Press release

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Press Release

Keio University
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The Characteristics of Protein Profiles in Extracellular Vesicles Derived from ALS Patients' Body Fluids and the Changes Caused by Ropinirole Hydrochloride Treatment

-Exploring and Unraveling the Mechanisms of ALS Pathogenesis and Treatment-

A research group has conducted comprehensive longitudinal analysis of the protein profiles of extracellular vesicles (EVs)[5] in the blood and cerebrospinal fluid (CSF)[4] of amyotrophic lateral sclerosis (ALS)[3] patients who participated in the Ropinirole Hydrochloride Remedy for Amyotrophic Lateral Sclerosis (ROPALS)[2] trial for the drug ropinirole hydrochloride (ROPI).[1] The research team included Professor Hideyuki Okano (now director of the Keio Regenerative Medicine Research Center) and Senior Lecturer Satoru Morimoto (now Associate Professor at the Keio Frontier Research and Education Collaborative Square at Tonomachi) from the Department of Physiology at the Keio University of Medicine; fifth-year Keio medical student Kusuri Kato; and Koji Ueda, Project Leader at the Cancer Precision Medicine Center of the Japanese Foundation for Cancer Research. Their analysis found that the protein composition of EVs from body fluids in sporadic ALS (SALS) patients differs from that of healthy individuals, and that these changes occur in conjunction with the progression of SALS. Moreover, they found that ROPI appeared to suppress these changes. Additionally, research using astrocytes (iPasts)[7] derived from induced pluripotent stem cells (iPSCs)[6] suggested that ROPI might activate the D2R-CRYAB pathway,[8] which suppresses neuroinflammation. Furthermore, researchers identified protein groups that could serve as useful biomarkers for ALS prognosis prediction and diagnosis using machine learning models.[9]

EVs are secreted into bodily fluids like blood and CSF by most kinds of cells and contain proteins and nucleic acids, leading scientists to posit that they are involved in intercellular communication. Scientists also believe that EVs are involved in the pathogenesis of malignant tumors and neurodegenerative diseases, including SALS, pointing to their potential as biomarkers. However, scientists had yet to conduct comprehensive longitudinal analysis of EV protein composition in SALS, or fully grasp the clinically useful biomarkers for prognosis prediction. Moreover, while findings from the ROPALS trial suggested that ROPI suppressed disease, little was understood about the changes it induced in EV protein composition. In this study, collaborators examined the ROPALS trial via reverse translational research (rTR)[10], comprehensively investigating the protein profiles of EVs using samples collected over time from patients to clarify these aspects.

The findings of this study shed light on an element of the pathogenesis of SALS and the mechanisms by which ROPI exerts therapeutic effects, providing important insights into understanding the disease and developing treatment strategies for SALS. Research findings were published in the official international journal of the Japanese Society of Inflammation and Regeneration, *Inflammation and Regeneration*, at 7:00 PM JST on July 12, 2024.

1. Main Points of Research

- Researchers conducted comprehensive proteomic analysis of the EV protein composition using blood and CSF samples taken at fixed points from healthy individuals as well as over time from SALS patients.
- The profiles of EV proteins taken from SALS patients contained more proteins related to inflammation and fewer proteins involved in protein quality control mechanisms compared to healthy individuals.
- The longitudinal changes in EV protein composition in SALS patients were generally consistent with the changes observed when comparing healthy individuals to SALS patients.
- ROPI administration suppressed the changes observed in the comparison between healthy individuals and SALS patients and the longitudinal changes in SALS patients.
- Research using iPasts suggested that ROPI might activate the D2R-CRYAB pathway, which is crucial in suppressing neuroinflammation, leading to changes in the EV protein composition.
- Machine learning models identified protein groups presumed to be useful for ALS prognosis prediction and diagnosis.

2. Background of Research

EVs, are secreted into body fluids like blood and CSF by most kinds of cells and contain proteins and nucleic acids, leading scientists to posit that they are involved in intercellular communication. Experts also believe that EVs are involved in the pathogenesis of malignant tumors and neurodegenerative diseases, including SALS, pointing to their potential as biomarkers. However, scientists had yet to fully understand the comprehensive longitudinal EV protein composition in SALS.

ROPI, a dopamine D2 receptor (D2R) agonist[11] used as a treatment for Parkinson's disease, was identified as a potential candidate for treating ALS through a drug screening[13] conducted by the Keio University School of Medicine (Fujimori, K., et al., *Nat Med,* 2018) of 1,232 existing drugs using lower motor neurons[12] derived from ALS patients' iPSCs. The ROPALS trial, a Phase I/IIa investigator-initiated clinical trial involving 20 SALS patients, was conducted in collaboration with the Department of Neurology at Keio University School of Medicine. The trial's outcomes suggested that ROPI significantly suppressed disease progression in SALS patients, indicating its potential as an effective ALS treatment (Morimoto, S., et al., *Cell Stem Cell,* 2023).

In this study, the collaborators aimed to investigate the role of EVs in SALS onset and progression, understand the pathogenesis of SALS, and elucidate the mechanism of action of ROPI by conducting a comprehensive analysis of EV protein composition using blood and CSF samples collected over time from 20 SALS patients who participated in the ROPALS trial.

3. Research Design and Findings

i. The composition of EV proteins derived from SALS patients differed from that of healthy individuals.

Uniform Manifold Approximation and Projection (UMAP)[15] analysis was performed on protein groups detected from all samples in blood-derived EVs (serum-EVs; sEVs)[14] and CSF-derived EVs (CSF-EVs; cEVs). The results showed that while there was a significant difference between

sEVs and cEVs, the distribution between healthy individuals and SALS patients differed within the groups of sEVs and cEVs, respectively (A in Figure 1).

Next, researchers compared the protein composition of sEVs and cEVs between healthy individuals and SALS patients who had not been treated with ROPI (B in Figure 1). The proteins that increased in SALS patients' groups in both sEVs and cEVs were associated with complement coagulation pathways, while the proteins that decreased were related to actin polymerization and protein quality control mechanisms (C in Figure 1).

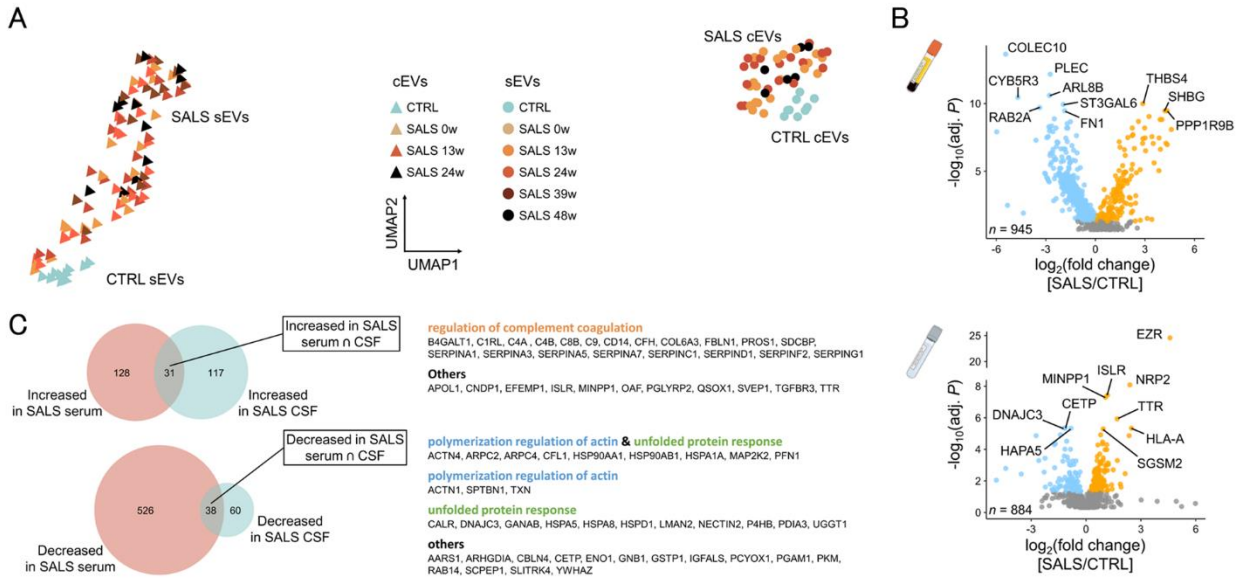


Figure 1. Comparing profiles of EV proteins derived from healthy individuals and SALS patients.

A. Scatter plot showing the results of UMAP analysis.
B. Comparative results of sEVs (upper plot) and cEVs (lower plot) between healthy individuals and SALS patients. Each plot represents a protein, with the vertical axis representing the -$\log_{10}$ transformed adjusted P-value, and the horizontal axis representing the $\log_2$ transformed fold change. EVs derived from serum and CSF are distinguished in subsequent figures using illustrations of collection tubes.
C. Venn diagram comparing the protein groups that increased or decreased in SALS patient-derived EVs in both sEVs and cEVs (left) and a list of protein groups included in each protein category (right).

ii. The profile of EV proteins in SALS patients changed over time, and these changes were suppressed by ROPI administration.

In the ROPALS trial, 13 patients were assigned to the ROPI group, and 7 patients were assigned to the placebo group. In this analysis, data from the placebo group were used to investigate the changes over time in EV protein composition. A clustering analysis was performed on the differential changes in proteins within EVs between week zero and each sampling point in the placebo group. The proteins were categorized into three groups as shown

in A and B in Figure 2: “increased over time” (red), “decreased over time” (blue), and “relatively unchanged over time” (gray).

Next, the changes in these protein groups were examined in the experimental ROPI group (A and B in Figure 2). The proteins that either increased or decreased over time in the placebo group saw that change suppressed by ROPI administration (both P<0.001). On the other hand, the protein group categorized as “relatively unchanged over time” showed little change with ROPI administration (P=0.7154 for sEVs and P=0.4247 for cEVs).

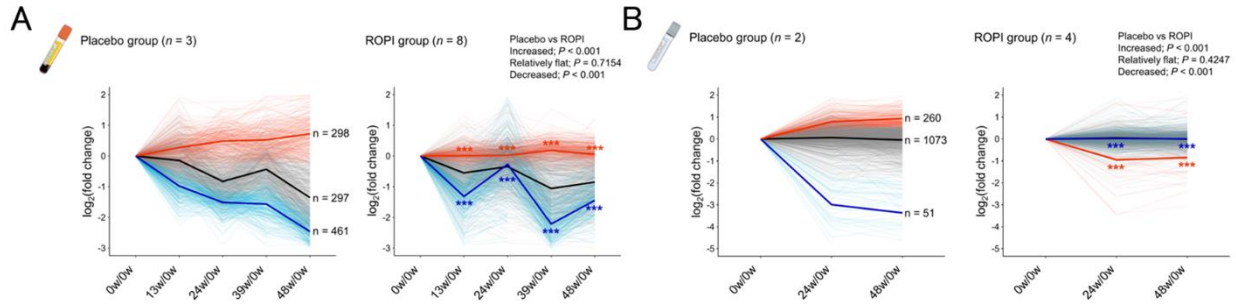


Figure 2. Longitudinal comparison of EV protein profiles derived from SALS patients

A, B. Graphs showing the increase or decrease of each protein in EVs. The thin lines indicate the transition of each individual protein, while thick lines indicate the average transition within each group. Based on the clustering analysis of temporal changes in the placebo group, all proteins were categorized into three groups as shown in Figure 2: “increased over time” (red), “decreased over time” (blue), and “relatively unchanged over time” (gray). The results are shown separately for the placebo group (left) and the ROPI group (right). ***; P<0.001.

iii. ROPI administration relatively and specifically suppressed the differences between healthy individuals and SALS patients, as well as the longitudinal changes in SALS patients.

To understand the changes in protein composition due to ROPI administration, researchers first compared the placebo group and the ROPI group. Subsequently, they performed a cosine similarity analysis[16] to compare the protein groups identified in the healthy vs. SALS patient comparison and the longitudinal analysis among SALS patients. The results showed that ROPI suppressed the protein groups that were increased in SALS patients compared to healthy individuals, or those that increased over time. Conversely, ROPI increased the protein groups that were decreased in SALS patients compared to healthy individuals, or those that decreased over time (A in Figure 3). Further functional analysis of these protein groups revealed that the proteins that increased in SALS patients and decreased with ROPI were related to inflammation, while the proteins that decreased in SALS patients and increased with ROPI were associated with the protein quality control mechanism (both P<0.05). In SALS, it is known that a protein called TDP-43 forms aggregates in the cytoplasm of motor neurons, and one proposed cause of this is the breakdown of the protein quality control mechanism (proteostasis disruption)[17]. Additionally, it has been reported that proteins involved in the protein quality control mechanism can be supplied to other cells via EVs and exert their effects in the recipient cells, contributing to the degradation of aggregated proteins (Takeuchi, T., et al., *Proc Natl Acad Sci U S A.,* 2015). Therefore, the decrease in proteins involved in the protein quality control

mechanism contained in EVs might lead to a reduced supply to motor neurons, potentially promoting TDP-43 aggregation and contributing to the onset and progression of SALS.
A scatter plot was created to examine the amounts of the protein groups that increased or decreased over time in SALS patients compared to healthy individuals in each sample (B in Figure 3). In the placebo group, the data distribution gradually diverged from that of healthy individuals over time (gray plots in B in Figure 3), but in the ROPI group, this divergence from the healthy data distribution was suppressed (red plots in B in Figure 3). Therefore, it was found that ROPI has a suppressive effect on changes in EV protein composition in SALS, suggesting its potential anti-ALS effects.

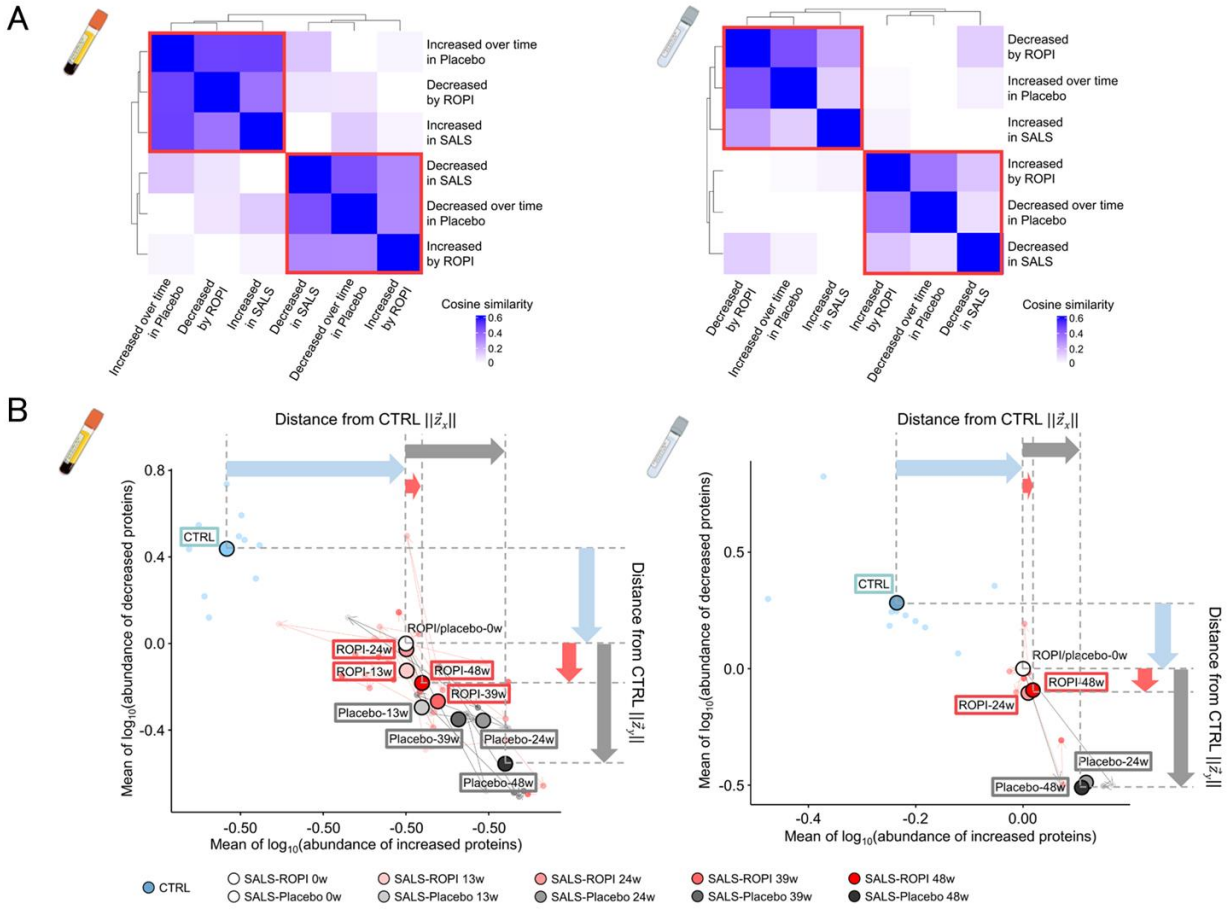


Figure 3. Comparison of healthy vs. SALS patients, longitudinal changes within SALS patients, and changes due to ROPI administration

A. A heatmap showing the results of cosine similarity analysis for each protein group, comparing healthy vs. SALS patients, longitudinal changes within SALS patients, and changes due to ROPI administration.
B. A scatter plot showing the amounts of protein groups that increased or decreased over time in SALS patients compared to healthy individuals. Each plot represents a sample, and the larger plots represent the average value for each group.

iv. Research using iPasts suggested that ROPI might activate the D2R-CRYAB pathway, which suppresses neuroinflammation.

iPasts derived from healthy individuals were created, cultured under ROPI-treated/untreated conditions, and subjected to RNA-seq[18] for comparison. The results showed that with iPasts, ROPI treatment significantly increased the expression of CRYAB and decreased the expression of CXCL14 and CCN2, which are involved in neuroinflammation (A in Figure 4). Since ROPI is a D2R agonist, it is suggested that ROPI may activate the D2R-CRYAB pathway, which is believed to contribute to the suppression of neuroinflammation downstream of D2R (B in Figure 4).

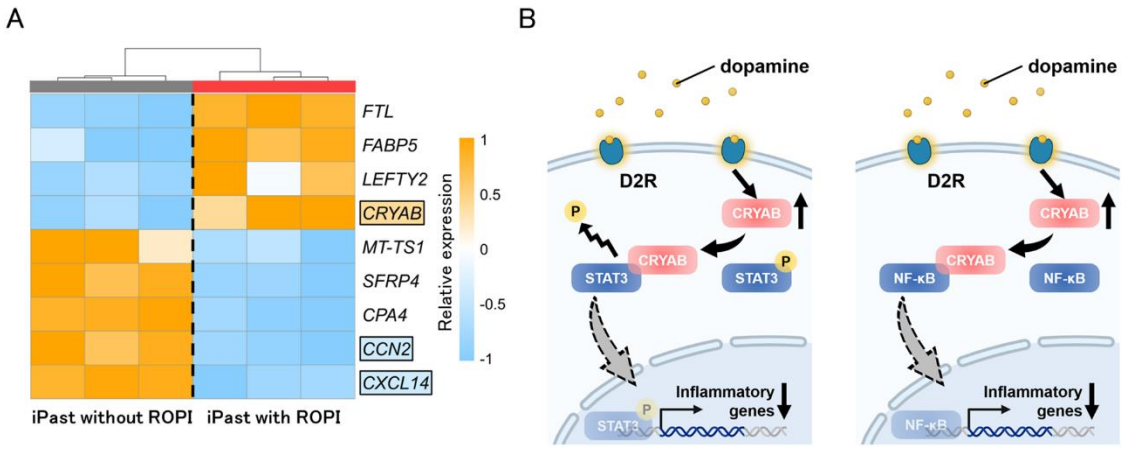


Figure 4. The potential for ROPI to activate the D2R-CRYAB pathway in iPasts

A. A heatmap showing the transcriptomic comparison between ROPI-treated and untreated conditions with iPasts.
B. A diagram illustrating the D2R-CRYAB pathway. CRYAB is thought to bind to STAT3 and NF-κB upon D2R stimulation, suppressing their nuclear translocation and thereby inhibiting the expression of inflammation-related genes, leading to the suppression of neuroinflammation (Qiu, J., et al., *Journal of Neuroinflammation, 2*016; Zhang, Y., et al., *Stroke,* 2015).

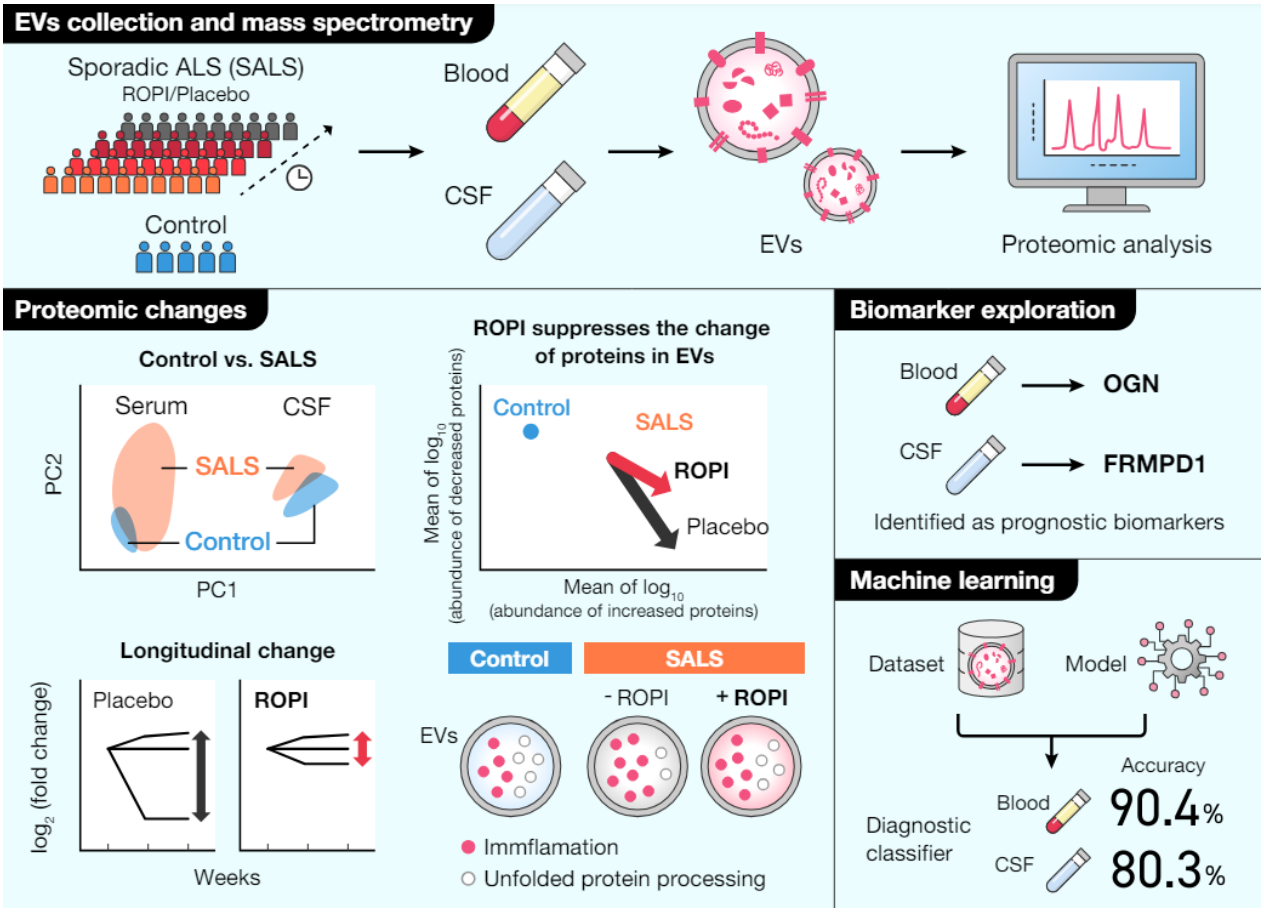
v. Machine learning model identifies protein groups useful for prognosis prediction.

Based on detailed clinical data collected in the ROPALS trial, researchers explored biomarkers for ALS clinical indicators using proteins within EVs. As a result, osteoglycin (OGN) in sEVs and FERM And PDZ Domain Containing 1 (FRMPD1) in cEVs at week 0 of the trial were identified as the most reflective prognosis prediction markers.

Additionally, biomarker exploration using a machine learning model selected 155 protein groups in sEVs, an accuracy of 90.4%, and 19 protein groups in cEVs, an accuracy of 80.3%.
Figure 5. Overview of the study outcomes

4. Future Developments

The findings of this study suggest that protein groups contained within EVs may contribute to the onset and progression of SALS and that these pathological changes may be suppressed by ROPI. In the future, the use of EVs for ALS treatment and the suppression of neuroinflammation or enhancement of proteostasis could potentially expand as viable ALS treatment strategies.



5. Notes

This research was made possible by the support of several grants from the Japan Agency for Medical Research and Development (AMED) and the Japan Society for the Promotion of Science (JSPS). Research was also supported by organizations, including the Yukihiko Miyata Memorial Trust for ALS Research, Japan Amyotrophic Lateral Sclerosis Association, Daiichi Sankyo Foundation of Life Science, and the UBE Foundation. Specific grant titles are available in Japanese in the special notes section at the bottom of the Japanese version of this press release.

A sincere thanks goes out to all the patients and their families who participated in the ROPALS trial.

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Details of Journal Article

Title: Proteomic Insights into Extracellular Vesicles in ALS for Therapeutic Potential of Ropinirole and Biomarker Discovery

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Glossary and References

[1] Ropinirole hydrochloride (ROPI): a drug that binds to dopamine D2 receptors and activates them. It is widely used as a treatment for Parkinson's disease, which is characterized by dopamine deficiency.

[2] ROPALS trial: a phase I/IIa investigator-initiated clinical trial for ROPI. It involved 20 patients with SALS, where an experimental group of 13 patients received ROPI and a control group of 7 received a placebo for 24 weeks, followed by an additional 24 weeks of ROPI administration to all participants for evaluation.

[3] Amyotrophic lateral sclerosis (ALS): a neurodegenerative disease abbreviated as "ALS." It is characterized by the selective degeneration and loss of motor neurons, leading to muscle weakness throughout the body. Without treatment, the prognosis is two to five years post-diagnosis due to the weakening of respiratory muscles.

[4] Cerebrospinal fluid (CSF): the fluid that surrounds the brain and spinal cord. In SALS, motor neurons that degenerate and die are located in the brain and spinal cord, making CSF prone to pathological changes.

[5] Extracellular vesicles (EVs): lipid bilayer-enclosed vesicles that circulate in body fluids. They contain proteins, nucleic acids, and lipids. They are secreted by almost all cells and transferred to other cells, playing a role in intercellular communication within organisms.

[6] Induced pluripotent stem cells (iPSCs): cells synthesized specifically by scientists that have pluripotency, meaning they can differentiate into almost all cell types in the body, and stemness, meaning they can proliferate indefinitely.

[7] Astrocytes: a type of cell in the brain and spinal cord that surrounds neurons. They are known to supply nutrients to neurons and maintain neuronal shape. In recent years, scientists have taken an increased interest in their role in neuroinflammation.

[8] D2R-CRYAB pathway: a downstream pathway of dopamine D2 receptors (D2R), important for suppressing neuroinflammation. It has been reported that CRYAB, when stimulated by D2R, binds to proteins like STAT3 and NF-κB in the cytoplasm, inhibiting their nuclear translocation, thereby suppressing the expression of chemokines involved in neuroinflammation (see Figure 4B).

[9] Machine learning model: a system that processes input data and makes judgments or evaluations accordingly.

[10] Reverse translational research (rTR): a research approach by which insights gained in clinical settings are used to advance basic research. It is considered a powerful driver for understanding diseases and developing treatments.

[11] Agonist: a drug that binds to a receptor and activates it.

[12] Lower motor neurons: motor neurons mainly located in the spinal cord. In humans, information is transmitted from upper motor neurons in the brain to lower motor neurons in the spinal cord, which then connect to muscles to relay movement commands from the brain to the muscles.

[13] Drug screening: the process in drug discovery research of selecting substances from existing drugs or new compounds that exhibit the desired pharmacological effects.

[14] Serum-EV: in this study, EVs were extracted from serum obtained from patient blood. To avoid confusing readers, “blood” is used in place of “serum” in this press release. Serum is the supernatant obtained by centrifuging blood.

[15] Uniform Manifold Approximation and Projection (UMAP): a dimension reduction technique that projects high-dimensional data (in this case protein composition) into low dimensions (2D in this study). Data that are similar in high dimensions are plotted close together in low dimensions.

[16] Cosine similarity analysis: a method using cosine similarity, a measure of the similarity between two vectors. A value of 1 indicates perfect agreement between the two vectors (in this study, protein groups), while a value of -1 indicates complete disagreement.

[17] Proteostasis: Homeostasis in proteins, or the maintenance of the stability and functionality of proteins within and outside cells.

[18] RNA-seq: The abbreviation for ribonucleic acid sequencing. RNA-seq is a method for comprehensively reading RNA sequences, which are produced from DNA and then translated into proteins. It allows for quantifying the expression levels or the abundance of various strands of RNA .

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