Unique distribution of antisense oligonucleotides in the brain by the intrathecal administration

○Sayuri Nakamura, Koji Murakami, Hitoshi Kandori, Kotaro Yokoyama

Pharmacology Business Unit, Axcelead Drug Discovery Partners, Inc.

Purpose

In the preclinical studies for antisense oligonucleotide (ASO) targeting central nervous system (CNS), visualizing and identifying brain regions and brain cell types where ASO distributes are quite important. In this study, we evaluated the biodistribution of ASO and knockdown (KD) of *Malat-1* in various CNS regions of mice using *in situ* hybridization (ISH). In addition, we developed a new panel of multiplex immunohistochemistry (mIHC) to detect various brain cell types where ASO distributes. ASO was administered via three different routes, and differences in distribution were analyzed.

Summary

ASO for *Malat-1* was once administered to mice by intracerebroventricular (ICV), intrathecal (IT), or subcutaneous injection (SC), and two weeks after administration, brain and spinal cord were collected as we reported in the last annual meeting (Sano *et al.* 2023) . The distribution of ASO and KD of *Malat-1* in multiple brain regions and the spinal cord were evaluated by *in situ* hybridization (ISH). In addition, we developed a new mIHC panel, and the ASO distribution of each cell types (neuron, astrocyte, oligodendrocyte, microglia, and endothelial cell) in each brain region was visualized. As a result, ASO was widely observed in the brains of ICV and IT groups, but slightly detected in the SC group except for areas without blood-brain barrier (BBB). Distribution of KD was correlated with the biodistribution of ASO. mIHC revealed that ASO distributed mainly in endothelial cells in the SC group, and in various cell types of brain in the ICV and IT groups. Additionally in the IT group, ASO was specifically observed in the neurons in the layer V of parietal cortex (around motor cortex). Our results suggested that the new panel of mIHC which we developed is very useful for detecting differences in cell types depending on the administration method when ASO is administered to the CNS.

Materials & Methods

・ASO
Mouse *MALAT1* ASO[1)]
5'-GGGTmCAGCTGCCAATGmCTAG-3'
ASO has fully phosphorothioate backbone.
Underlining; 2'-O-methoxyethyl modified base ASO
mC; 5-methyl cytosine
ASO was purchased from GENEDESIGN, Ajinomoto Bio-Pharma Services.

・Animal study
C57BL/6J mice at 8 weeks of age were administered *Malat1* ASO at 37.5 μg/animal (approximately 1.5 mg/kg, ICV and IT) , 50 mg/kg or saline (SC) (n = 3/group). Mice were euthanized 14 days after administration and tissues were collected.
All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Shonan Health Innovation Park.

・Histology analyses
Collected tissues were fixed by 10% neutral buffered formalin and embedded in paraffin. Coronal sections were prepared at 4 μm-thickness from 5 brain regions (Bregma approx.+3.92, -0.98, -1.94, -3.52, and -6.36 mm) and 3 spinal regions (cervical, thoracic and lumber) . These sections were stained by *in situ* hybridization (ISH) with the probe for mouse *Malat1* ASO and mouse *Malat-1*. Coronal sections were prepared at 4 μm-thickness from 2 brain sections (Bregma approx. -0.98 and -1.94 mm). These sections were stained by multiplex immunohistochemistry with anti-PS-ASO (phosphorothioate - antisense oligonucleotide), anti-NeuN, anti-GFAP, anti-TPPP, anti-Iba-1, anti-CD31 antibodies.

Methods of staining of ASO for *Malat1*-ISH
- Staining equipment: Leica BOND RX
- Prestreatment: Bond ER Solution 2, RNAscope 2.5 LSx Protease
- Detection: miRNAscope 2.5 LS Reagent Kit-Red, BOND Polymer Refine RED Detection
- probe RNAscope® LS 2.5 Probe- SR-ASO-Mm-Malat1

Methods of staining of *Malat1*-ISH
- Staining equipment: Leica BOND RX
- Pretreatment: Bond ER Solution 2, RNAscope 2.5 LSx Protease
- Detection: RNAscope 2.5 LS Reagent Kit-Brown, BOND Polymer Refine Detection
- probe RNAscope® LS 2.5 Probe- Mm-Malat1

Methods of multiplex immunohistochemistry
- Staining equipment: Leica BOND RX
- Antigen retrieval: Bond ER Solution 1 and 2
- Detection kit: Opal 6-plex Detection Kit for Whole Slide Imaging
- Antibodies:
  - PS-ASO, Anti-asO(M1-27), ADDP-2), 1:5000
  - NeuN, EPR12763, abcam, ab177487, 1:5000
  - GFAP, Dako, IR524, 1:4
  - Iba1, EPR16589, abcam, ab178847, 1:5000
  - TPPP, EPR3316, abcam, ab92305, 1:10000
  - CD31, D8V9E, Cell Signaling Technology, 77699S, 1:200

2) Originated from Takeda Pharmaceutical Company Limited.

Reference
1) G. Hung, et al. Al. 2013. Nucleic Acid Ther 23:6, 369-378.

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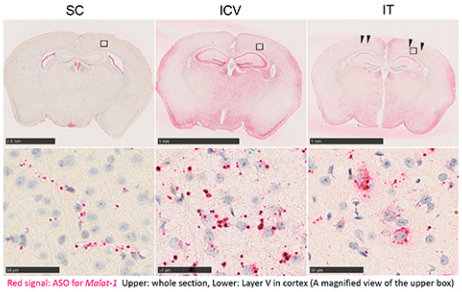
Evaluation of tissue distribution by ISH

1. Biodistribution of ASO for *Malat-1* by ISH



Red signal: ASO for *Malat-1* Upper: whole section, Lower: Layer V in cortex (A magnified view of the upper box)

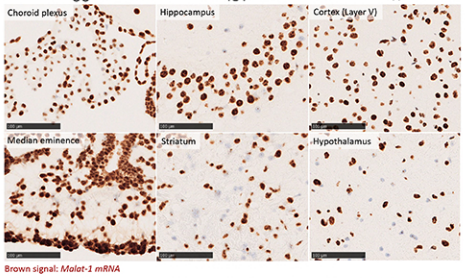
Table 1. Histological evaluation of ASO for *Malat-1*

Region	SC（PBS）	SC(ASO)	ICV(ASO)	IT(ASO)
Brain				
Olfactory bulb	0	1	3-4	4
Cortex（Parietal）	0	1	3	2*1
Cortex (Temporal-Basal)	0	1	3-4	3-4
Striatum	0	1	3	2
Hippocampus	0	1	4	2-3
Amygdala	0	1	3	3
Thalamus	0	1	4	2
Hypothalamus	0	2	4	4
Midbrain (Substantia nigra)	0	1	4	4
Cerebellum	0	1	3-4	3
Medulla oblongata	0	1	4	4
Corpus callosum	0	1	3	1-2
Choroid plexus (Lateral-3rd ventricle)	0	4	3	1
Choroid plexus (4th ventricle)	0	4	3	1
Spinal cord				
Cervical	0	1	3-4	4
Thoracic	0	1	3-4	4
Lumber	0	1	3-4	4

0: no signal, 1: a few signals, 2: More signal than score 1, 3: Many signals were focally observed, 4: Many signals were diffusely observed
*1: Layer V showed specifically positive.

A smaller amount of ASO was detected in the SC group compared to the ICV and the IT groups throughout brain and spinal cord, but clearly detected in areas without BBB which were median eminence of hypothalamus and choroid plexus. In the ICV group, ASO was widely detected especially in the brain regions around ventricles (e.g. hippocampus, cortex, striatum, thalamus, hypothalamus and cerebellum). In the IT group, ASO was observed mainly in the temporal to basal regions of brain (e.g. temporal to basal region of cortex, hypothalamus, basal midbrain and medulla oblongata) and in the layer V in parietal cortex (arrow heads).

2. Distribution of KD for *Malat-1* by ISH



Brown signal: *Malat-1 mRNA*

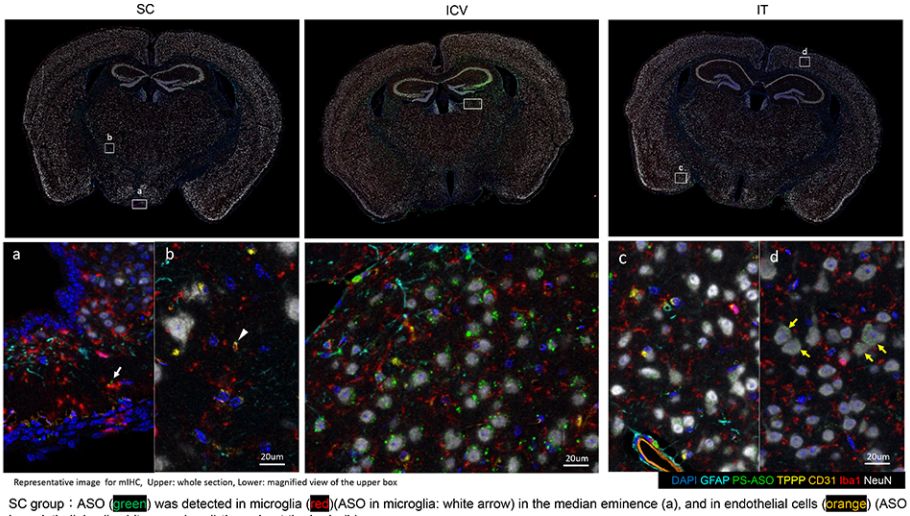
Table 2. Histological evaluation of KD for *Malat-1*

Region	SC（PBS）	SC(ASO)	ICV(ASO)	IT(ASO)
Brain				
Olfactory bulb	0	1	2	2
Cortex（Parietal）	0	1	3	0-1
Cortex (Temporal-Basal)	0	1	3	3
Striatum	0	1	3	1
Hippocampus	0	1	3	2
Amygdala	0	1	2	2
Thalamus	0	1	3	1
Hypothalamus	0	1	3	3
Midbrain (Substantia nigra)	0	1	3	3
Cerebellum	0	1	3	2
Medulla oblongata	0	1	2-3	2
Corpus callosum	0	1	4	1-2
Choroid plexus (Lateral-3rd ventricle)	0	2-3	1-2	1
Choroid plexus (4th ventricle)	0	2	1	1
Spinal cord				
Cervical	0	1	3	3
Thoracic	0	1	3	3
Lumber	0	0	3	3

0: KD was not observed, 1: KD was observed in a few cells, 2: Small foci of KD cells was observed, 3: Large number of cells showed KD, 4: KD was diffusely observed

KD of *Malat-1* was observed as a pale blue nucleus without positive signal for *Malat-1* by ISH. The distribution of KD correlated with the biodistribution of ASO. However, KD was rarely observed in the layer V of parietal cortex in the IT group.

Evaluation of cell-type distribution by mIHC



Representative image for mIHC, Upper: whole section, Lower: magnified view of the upper box

SC group：ASO (green) was detected in microglia (red)(ASO in microglia: white arrow) in the median eminence (a), and in endothelial cells (orange) (ASO in endothelial cell: white arrowhead) throughout the brain (b).
ICV group : ASO was detected in all cell types (neurons (white), astrocytes (cyan), oligodendrocytes (yellow), microglia) throughout the brain.
IT group : ASO was detected in all cell types in the temporal to basal area (c). In the layer V of the parietal cortex (d), ASO was observed almost exclusively in neurons (yellow arrows).

Conclusion

Our results indicated that it was difficult to distribute ASO across Blood-Brain Barrier by SC administration in this study, and ASO was taken up diffusely by all cell types by ICV and IT administration. In addition, it was speculated that ASO by IT administration was delivered by retrograde transport from motor neurons in the ventral horn of the lumber spinal cord to neurons in layer V of the parietal. Our results also suggested that the new panel of mIHC is very useful for detecting differences in cell types depending on the administration method when ASO is administered to the CNS.