**ABSTRACT**

Myotonic dystrophy type 1 (DM1) is the most common adult-onset muscular dystrophy and affects 1 in 8000 individuals. 1 DM1 is an autosomal dominant disorder characterized by systemic symptoms, including progressive muscular atrophy, muscular weakness, myotonia, cardiac arrhythmia, insulin resistance, gastrointestinal dysfunctions, cataract, and cognitive impairment.1 It is caused by the expansion of CTG repeat in the 3’ untranslated region (UTR) of dystrophia myotonica protein kinase (*DMPK*) gene. 2, 3 Healthy subjects have 5 to 37 CTG repeats, whereas DM1 symptoms are identified in individuals with more than 50 CTG repeats. 2-4 The larger repeat sizes tend to associate with worse clinical manifestation and several thousand repeats are observed in severe congenital DM1 patients.5, 6 The expanded CTG repeats exhibit somatic and intergenerational instability with a bias toward expansion.7, 8

**INTRODUCTION**

Myotonic dystrophy type 1 (DM1) is the most common adult-onset muscular dystrophy and affects 1 in 8000 individuals. 1 DM1 is an autosomal dominant disorder characterized by systemic symptoms, including progressive muscular atrophy, muscular weakness, myotonia, cardiac arrhythmia, insulin resistance, gastrointestinal dysfunctions, cataract, and cognitive impairment.1 It is caused by the expansion of CTG repeat in the 3’ untranslated region (UTR) of dystrophia myotonica protein kinase (*DMPK*) gene. 2, 3 Healthy subjects have 5 to 37 CTG repeats, whereas DM1 symptoms are identified in individuals with more than 50 CTG repeats. 2-4 The larger repeat sizes tend to associate with worse clinical manifestation and several thousand repeats are observed in severe congenital DM1 patients.5, 6 The expanded CTG repeats exhibit somatic and intergenerational instability with a bias toward expansion.7, 8

The expanded CTG repeats in 3’UTR of *DMPK* are transcribed to mRNA as CUG repeats. It was demonstrated that the expanded CUG repeat forms stable hairpin structure that aggregates as RNA foci. 9-11 The intranuclear RNA foci sequester RNA binding proteins including muscleblind-like 1 (MBNL1), a known splicing regulator. 12-14 This in-turn causes depletion of soluble MBNL1 with normal regulatory function. 15 In addition, the RNA foci up-regulate the activity of another splicing regulator, CUGBP and Elav-like family member 1 (CELF1), by activating protein kinase C pathway and suppressing the expression of specific microRNAs for CELF1. 16, 17 Altered function of these splicing regulators result in abnormal splicing of many genes such as *CLCN1*, *BIN1* or *IRa*, which explain some aspects of the systemic features in DM1. 18-21

“This paper is organized as follows….”1

**BACKGROUND**

Although CRISPR/Cas9 is such an innovative technology, we have to be strictly careful about the collateral undesired mutations to apply it for the therapeutic purpose. 27 One possible candidate for this is to use double nicking strategy. 28 In this system, Cas9 nickase, a D10A mutant of Cas9,is utilized with a pair of offset sgRNAs complementary to opposite strands of the target site. The nicks of both DNA strands lead to a DSB with 5' overhang. Dramatic reduction of off-target cutting is expected by the requirement of two sgRNAs, because it is unlikely that two off-target nicks will be generated by chance in close proximity. 28, 29 Importantly, by dual DSBs, the encompassed region up to several Mb can be removed and the 5' and 3' cut ends are rejoined by the NHEJ or HDR repair systems. 30 Another candidate is CRISPR interference (CRISPRi), a methodology in which the transcription of any gene is downregulated without inducing DSBs. 31, 32 This utilizes catalytically inactive Cas9 (dCas9) fused with transcription suppressor, KRAB, and sgRNA designed at the vicinity of transcription start sites (TSSs). This DSB free method is expected to be much safer than DSB dependent genome editing.

In the present study, we demonstrated that both a conventional Cas9 nuclease and a double nicking strategy using Cas9 nickase successfully excised the CTG repeat tract by designing sgRNAs at 5’ and 3’ flanking regions. By these procedures, the formation of RNA foci was strikingly inhibited. However, unbiassed detection of genomic alterations using linear amplification-mediated high-throughput genome-wide translocation sequencing (LAM-HTGTS) 33, 34 revealed unexpected on- and off-target mutations by these procedures. Finally, we showed that the downregulation of *DMPK* transcription by CRISPRi significantly suppressed the formation of RNA foci. Based on these observations, we propose that the alternative approaches independent of a DSB formation such as CRISPRi should be also considered, when applying the CRISPR/Cas9 technologies for the therapeutic purpose in the future.

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**DATA**

CRISPR/Cas9 system was first discovered as a microbial adaptive immune system. 22 Then, it has been successfully applied to genome editing of eukaryotic cells and utilized in a variety of research fields. 23 -25 Most basic CRISPR/Cas9 system uses Cas9 nuclease derived from Streptococcus pyogenes and a single-guide RNA (sgRNA) which has complementary sequence to the target region of interest. These two components form complex and induce a double strand break (DSB) at the target site. After the cleavage, DSBs are repaired by one of the two major repair pathways i.e. non-homologous end joining (NHEJ) or homology-directed repair (HDR). 26

Although CRISPR/Cas9 is such an innovative technology, we have to be strictly careful about the collateral undesired mutations to apply it for the therapeutic purpose. 27 One possible candidate for this is to use double nicking strategy. 28 In this system, Cas9 nickase, a D10A mutant of Cas9,is utilized with a pair of offset sgRNAs complementary to opposite strands of the target site. The nicks of both DNA strands lead to a DSB with 5' overhang. Dramatic reduction of off-target cutting is expected by the requirement of two sgRNAs, because it is unlikely that two off-target nicks will be generated by chance in close proximity. 28, 29 Importantly, by dual DSBs, the encompassed region up to several Mb can be removed and the 5' and 3' cut ends are rejoined by the NHEJ or HDR repair systems. 30 Another candidate is CRISPR interference (CRISPRi), a methodology in which the transcription of any gene is downregulated without inducing DSBs. 31, 32 This utilizes catalytically inactive Cas9 (dCas9) fused with transcription suppressor, KRAB, and sgRNA designed at the vicinity of transcription start sites (TSSs). This DSB free method is expected to be much safer than DSB dependent genome editing.

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**ANALYSIS**

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**SUMMARY AND CONCLUSIONS**

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**BIBLIOGRAPHY**

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**APPENDICES**

Not counted in word count.