# 無断転載禁止

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PK/PD evaluation of antisense oligonucleotides

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## Purpose

Distribution and activity of antisense oligonucleotides (ASOs) are limited to some tissues when administered systemically, which is a barrier to use as a therapeutic drug for a wide range of diseases. Now that, various drug delivery systems have been developed to overcome these problems. It is known that the distribution of ASO is not evenly in tissues and cells, evaluation method for distribution of ASOs has not been sufficiently constructed. Detailed evaluation of the distribution of ASO in tissues and cells would be needed to select appropriate targets, evaluate the efficiency of drug delivery systems, and validate their concepts. In this study, we constructed methods for evaluating PK/PD in more detail *in vivo* and *in vitro*.

# Summary

First, we developed a double staining method of IHC with anti-PS-ASO antibody and ISH of target mRNA. Using this method, the distribution of ASO and mRNA knockdowr could be simultaneously assessed in the histological section. Distribution and knockdown activity in specific cells and regions in tissue

Second, we developed hybridization ELISA methods to measure the concentration of ASO in cultured cells and nuclei. Using this method we can know the relationship between the number of nucleic acids and mRNA knockdown

These methods make it possible to select appropriate targets, evaluate the efficiency of drug delivery systems and validate their

### **Material method**

· ASO
Mouse NALAT1 ASO\*(Fig. 1-6)
5\*-GGETmCAGCTGCCAATGmCTAG-3\*
Human NALAT1-ASO\*(Fig. 8-10)
5\*-AGATCATNAGCATMCTG-3\*
Fig. 8-10 5\*-AGALACTATAGCATMCTG-3\*
5\*-GGMCAMCAMCAMCATAGCAT-3\*
5\*-GGMCAMCAMCAMCATAGCAT-3\*
5\*-GGMCAMCAMCAMCATAGCAT-3\*
Human APOB ASO\*(Fig. 11)
Human APOB ASO

# Animal study

CSTBL/6J mice at 9 weeks of age were treated subcutaneous With MALAT1 ASO at 50 mg/kg or saline (n = 2/group). Mice w cuthanized 24 hours after dosing and tissues were harvested. All animal experiment protocols were approved by the Instituti Animal Care and Use Committee of Shonan Health Innovation

- Histology analyses
   Histological sections were stained by immunohistochemical staining (IHC) with anti-PS-ASO (phosphorothioate antisense oligonucleotide) antibody and in-situ hybridization (ISH) of target mcMA
- Methods of IHC

  Staining equipment: Leica BOND RX
  Antigen retrieval: Bond ER Solution 1
  Primary anti body: AMCC-11975\_1A, Anti-asO(M1-27),
- Staining kit: BOND Polymer Refine Detection
- Methods of double staining of Malat1-ISH and PS-ASO-IHC

  Staining equipment: Leica BOND RX

  Antigen retrieval: Bond ER Solution 2, RNAscope 2.5 LSx
- Protease
  Staining kit for ISH: RNAscope 2.5 LS Reagent Kit-Brown
  probe for ISH: RNAscope® LS 2.5 Probe- Mm-Malat1
  Primary anti body for IHC: AMCC-11975\_1A, Anti-asO(M1-27),
- ning kit for IHC: BOND Polymer Refine RED Detection

# ASOs with or without Lipofectamine 1th 2000 Transtection Reagent were treated to Hep G2 (2 day) or A549 (1 day) cells and after incubation, cells were detached with trypsin and recovered. Nuclei were extracted from a part of cells using Nuclear/Cytosolic Fractionation Kit (Cell Biolabs).

Measurement of ASO concentration in tissue ASO concentration was measured by LC-MS/MS.

ASO concentrations in cells and nuclet were measured by hybridization ELISA/FILEISA, We use "one-step hybridization ELISA/FICEISA, We use "one-step hybridization ELISA," for 3-fluorescein labeled ASO and "two-step hybridization [Islash or unabeled ASO."] Images were taken using a confocal microscope (Cyfiva).

metastasis associated lung adenocarcinoma transcript 1 (Malat1) mRNA and apolipoproteinB (APOB) mRNA expression were quantified by quantitative real-lime polymerase chain reaction using GAPDH as a house keeping gene.

No. 1) G. Hung, et. Al. 2013. Nucleic Acid Ther 23:6, 369-378.
2) R. S. Geary, et al. 2015. Clin Pharmacokinet 54, 133-146.
3) X. Wei, et. Al. 2006. Pharm. Res. 23:6, 1251-1264.

We have no financial relationships to disclose

# Evaluation of distribution in tissue and mRNA knockdown

### 1. Distribution analysis of ASO by LC-MS/MS and knockdown analysis by qPCR

in tissues and cells

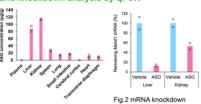


Fig. 1 ASO concentration (24 h) of

### 2. Distribution analysis of ASO by IHC (anti-PS-ASO ab)

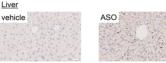


Fig. 3 Granular positive brown signals were observed in the cytoplasm of almost all the Kupffer cells and hepatocytes in ASO animals. Bar = 100 um



Fig. 4 Granular positive brown signals were observed in the cytoplasm of proximal tubules in ASO animals. Glomeruli and distal tubules (arrows) were almost negative. Bar = 100 um

### 3. Double staining of Malat1-ISH and PS-ASO-IHC

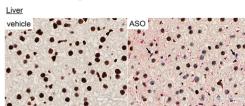


Fig. 5 In vehicle animals (left), almost all nuclei of hepatocytes and Kupffer cells showed positive (brown) for ISH for *Malat1*. In ASO animals (right), signals of PS-ASO (red) were observed in the cytoplasm of many hepatocytes and Kupffer cells. Many nuclei of the hepatocytes and Kupffer cells turned out to be negative for ISH for *Malat1* and showing bluish hematoxylin color, which suggested knockdown of *Malat1* in these cells by ASO administration. Some Kupffer cells showing knockdown clearly contained PS-ASO signals (red) in their cytoplasm

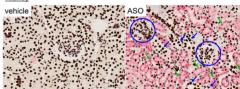
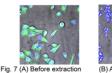


Fig. 6. In vehicle animals (left), almost all nuclei of renal tubules and glomeruli showed positive (brown) for ISH for *Malat1*. In ASO animals (right), PS-ASO signals (red) were observed almost exclusively observed in the proximal tubules Many nuclei of the proximal tubules turned out to be negative for ISH for *Malat1* and showing bluish hematoxylin color (green arrows), which suggested knockdown of *Malat1* in these cells by ASO administration. On the contrary, nuclei of distal tubules (blue arrows) and glomeruli (blue circle) showed positive for ISH for Malat1 (brown), which suggested knockdown by ASO administration did not occur in these cells. Bar = 100 um

# Measurement of concentration of ASO in cells and nuclei

3.5

### 1. Extraction of nuclei from cells



(B) After extraction

of nuclei

Hoechst 33342

of nuclei

molecules /cell nucleus (×10<sup>6</sup>) 2.5 60 2 1.5 ASO

2-1. Evaluation of the transfer rate to nucleus of ASO

fectamine 2000 (%)

0.0016 0.008 0.04

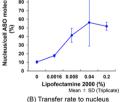
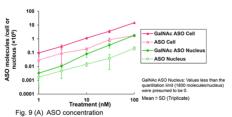
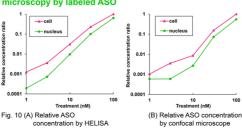


Fig.8 (A) ASO concentration

### 2-2. Cell concentration of GalNAc conjugated ASO



2-3. Comparison with the results using confocal microscopy by labeled ASO



### 2-4. Relationship between the number of ASO molecules and KD activity

(B) GalNAc ASO 100 nM (C) ASO 100 nf

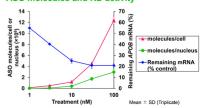


Fig. 11 ASO concentration and mRNA knockdown