SHIMADZU Kumamoto University

WP 096

Exploring novel biomarkers of COVID-19 diagnosis and aggravation by comprehensive analysis of modified nucleosides, and development of automated analysis system

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1. Overview

Several modified nucleosides have been found to be useful for prediction of COVID-19 infection or prognosis. The fully automated analytical system for modified nucleosides in biological samples was developed .

2. Introduction

COVID-19 is now having an explosive epidemic and social impact around the world. The diagnosis of COVID-19 is mainly made by PCR, but there are problems such as the time required for the determination, the risk of infection in the specimen, and the inability to determine the severity of the disease.

Ribonucleic acid (RNA) is an important molecule in protein synthesis, and it has come to be known that some RNA is modified by enzymes (Figure 1). Such modified RNA is degraded in the cytosol, and the original nucleobases are recycled. The modified portions are then secreted from the cytosol and discharged from the body as urine or blood (Figure 2). In recent years, the measurement of modified nucleosides in urine has come to be regarded as extremely important in the research of various infectious diseases.

In previous studies, we searched for biomarkers of various diseases and infections by measuring modified nucleosides in serum and urine (low risk of infection) with LC-MS/MS. In this study, we discovered novel biomarkers for diagnosis and assessment of severity by comprehensive analysis of modified nucleosides in the serum and urine of patients with COVID-19. In addition, we developed a fully automated system that could rapidly analyze them.

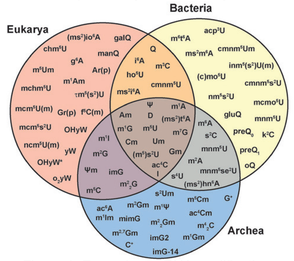

Figure 1. Examples of RNA modification.

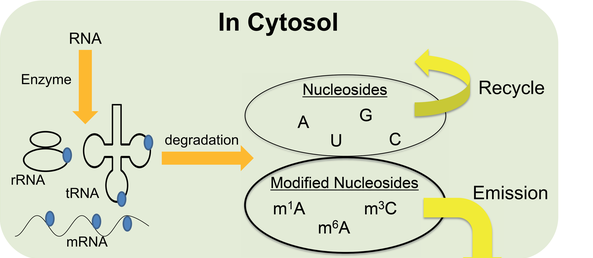

Figure 2. Intracellular metabolism of RNA.

3. Methods

3-1. Evaluation of modified nucleosides contained in COVID-19 infected cells

SARS-CoV-2 infected cells were prepared and the modified nucleosides contained within the cells were evaluated. The modified nucleosides in the serum and urine of COVID-19 patients and healthy individuals were then analyzed using LC-MS/MS to determine specific biomarkers.

Figure 3. Evaluation of COVID-19 infected cells.

3-2. Investigation of the relationship with disease prognosis by measuring modified nucleosides

The relationship between modified nucleosides and COVID-19 was investigated using the serum of the patients and healthy subjects. In addition, we measured the modified nucleosides in the serum of patients after positive PCR tests and investigated their association with subsequent disease.

Analytical Conditions

UHPLC conditions (Nexera™ X2)

Column:	ODS Column
Mobile phase:	A: Formic acid /Water
	B: Formic acid /Acetonitrile
Flow rate:	0.3 mL/min
LC Mode:	Gradient mode
Injection vol.:	2 μL
Column temp.:	40°C

MS conditions (LCMS™-8050)

Ionization:	ESI Positive
DL temp.:	250 °C
HB temp.:	400 °C
Interface temp.:	300 °C
Nebulizing gas:	3.0 L/min
Drying gas:	10 L/min
Heating gas:	10 L/min

3-3. Development of automated analysis system

We attempted to simplify and automate the extraction of modified nucleosides using a pretreatment apparatus (CLAM-2030). In addition, the analysis conditions in LC-MS/MS were optimized to improve the speed.

Figure 4. Automated analysis system for modified nucleosides in serum and blood.

4. Results

4-1. Relationship between COVID-19 and modified nucleosides

As a result of LC/MS/MS evaluation of modified nucleosides in coronavirus-infected cells and healthy control cells, there were significant differences in the amounts of two modified adenosine in serum and urine between healthy individual and COVID-19 patients (Figure 5). Sensitivity and specificity were similar to those of PCR.

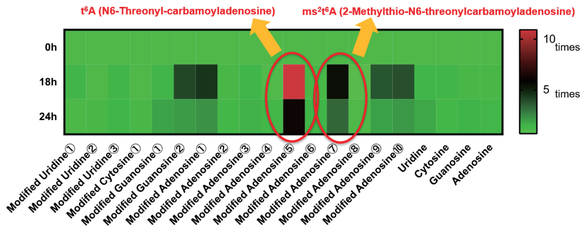

Figure 5. Ratio of modified nucleoside abundance in coronavirus-infected individuals to healthy individuals.

4-2. Aggravation prediction of COVID-19 patients

As a result of analyzing the serum of COVID-19 patients and healthy subjects, as shown in Figure 6, the amounts of t^6A and ms^2t^6A were low in healthy subjects, while they were higher in infected subjects. Similar results were obtained with the mutant strain.

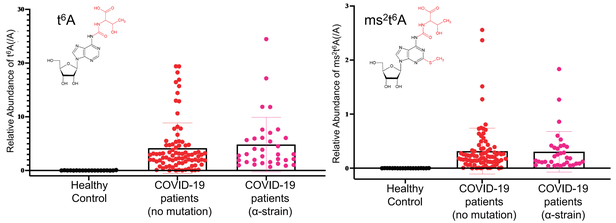

Figure 6. Results of serum analysis of COVID-19 patients and healthy subjects.

As a result of measuring the amount of ms^2t^6A in the serum of patients after a positive result of PCR test and examining the relation with the subsequent disease state, it was found that patients with high level of ms^2t^6A showed rapid changes afterwards even if they were mild at the time of measurement. The results suggest that it may be possible to predict whether symptoms will be severe before they appear. Therefore, this technique is considered to assist in the selection of treatment method and treatment location after PCR positive judgment.

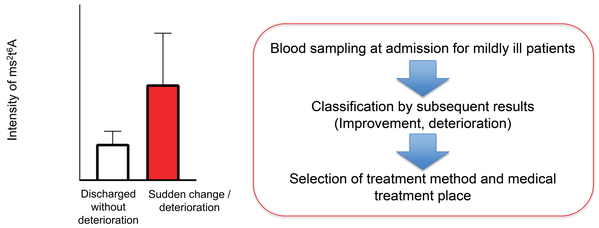

Figure 7. Relationship between prognosis of mildly ill patients and ms^2t^6A.

4-3. Evaluation of automated analysis system

In order to analyze serum by LC/MS/MS, pretreatment such as deproteinization, extraction of target components, and dilution is required. These operations take about 30 minutes manually, but by optimizing with CLAM-2030, it can be processed automatically in about 6 minutes. In addition, by optimizing the analysis conditions after pretreatment, the analysis time was reduced to 5 minutes.

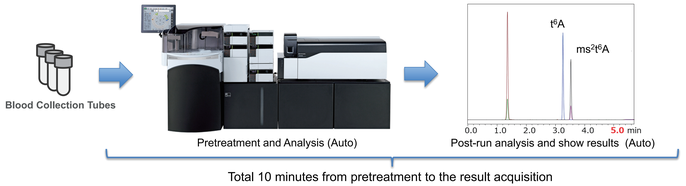

Figure 8. Fully automated analysis system for modified nucleosides.

The validation results such as linearity, reproducibility, and recovery rate using this system were good (Table 1). Furthermore, in various evaluations using serum of COVID-19 patients and healthy subjects, the same results as before automation were obtained (Figure 9). Therefore, we have succeeded in constructing a fully automatic modified nucleosides analysis system from pretreatment to analysis.

Table 1. Validation results of automated system.

	Results
linearity	・R^2 > 0.999 in the range 0.1 nM -100 nM ・Calibration point accuracy: within ± 25% at 0.1 nM; within ± 10% for others
reproducibility	・Retention time reproducibility: t^6A, ms^2t^6A% CV < 0.1, Adenosine %CV < 1 ・Area Reproducibility:% CV < 5
recovery rate	・Serum recovery within ± 25%,% CV < 6

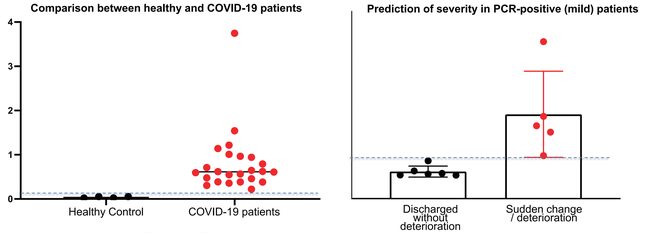

Figure 9. Results of serum analysis with automated system.

5. Conclusions

- We demonstrated that two modified nucleosides in the serum of COVID-19 patients are different from those of healthy individuals and that they are related to the aggravation of COVID-19.
- We have developed modified nucleosides analysis system, which enables fully automatic and rapid processing from pretreatment to analysis.

⇒ This system may assist in the selection of treatment method and treatment location for COVID-19 patients.

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