mRNA (B). The relative viable cell counts in the absence of sgRNAs (mock) are adjusted to 100.

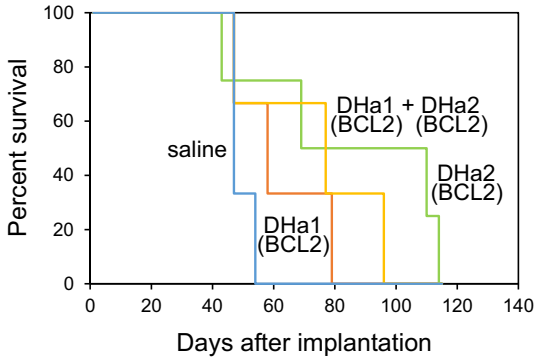

Fig. 4. Kaplan-Meier survival analysis. KMM-1 cells were implanted subcutaneously into SCID/NOD mice, and the naked DHa1(BCL2) and/or DHa2(BCL2) was administered into the implanted place. Saline was used as a control. They were sacrificed on the day when the tumor volume reached to 1500 mm^3.

Authorship contributions

TIshikawa, AH, and TIchiyanagi performed the experiments. MS analyzed the data. MN designed the experiments, analyzed the data, and wrote the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The author MN is an advisor of Veritas In Silico Inc., and owns stock of the company.

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