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Evaluation of the extrapolation about the off-target effects of antisense oligonucleotides from *in vitro* to human

○Shinya Asano[1)], Ikuma Yoshida[1)], Yuuichi Arakawa[1)], Kazuya Sakaguchi[1)], Takuro Kobori[2)], Kazuo Sekiguchi[2)], Takaaki Kawanobe[2)], Masaki Yamagami[2)], Tadashi Umemoto[2)], Ryo Fukuda[3)], Hiroyuki Higashiyama[1)]

1) Integrated & Translational Science, Axcelead Drug Discovery Partners, Inc. 2) Research and Development Division, Luxna Biotech Co., Ltd.
3) Consulting, Axcelead Drug Discovery Partners, Inc.

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Abstract

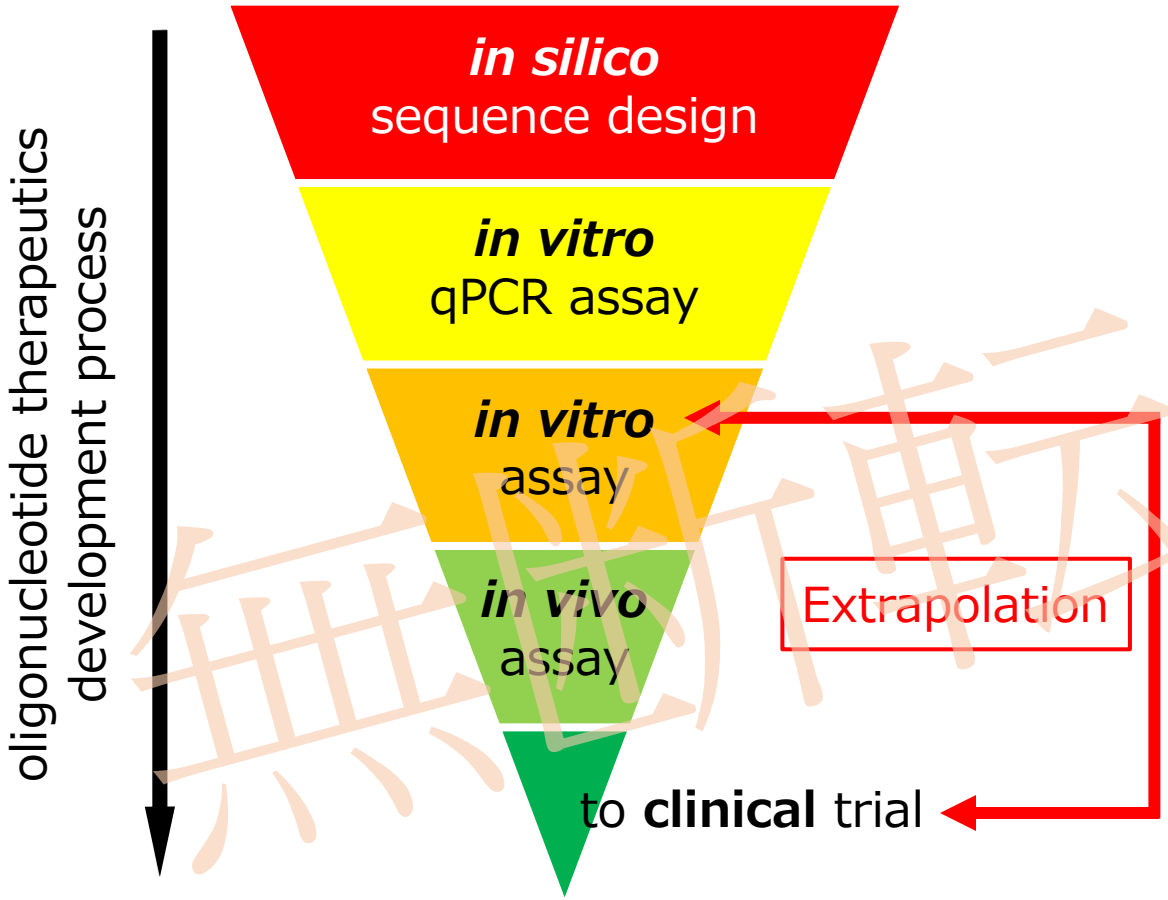
It is crucial for oligonucleotide therapeutics development to take the off-target effects into account from the early preclinical phase to improve the success rates in the clinical trials. To implement it, it is important to establish a high-throughput assay system *in vitro* which predicts the off-target effects and shows the extrapolation from in vitro to human.

In this study, we transfected antisense oligonucleotides (ASOs) of which clinical safety information were reported in HepaRG cells, identified from human hepatocarcinoma. The indicators of the cytotoxicity (AST and ALT) were measured from the cell culture supernatants. Furthermore, transcriptome analysis was performed using next generation sequencer.

Inotersen and other ASOs, which were approved and launched, didn't increase the AST and ALT levels over 1.5 times, and the identified differentially expressed genes (DEGs) of these ASOs were a few (about 50-100). On the other hand, EZN-4176, of which the toxicity was reported in the clinical trials, increased the AST and ALT levels, and a large number (>1000) of DEGs were identified.

The effects of these ASOs on the cytotoxicity and the number of DEGs in HepaRG cells were associated with their clinical safety information, suggesting that this assay system might be useful for predicting the toxicity in human. We also discuss about the off-target effects at the presentation.

Introduction: the extrapolation from *in vitro* to human



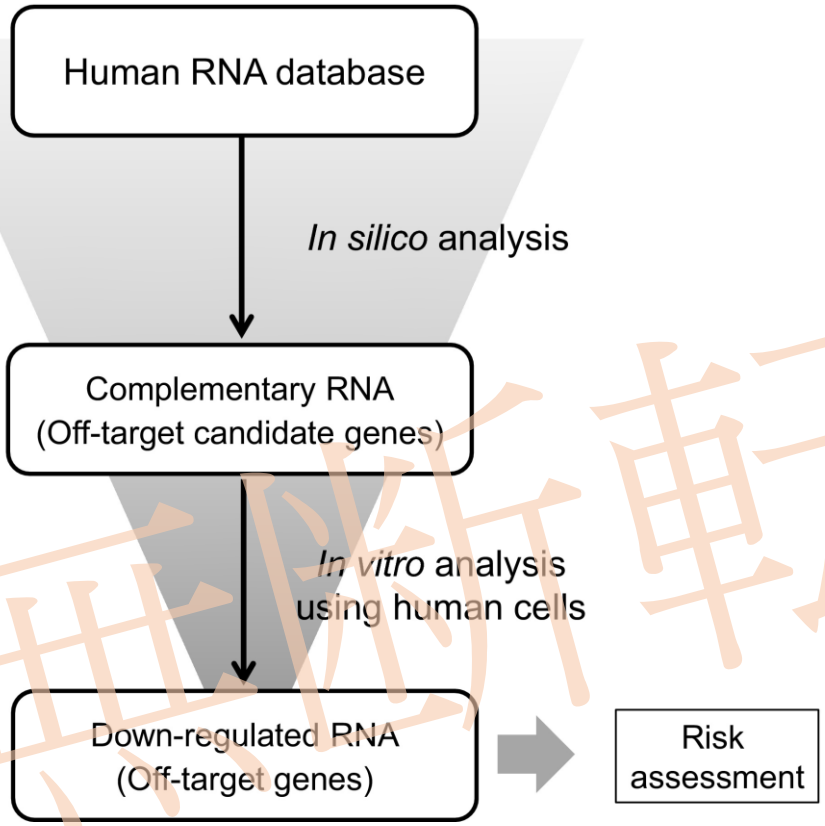
It is crucial for oligonucleotide therapeutics development to take the off-target effects into account from the early preclinical phase to improve the success rates in the clinical trials. To implement it, it is important to establish a high-throughput assay system *in vitro* which predicts the off-target effects and shows the extrapolation from *in vitro* to human.

We have established the off-target effects assessment platform using the next generation sequencer and *in vitro* assay using HepaRG cells, identified from human hepatocarcinoma (S Asano et al. 2019). However, the extrapolation to human of this assay remains unclear.

Here we performed the fundamental study to explore the extrapolation from *in vitro* to human using ASOs of which clinical information is reported.

References
- Asano, Shinya, et al. “Construction the off-target effects assessment platform using Ion AmpliSeq transcriptome technology.” Poster presented at the 5th Annual Meeting of the Nucleic Acids Therapeurics Sciety of Japan.

Introduction: the evaluation of the off-target effects



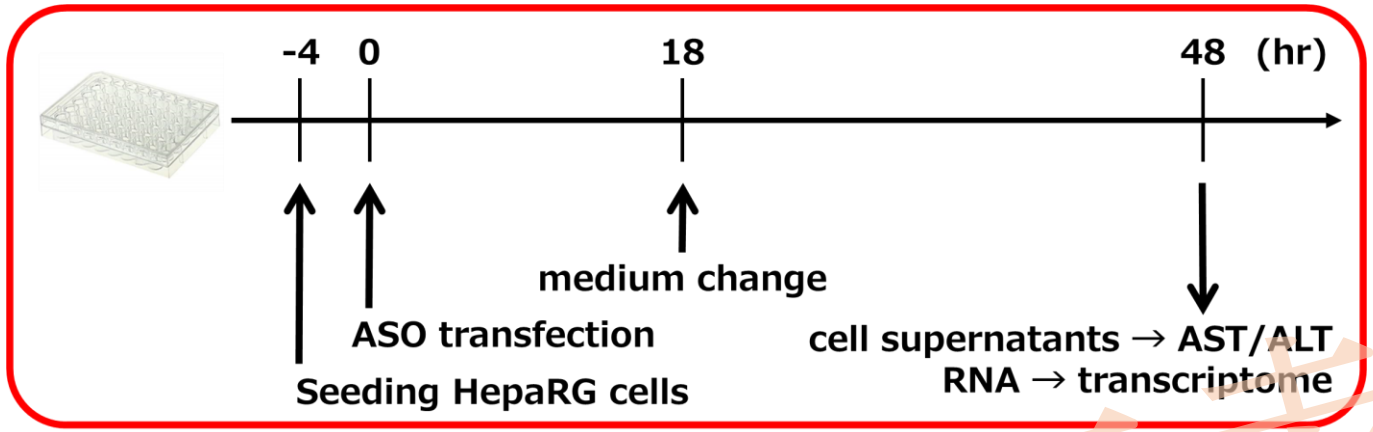
The scheme for the assessment of hybridization-dependent off-target effects of gapmer ASOs. (T Yoshida et al. 2019)

To assess the hybridization-dependent off-target effects, which are the results of complementary binding between the ASO and unintended RNA, the scheme of *in silico* and *in vitro* analysis is proposed (T Yoshida et al. 2019). And we have considered the criteria for predicting the potential off-target genes (S Asano et al. 2019). However, the standard protocol of *in silico* analysis to select the off-target candidate genes remains to be established.

Here we examined a method for predicting the off-target genes and evaluated the off-target effects induced by gapmer ASOs with clinical information in this assay.

References
- Yoshida, Tokuyuki, et al. "Evaluation of off-target effects of gapmer antisense oligonucleotides using human cells." Genes to Cells 24.12 (2019): 827-835.
- Asano, Shinya, et al. “Construction the off-target effects assessment platform using Ion AmpliSeq transcriptome technology.” Poster presented at the 5th Annual Meeting of the Nucleic Acids Therapeurics Sciety of Japan.

Materials and Methods: the extrapolation from *in vitro* to human



In vitro assay

HepaRG cells were transfected with ASOs. After 48 hours post-transfection, the AST and ALT levels were measured from the cell supernatant. Total RNA was prepared from the cells Then transcriptome analysis and TaqMan PCR assay were performed.

ASO	Concentration	Type	Modification	Length	Target	Phase	Reference
Inotersen	30 nM	gapmer	2'-MOE	20 mer	TTR	launched	MD Benson et al. 2018
Mipomersen	30 nM	gapmer	2'-MOE	20 mer	APOB	launched	MP McGowan et al. 2012
EZN-4176	30 nM	gapmer	LNA	16 mer	AR	halted at the Phase 1a	D Bianchini et al. 2013
Nusinersen	100 nM	exon inclusion	2'-MOE	18 mer	SMN2	launched	BT Farras et al. 2019

Information of the evaluating ASOs.

The evaluating ASOs

We examined 4 ASOs of which clinical information was reported. Inotersen, Mipomersen, and Nusinersen were FDA approved ASOs. EZN-4176 was terminated in clinical study because of the hepatotoxicity.

References

- Benson, Merrill D., et al. "Inotersen treatment for patients with hereditary transthyretin amyloidosis." New england journal of medicine 379.1 (2018): 22-31.
- McGowan, Mary P., et al. "Randomized, placebo-controlled trial of mipomersen in patients with severe hypercholesterolemia receiving maximally tolerated lipid-lowering therapy." PloS one 7.11 (2012): e49006.
- Bianchini, Diletta, et al. "First-in-human Phase I study of EZN-4176, a locked nucleic acid antisense oligonucleotide to exon 4 of the androgen receptor mRNA in patients with castration-resistant prostate cancer." British journal of cancer 109.10 (2013): 2579-2586.
- Darras, Basil T., et al. "An integrated safety analysis of infants and children with symptomatic spinal muscular atrophy (SMA) treated with nusinersen in seven clinical trials." CNS drugs 33.9 (2019): 919-932.

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Materials and Methods: the evaluation of the off-target effects

GGGenome off-target candidate selection

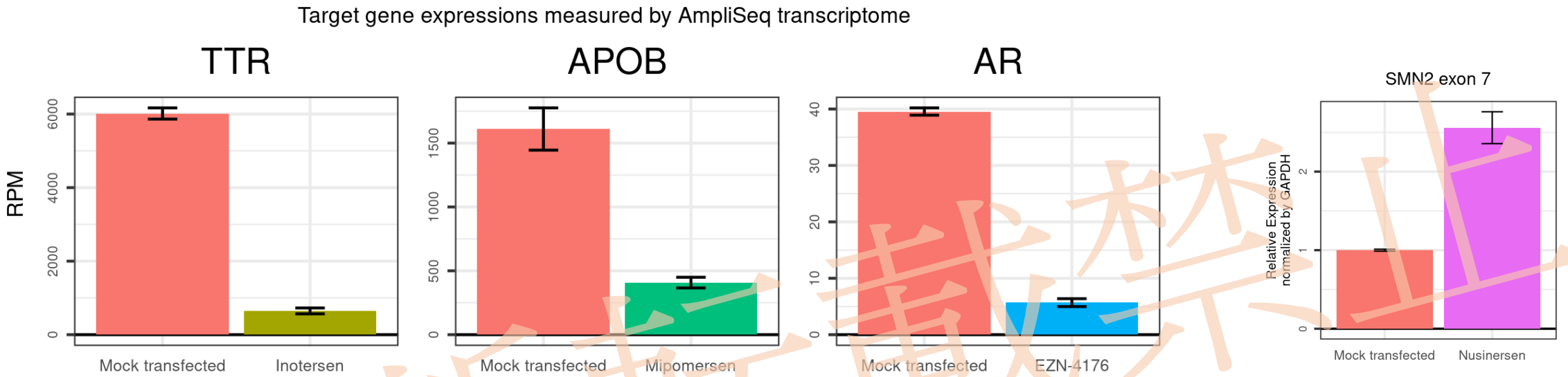
The off-target candidate genes of gapmer ASOs were selected using GGGenome software based on the D3G database. Both spliced mRNA and pre-mRNA of curated protein coding gene database were used for searching and we searched the off-target candidate genes as many mismatches/gaps as GGGenome software allowed.

In silico analysis for the off-target effects evaluation

We compared the off-target candidates selected by GGGenome software with transcriptome analysis results and we considered the approval method to select the off-target candidate genes.

After constructing the selection method, then we evaluated the selected off-target genes and DEGs.

Results: Effects of ASOs on target gene knockdown or splicing change



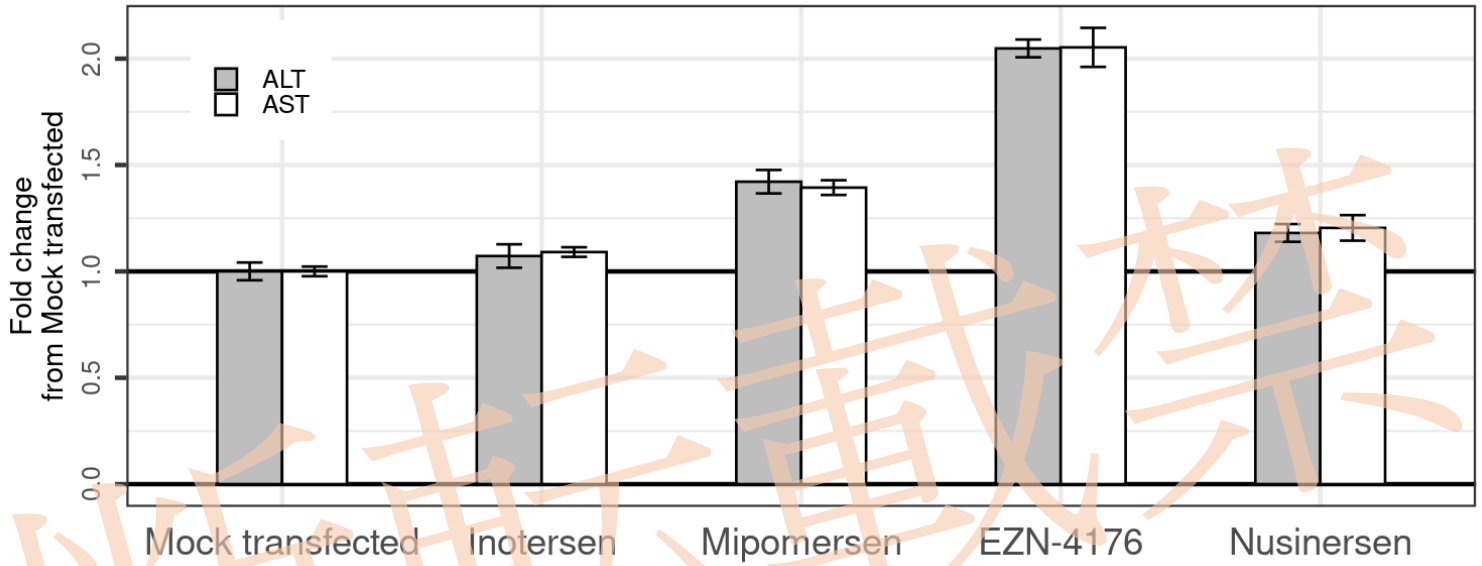
Bar graph of the gene/isoform expressions measured by transcriptome (TTR, APOB, and AR) or qPCR assay (SMN2 exon 7).

Gene-level target mRNA knockdowns were observed at all 3 gapmer ASOs. RPM, the expression quantity calculated from transcriptome, of these ASO's target genes were down-regulated over 50% compared with that of mock transfected.

For Nusinersen, splicing change of *SMN2* gene was measured by TaqMan PCR assay. Expression levels of exon 7 included *SMN2* gene were up-regulated over 2.5 times compared with that of mock transfected.

- These results indicate the on-target effects induced by each ASO were confirmed in this assay.

Results: Increase of AST and ALT levels induced by EZN-4176



Bar graph of the AST and ALT levels. y-axis represents the fold change compared with the AST and ALT levels of mock transfected.

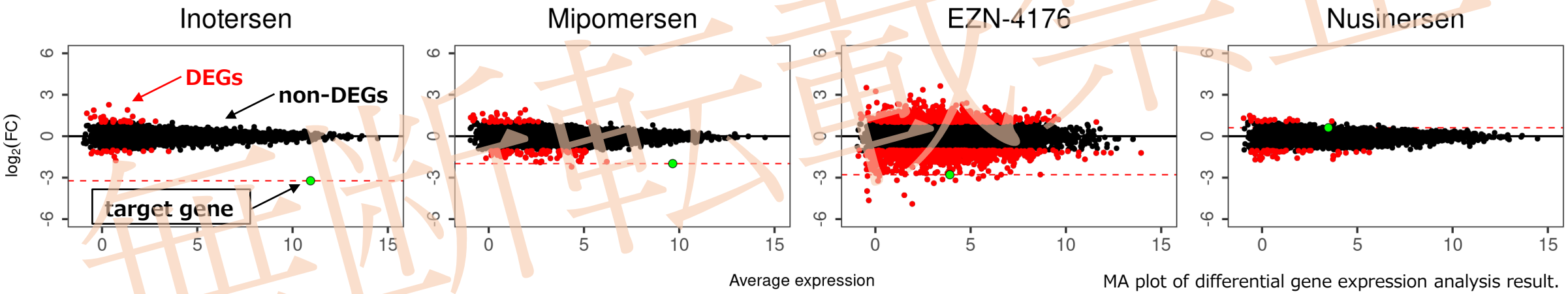
Inotersen, Mipomersen, and Nusinersen didn't increase the AST and ALT levels compared to that of mock transfected. On the other hand, EZN-4176, of which the toxicity was reported in the clinical trials, increase the AST and ALT levels over 1.5 times.

- The cytotoxicity induced by ASOs was associated with the clinical safety.

Results: Association of the number of DEGs with the clinical safety

ASO	# of DEGs	top 10 significant DEGs
Inotersen	**49**	**TTR**, UNC5C, SH3D21, KCNAB3, FRAS1, TREM2, KRT6C, TNFSF14, LOC100128361, TSPAN1
Mipomersen	**118**	MGEA5, **APOB**, CDK5RAP3, COMT, CDS2, PCDH7, GPC6, UST, LAMA5, TSHZ2
EZN-4176	**1283**	ACSM2A, ISCU, ADH1B, HAO2, RRM2, ACSL1, TF, SULT2A1, AKR1B10, GDF15
Nusinersen	**65**	POGZ, WDR70, FRK, C20orf72, MAGED2, CAPN7, GIT2, PITHD1, MAP1S, ITGAE

The result overview of differential gene expression analysis compared with mock transfected.
Blue letters represent down-regulated genes and red letters represent up-regulated genes.



MA plot of differential gene expression analysis result.

We compared the genome-wide gene expression of ASOs transfected HepaRG cells with that of mock transfected HepaRG cells. The identified differentially expressed genes (DEGs) of Inotersen, Mipomersen, and Nusinersen transfected HepaRG cells were a few. But the identified DEGs of EZN-4176 transfected HepaRG cells were large, suggesting that EZN-4176 altered many gene expressions.

➢ The genome-wide gene expression change induced by ASOs was associated with the clinical safety.

Results: Selection *in silico* off-target candidate genes using GGGenome

ASO	Length	Reference	# of mismatch/gap (≤) 0	1	2	3	4	5
Inotersen	20 mer	spliced mRNA	1	1	5	36	1095	12287
		pre-mRNA	1	1	19	1094	18762	(> 100000)
Mipomersen	20 mer	spliced mRNA	1	1	1	54	1759	25776
		pre-mRNA	1	1	15	1041	20732	(> 100000)
EZN-4176	16 mer	spliced mRNA	2	14	534	8069	87705	-
		pre-mRNA	4	221	6847	(> 100000)	(> 100000)	-

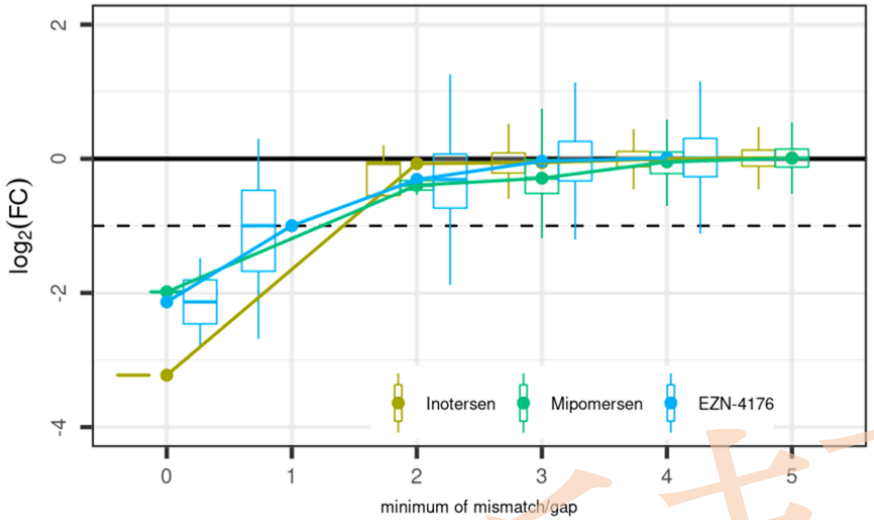
The number of predicted the off-target candidate transcripts (RefSeq ID)

The off-target candidate genes of gapmer ASOs were selected using GGGenome software based on the D3G database.

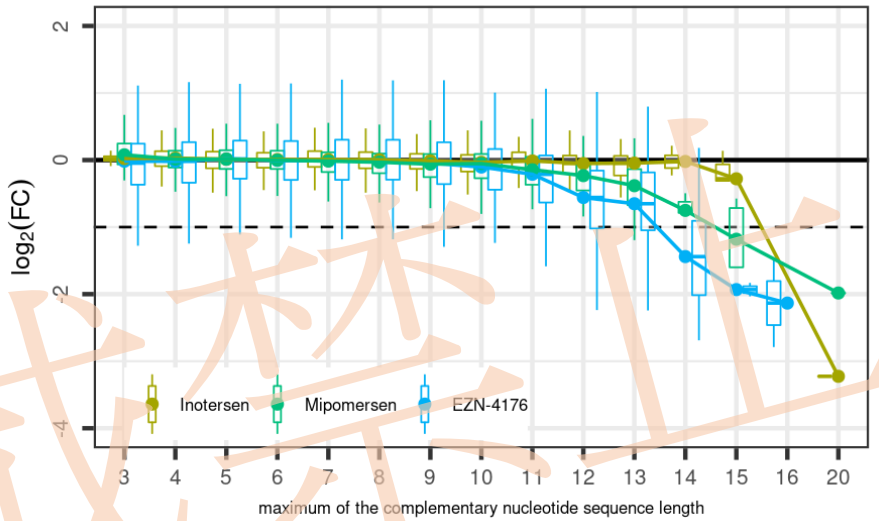
The number of candidate transcripts were increased exponentially as the more mismatch/gap allowed, and the number of candidates from pre-mRNA reference was larger than that of spliced mRNA reference.

We used all GGGenome search results and further analysis was performed.

Results: The better criteria for prediction -12 nt complementary match-



Relationship between the number of mismatch/gap of the off-target candidates and the expression fold change obtained from transcriptome analysis



Relationship between the number of complementary nucleotide sequence length of the off-target candidates and the expression fold change obtained from transcriptome analysis

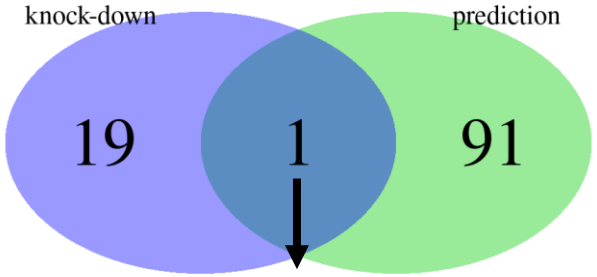
Next, we examined what parameters affect the off-target prediction accuracy by comparing with the transcriptome analysis result. Many genes within 1 mismatch/gap were down-regulated, but genes with 2 mismatches/gaps or more were not.

We found the tendency for the gene expression to down-regulated as the complementary length increased from 12 nt in Mipomersen (20 mer) and EZN-4176 (16 mer).

- The 12 nt complementary match or more might be better criteria for predicting the off-target genes regardless the ASO length.

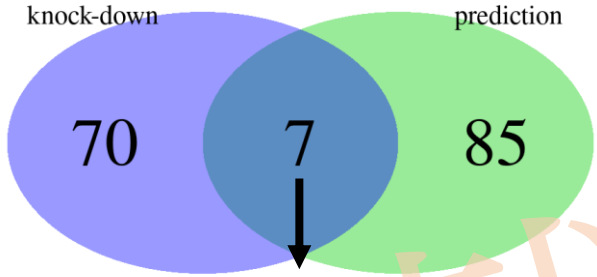
Results: Identification of reported off-target candidate gene (*PRKG2*)

Inotersen



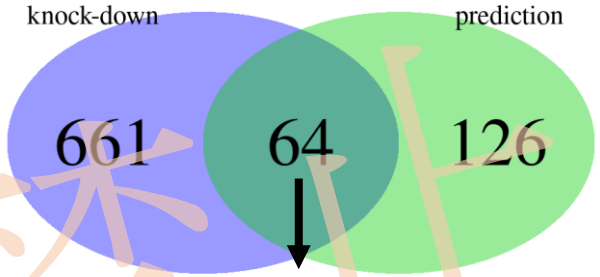
Reference	genes
Both references	**TTR**
spliced mRNA	-
pre-mRNA	-

Mipomersen



Reference	genes
Both references	**APOB**, **PRKG2**
spliced mRNA	MEGF8
pre-mRNA	PITPNM2, NKAIN2, DZIP3, FGF13

EZN-4176



Reference	genes
Both references	**AR**, DHX15, KIAA0100, BTAF1, ATAD2B, F8
spliced mRNA	KDM5C, CRIPT, ENOPH1, ZFC3H1, NEB
pre-mRNA	(53 genes)

Identified off-target genes which overlapped between the transcriptome knockdown genes and *in silico* predicted off-target candidate genes

Red bold letters represent the on-target genes.

Finally, we examined the overlap between the knockdown genes identified from transcriptome analysis and the off-target candidate genes which match 12 nt complementary length or more.

No off-target genes were detected in Inotersen, and several off-target genes were identified in Mipomersen and EZN-4176.

PRKG2 gene was identified as the off-target genes of Mipomersen, and this gene was also listed in the review report of Mipomersen as the candidate off-target genes.

- Although further investigation was needed, the criteria of selecting the off-target candidates which match 12 nt complementary length or more could detect the potential off-target genes.

Summary

[The extrapolation from *in vitro* to human]

The effects of ASOs on the cytotoxicity and the number of DEGs in HepaRG cells were associated with their clinical safety information. That is, EZN-4176 increased the AST and ALT levels and yielded the large number of DEGs. In contrast to EZN-4176, Inotersen, Mipomersen, and Nusineren didn't increased the AST and ALT levels and the identified DEGs were a few.

- ✓ These results suggest that this assay may be useful for predicting the clinical toxicity from *in vitro.*

[The evaluation of the off-target effects]

We found that the 12 nt complementary match might be better criteria for predicting the off-target genes regardless the ASO length. And *PRKG2* gene was identified as the off-target genes of Mipomersen, and this gene was also listed in the review report of Mipomersen as the candidate off-target gene.

- ✓ These results suggest that the selection criteria we shown may be useful for predicting the hybridization-dependent off-target genes precisely.

- ➢ Our assay system may be useful for the oligonucleotide therapeutics development by predicting the toxicity in human and predicting the potential risk of hybridization-dependent off-target effects from the early preclinical phase.