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Original Article

Analysis of the Correlation between Cholesterol Levels in Blood Using Clinical Data and Hair Using Mass Spectrometry Imaging

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Mass spectrometry imaging (MSI) is a technique that visualizes the distribution of molecules by ionizing the components on the surface of a sample and directly detecting them. Previously, MSI using hair has primarily been used in the forensic field to detect illegal drugs. On the other hand, there are few examples of using this technology for health monitoring. In this study, hair and clinical data were collected from 24 subjects, and the correlation between blood cholesterol levels and cholesterol detected from cross-sectional hair slices was analyzed. As a result, a positive correlation with a correlation coefficient of 0.43 was observed between blood cholesterol and cholesterol detected from hair. Furthermore, when comparing the results of fluorescence staining (FS) of hair cholesterol with Filipin III and the MSI results, it was found that while FS could visualize detailed hair structures, there were cases where the results differed from MSI, possibly due to some cholesterol loss during the staining process. In the future, if various disease biomarkers can be detected using hair MSI, it could potentially become a non-invasive diagnostic method.

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INTRODUCTION

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is a visualization technique for surface components of samples and is an effective method for visualizing biological phenomena and dynamics. To date, samples such as animal tissues, human clinical samples, plants, food, and insects have been used. The greatest advantage of MSI is that, unlike traditional mass spectrometry methods such as liquid chromatography-mass spectrometry (LC-MS), there is no need to homogenize the sample, allowing one to see "where" the target molecule is located. However, measuring samples such as blood and urine is challenging, even for biological samples. Nevertheless, previous studies have reported that hair is effective as a substitute for blood, and research using hair as an alternative sample to blood has been increasing.[1)] Blood and urine are commonly used clinical samples for early disease detection, diagnosis, and confirmation of therapeutic effects and are considered important samples that reflect the overall health condition.[2–5)] Blood is taken up by hair matrix cells through capillaries in the scalp, and hair grows outward from the scalp. In other words, blood components are thought to accumulate in hair from the root to the tip over time. With MSI, it is possible to observe the temporal changes in blood components by measuring a single strand of hair. While drug identification research using hair has been mainly conducted by forensic research institutes,[6–19)] recent research on hair samples has been actively conducted to measure bioactive substances[20,21)] and toxic components,[22–24)] biomarker exploration,[25–33)] and pharmacokinetic analysis.[20,34)]

In this paper, we focused on cholesterol,[35)] one of the causes of lifestyle-related diseases, and analyzed the correlation between total cholesterol (TC) value in the blood of 24 subjects and the intensity of cholesterol detected from hair using MALDI-MSI. As a result, a positive correlation with a correlation coefficient of 0.43 was observed between blood TC values and the intensity of cholesterol detected from hair. In addition, we attempted to visualize hair cholesterol

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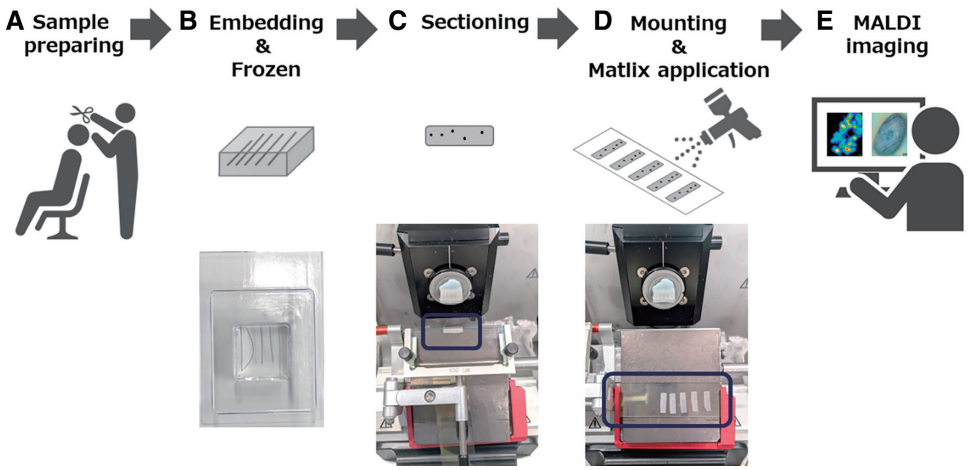

Fig. 1. Workflow of MALDI-MSI analysis of cholesterol in hair. (A) Hair was collected from the occipital region of the subjects. (B) The hair, cut to 10 mm from the root, was embedded in a cryomold filled with 3% CMC and completely frozen at −80°C. (C) Cryosections with a thickness of 12 μm were prepared using a microtome. (D) The prepared cryosections were attached to ITO glass and supplied with the matrix. (E) MALDI-MSI measurements were performed. CMC, carboxymethyl cellulose; ITO, indium-tin-oxide; MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging.

using fluorescence staining (FS) with Filipin III. While it was possible to visualize the detailed structure of hair, it was also found that some cholesterol might be lost during the washing process, leading to different results from MSI. In other words, it was difficult to find a correlation with blood concentration using FS. The method proposed in this paper using MALDI-MSI can indicate the relationship between blood components and the intensity values of hair components, suggesting the possibility of developing a non-invasive testing method using hair by advancing research on the influence of hair characteristics, reproducibility, and quantitativeness.[36)]

MATERIALS AND METHODS

Chemicals and reagents

α-Cyano-4-hydroxycinnamic acid (CHCA, C8982) and 2,5-dihydroxybenzoic acid (DHB, 98% purity, 149357) were purchased from Merck (Darmstadt, Germany). Cholesterol (034-03002) for use as standard and carboxymethyl cellulose sodium salt (CMC, O001) were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All solvents used in this experiment were of LC-MS grade and purchased from Fujifilm Wako Pure Chemical Industries, Ltd.

Hair samples

The workflow for hair collection to analysis is shown in Fig. 1. Hair samples were collected from the occipital region of 24 men and women aged 20 and over (17 men and 7 women) and the 0–10 mm segment from the root was used (Fig. 1A). The range of blood TC values of the subjects was 138–238 mg/dL, consisting of 1 subject with low TC (<140 mg/dL), 4 subjects with high TC (>219 mg/dL), and 19 subjects with intermediate TC (141–218 mg/dL). After collecting the hair sections for MSI analysis, the remaining hair sample was then washed with 5% *n*-Octyl-β-D-glucoside (340-05031; DOJINDO, Kumamoto, Japan) at 40°C for 30 min, followed by ion-exchanged water at 40°C for 30 min to remove an embedding media. To avoid ion suppression, we selected *n*-Octyl-β-D-glucoside as a non-ionic detergent. This study was approved by the ethics review board to collect hair samples (approval number: TB-RER-2101).

Tissue sectioning and mounting

The collected five hair samples were placed in a Cryomold No. 2 (Sakura Finetek, Tokyo, Japan) filled with 3% CMC and frozen at −80°C to prepare the frozen block (Fig. 1B). An optimal cutting temperature (OCT) compound was used to fix each tissue block on a holder. The frozen tissue block was placed on the surface of the OCT compound and stored at −80°C for 5 min. Frozen 12-μm sections were sliced at −20°C with a microtome (Leica CM 1950, Leica Microsystems, GmbH, Nussloch, Germany) (Fig. 1C). The frozen sections were thaw-mounted on an indium-tin-oxide (ITO) coated glass slide (SI0100N; Matsunami, Osaka, Japan) and allowed to dehydrate in a 50-mL conical tube containing silica gel. The glass slides were stored at room temperature in the tube until matrix application.

Matrix application

CHCA was deposited at 250°C with a thickness of 0.7 μm on each ITO glass loaded with hair sections using a vacuum deposition system (iMLayer; Shimadzu Corporation, Kyoto, Japan) (Fig. 1D). This process deposited nanometer-sized matrix crystals.

MALDI-MSI analysis

MALDI-MSI analysis was performed on iMScope TRIO (Shimadzu, Kyoto, Japan) equipped with a 1-kHz Nd: YAG laser (λ = 355 nm). The laser spot size was approximately 10 μm, and each pixel was irradiated 20 times at a repetition rate of 1 kHz. For *m/z* calibration, DHB was used. All MSI images were acquired with a 3 μm pitch. Mass spectra were acquired in the *m/z* range of 364–374 in the positive ion detection mode for cholesterol, and the target *m/z* 369.35 was obtained. We compared product ion mass spectra obtained from the cholesterol standard sample and hair sample. We could confirm the *m/z* 369.35 was derived from cholesterol. After measurement, the selected ion images were obtained using the analysis software IMAGEREVEAL (Shimadzu, Kyoto, Japan) (Fig. 1E).

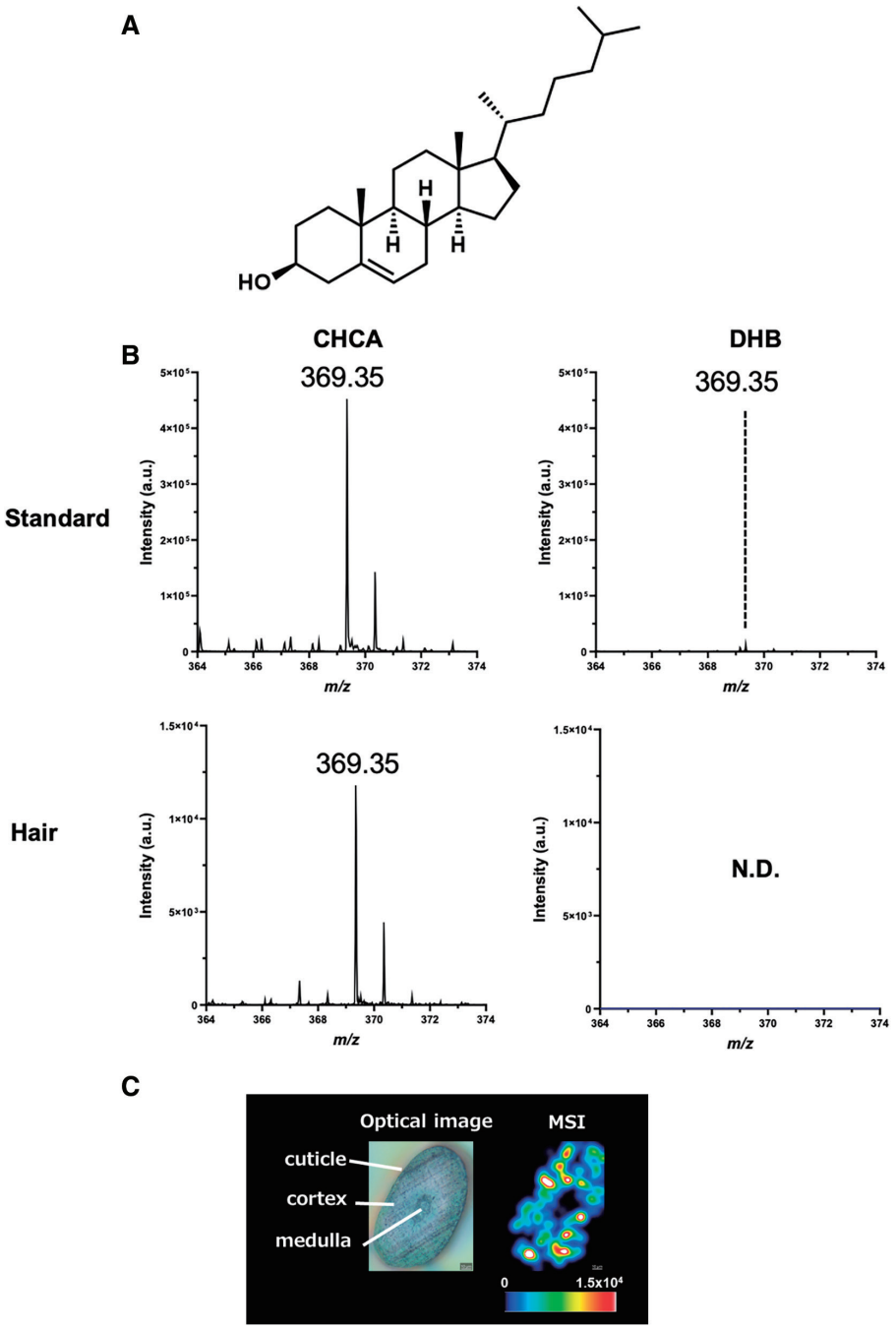

Fig. 2. Selection of matrices. The peak intensities of cholesterol measured after the deposition of CHCA or DHB were compared. (A) Structure of cholesterol. Cholesterol is detected at *m/z* 369.35 ($[M-H_2O+H]^+$). (B) In the standard solution (0.1 mg/mL), the peak intensity of cholesterol was higher with CHCA than with DHB. In the hair sample, the cholesterol peak was detected with CHCA but not with DHB. (C) Typical optical and imaging images of cholesterol analyzed from the mass spectrum of a hair sample using CHCA as the matrix from (B). Hair consists of three layers called the cuticle, cortex, and medulla, from the outside to the inside. Microscope magnification ×40, scale bar 10 μm. CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid.

Cholesterol FS

The hair cross-sections of 16-μm thickness were prepared under freezing conditions using a sliding microtome (SM2000R; Leica Microsystems, GmbH, Nussloch, Germany). Cholesterol staining of each hair section was then performed using the Cholesterol Cell-Based Detection Assay Kit (10009779; Cayman Chemical, MI, USA). After staining, sections were observed with a fluorescence microscope (BZ-X800; Keyence, Osaka, Japan) with DAPI-V filters (EX 395/25, DM 425, BA 460/50).

Statistical analysis

For comparison with blood test results, five hair samples and clinical data per person were collected. A region of interest (ROI) analysis was performed using IMAGEREVEAL to calculate the mean intensity of cholesterol values in the hair. The values were compared with the blood test results for TC (mg/dL).

The obtained raw data were converted to IMDX format using IMDX Converter. The converted IMDX files were processed using IMAGEREVEAL. Mass spectra were extracted

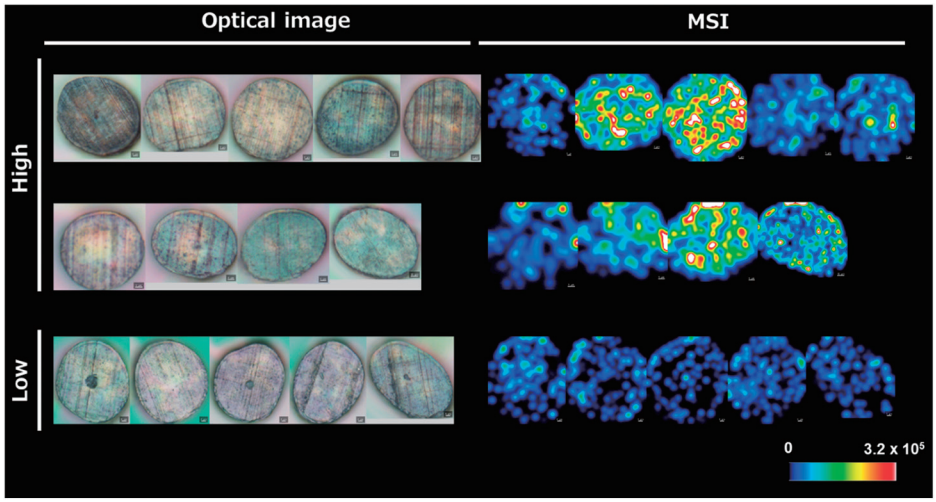

Fig. 3. MSI results by differences in blood TC levels. Cross-sections were prepared from 4 to 5 hairs collected from two subjects with high blood TC levels (>219 mg/dL), and one subject with low blood TC levels (<140 mg/dL), and MSI was performed. The hair from subjects with high blood TC levels showed a tendency for higher cholesterol intensity compared to the hair from subjects with low blood TC levels. MSI, mass spectrometry imaging; TC, total cholesterol.

from the ROI set on the section of total ion current (TIC) images, and data normalization (TIC normalization) was performed on all ROIs to obtain mass spectra independent of TIC and technical bias. From this spectrum, cholesterol (*m/z* 356.35) was selected and MS images were obtained. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., CA, USA), and the correlation coefficient between the average ion intensity value of hair cholesterol in ROI (target ion intensity of the entire ROI/number of measurement points) and the blood TC value was calculated.

RESULTS AND DISCUSSION

Matrix selection and cholesterol imaging of cross-sectioned hair

Cholesterol has a structure with a carbonyl group on the side chain of the steroid skeleton (Fig. 2A). Compounds with carbonyl groups are often detected as positive ions; therefore, the ionization was investigated using CHCA and DHB, which are commonly used matrices for positive ion mode measurements by MALDI. In MALDI-MSI, optimizing ionization conditions is a crucial step, especially for samples like hair, which are expected to contain very low amounts of the target component. Figure 2B shows the mass spectra obtained from standard solutions and cross-sectional hair slices using CHCA and DHB. From these spectra, it was found that cholesterol is detected at $[M-H_2O+H]^+$ = 369.35. In the standard solution, the cholesterol peak was detected with both matrices, but the peak intensity was significantly lower with DHB compared to CHCA. Furthermore, when DHB was used on cross-sectional hair slices, the cholesterol peak could not be detected. This was thought to be due to the inherently low ionization efficiency of DHB and the impact of the biological matrix (ion suppression). To validate this result, we applied product ion mass spectrometry of *m/z* 369.35 (Fig. S1). From the product ion mass spectra obtained from the cholesterol standard and *m/z* 369.35 on the hair surface, we could confirm that the *m/z* 369.35 was derived from cholesterol. On the other hand, when CHCA was used, the cholesterol peak could be detected even on cross-sectional hair slices, allowing the visualization of cholesterol distribution within the hair (Fig. 2C). It was also found that cholesterol is more abundant in the cuticle and cortex compared to the medulla. From the results of the ionization study, it was concluded that CHCA is preferable for detecting cholesterol. Interestingly, it was also found that detecting cholesterol in hair requires vacuum deposition of CHCA rather than applying the matrix solution (data not shown).

Correlation between blood TC values and hair cholesterol intensity values measured by MSI

To confirm whether there is a relationship between blood TC values and the peak intensity values of cholesterol obtained by MSI, cross-sectional slices were prepared from 4 to 5 hairs collected from two subjects with high blood TC values (>219 mg/dL) and one subject with low blood TC values (<140 mg/dL), and MSI was performed. As a result, cholesterol was detected in all subjects, but in subjects with high blood TC values, regions with higher cholesterol peak intensity values were observed compared to subjects with low blood TC values (Fig. 3). These results suggested the possibility of a correlation between blood TC values and hair cholesterol peak intensity values. In addition, even among subjects with high blood TC values, there was variability in cholesterol peak intensity values. This was considered to be due to the very limited area of the hair section being analyzed and differences in the transfer amount of blood components in the collected hairs. Therefore, multiple measurements from the same subject are necessary to obtain reproducible data due to variability in the measured hair and the measured area.

Subsequently, the correlation between blood TC values and hair cholesterol intensity was investigated in 24 subjects. The subjects were 24 men and women aged 20 and over (7 aged 20–29, 13 aged 30–39, 2 aged 40–49, and 2 aged 50 and over), with blood TC values ranging from 138 to 283 mg/dL (17 men and 7 women). When blood TC values were categorized as low (<140 mg/dL), intermediate (141–218 mg/dL), and high (>219 mg/dL), there was 1 subject with low values, 19

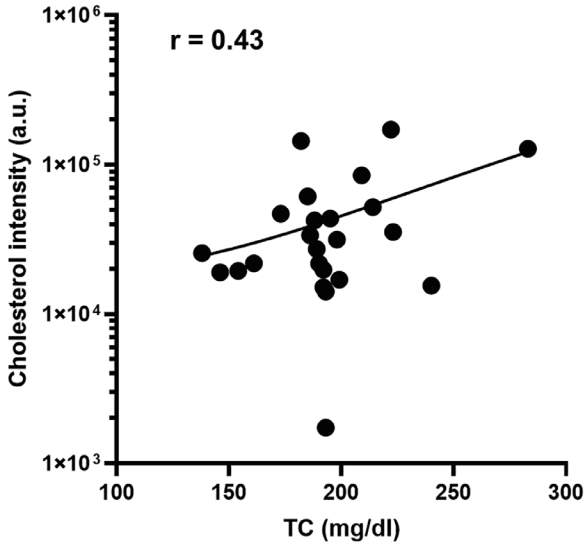

Fig. 4. Correlation between blood TC levels and hair cholesterol intensity. Scatter plot and regression line comparing blood TC levels and hair cholesterol intensity values. The correlation between TC and hair cholesterol intensity values showed a positive correlation with $r = 0.43$. $n = 24$. TC, total cholesterol.

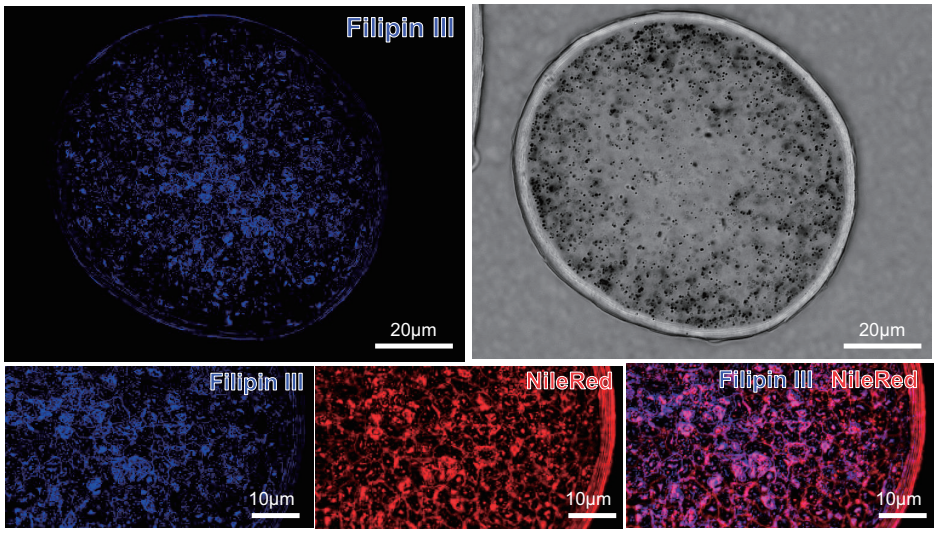

Fig. 5. Fluorescence staining of hair cholesterol with Filipin III. In hair sections, Filipin III fluorescence was mainly detected in the cortex (upper image). The combination of Filipin III and Nile Red staining, which visualizes hydrophobic regions such as the cell membrane complex, showed that cholesterol is distributed in the cell membrane complex and granular forms.

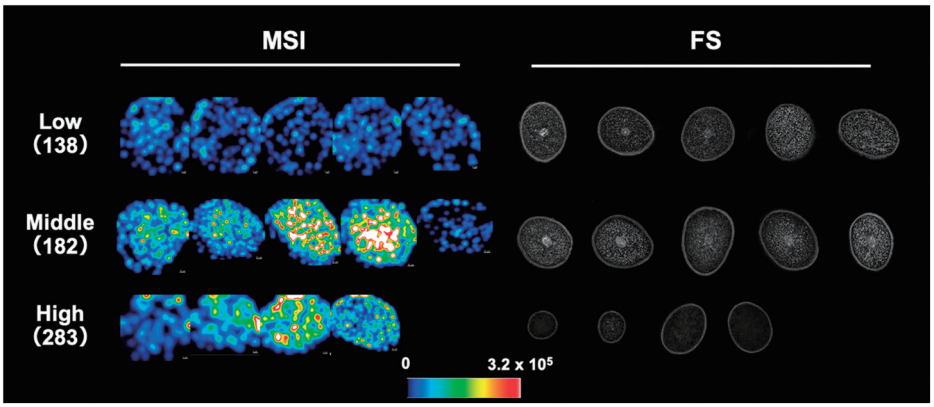

Fig. 6. Comparison of hair cholesterol MSI and FS. Cross-sections were prepared from 4 to 5 hairs of three subjects with low (<139 mg/dL), high (≥219 mg/dL), and intermediate (182 mg/dL) blood TC levels, and both MSI and FS were performed on the same hair. While FS barely detected cholesterol, it could visualize detailed structures of the hair. MSI showed differences in detection intensity among low, intermediate, and high values, and images corresponding to blood TC levels were obtained. (actual blood TC values are shown in parentheses.) FS, fluorescence staining; MSI, mass spectrometry imaging; TC, total cholesterol.

with intermediate values, and 4 with high values. The correlation coefficient between blood TC values and hair cholesterol intensity values obtained by MSI was 0.43, indicating a positive correlation (Fig. 4). This suggests that the ion intensity of cholesterol in hair reflects the blood TC values.

Comparison of cholesterol intensity and distribution between MSI and cholesterol FS

In tissue staining, the distribution of cholesterol can be visualized by FS using Filipin III, and there have been reports on cholesterol distribution in hair roots.[37] Using Filipin III, the distribution of cholesterol in the hair shaft was visualized in this study (Fig. 5, Upper panel), with granular and complex fluorescence of Filipin III observed, particularly in the cell membrane complex of the cortex (Fig. 5, Lower panel).

Next, FS was performed using consecutive sections prepared from the hair of 24 subjects measured by MSI. The results for samples with high, intermediate, and low TC values are shown in Fig. 6. Comparing the results of MSI and Filipin III in Fig. 6, it was found that while MSI showed a tendency for higher cholesterol intensity values in subjects with high blood TC values, Filipin III showed a tendency for lower fluorescence intensity (Fig. 6, upper panel). This difference suggests that the state of cholesterol within the hair may not be uniform. Cholesterol is said to be incorporated into the cell membrane complex between cortex cells within the hair.[38] However, it is possible that the ease of release of cholesterol differs within the cell membrane complex, or that cholesterol exists in other states, and that the washing process during section preparation led to the loss of cholesterol in tissue staining with Filipin III. On the other hand, in MSI, such cholesterol in different states can be detected without loss since the washing process is not involved, suggesting that MSI is useful for imaging specific components within the hair.

CONCLUSION

This study demonstrated a positive correlation between blood TC values obtained from clinical blood tests and hair cholesterol intensity values measured by MALDI-MSI. On the other hand, the variety of intensity was also confirmed. Even under the variety, the ability to indicate the relationship between blood component values and hair component ion intensity values using MALDI-MSI suggests the potential for developing a non-invasive testing method using hair by advancing research on the effects of hair characteristics, reproducibility, and quantitativeness. Furthermore, since the correlation was obtained in cross-section, it is expected that hair can be used as a sample for long-term health monitoring.

CONFLICT OF INTEREST

This study was designed and funded by Miruion Inc. and Takara Belmont Corp. Erika Nagano, Kazuki Odake, and Shuichi Shimma are employees of Miruion Inc. Tetsuya Mannari and Munekazu Kuge are employees of Takara Belmont Corp.

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