〈Research Article〉

# High-performance liquid chromatography-fluorescence detection of compounds containing keto and hydroxy groups in urine for cancer screening

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**Summary** The cancer-related mortality rate has increased with the rapid aging of society. Several biomarkers for the early detection of cancer using serum samples have been reported. Since urine samples are easy to collect, the development of a method to screen a lot of samples using widely available high-performance liquid chromatography devices has important practical implications. We examined the levels of the keto group-containing substances 2,3-butanedione and 2-butanone and the hydroxy group-containing substances 1-octanol and 2-butanol in urine samples from patients with cancer and healthy adults. We detected 2,3-butanedione and 2-butanone. In particular, there was a significant difference in the level of 2,3-butanedione between the cancer and healthy control groups. In particular, 2,3-butanedione was an effective marker of liver and colorectal cancer. Accordingly, low-molecular-weight compounds containing keto groups are promising for the development of a useful primary screening test.

**Key words:** Urine, High-performance liquid chromatography, Fluorescent label, Cancer screening

## 1. Introduction

In Japan, the cancer-related mortality rate has increased with the rapid aging of society, thereby increasing health expenditures. Several biomarkers for the early detection of cancer using serum samples have been developed. However, this approach requires blood sampling at medical institutions. Samples that can be readily collected at home, including saliva[1], expired air[2], and urine[3], can expand testing. Cancer biomarkers are currently being developed for expired air and urine. To analyze expired air, gas chromatography-mass spectrometry

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is utilized, but devices for this procedure are not routinely available in the clinical laboratories of hospitals. With respect to saliva, metabolome analyses are performed by two-dimensional electrophoresis or flight-time-type mass spectrometry, but few biomarkers are available. Therefore, these sample types are not appropriate for routine examinations. Urine samples are conventionally evaluated by diacetylspermine enzyme immunoassays[4] and high-performance liquid chromatography (HPLC) mass spectrometry[5]. Furthermore, urinary component screening using nematodes or dogs has been established[6]. However, these measurement methods require expensive mass spectrometers, special devices for observing nematode motions, or dog acclimation; they are not accepted as routine examination methods. Therefore, a measurement method for screening a lot of samples using HPLC devices, which are widely available in clinical laboratories, should be developed from the viewpoint of practical use.

Many low-molecular-weight biomarkers for cancer have been reported, including substances that can be easily measured by HPLC. Amino-[7], hydroxy-[8], aldehyde-, carboxyl-, hydroxy-, or keto-group[9] compounds are useful for the measurement of urinary components by fluorescence labeling. In this study, we focused on low-molecular-weight compounds containing hydroxy or keto groups, and performed reversed-phase chromatography with fluorescence labeling. We confirmed the presence of compounds containing keto groups among metabolites in urine from patients with cancer, supporting the utility of this new screening method.

## 2. Materials and Methods

Fluorescence labeling with 1,2-di-amino-4,5-methylenedioxybenzene dihydrochloride (MDB) for keto-group detection[10] For labeling, an MDB reagent (Dojin Chemistry, Tokyo, Japan), sodium dithionite ($Na_2S_2O_4$), and 2-mercaptoethanol were dissolved in ion-exchanged water and used as a labeling reagent. This MDB solution at 200 μL was added to 200 μL of the urine sample and mixed. The solution was then heated in a warm bath at 60°C for 2.5 hours and cooled with ice-cold water. After centrifugation at 3000 rpm for 10 minutes, 10 μL of the supernatant was injected into the HPLC for measurement.

Fluorescence labeling with 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (DMEQ-COCl) for hydroxy-group detection[11]

A DMEQ-COCl reagent (Dojin Chemistry) was dissolved in benzene and used as a labeling reagent. A urine sample of 200 μL was placed in a glass vial and mixed with 200 μL of the DMEQ-COCl solution. After the vial was sealed, it was heated at 100°C for 40 minutes and cooled with running water. After centrifugation at 3,000 rpm for 10 minutes, 990 μL of methanol was added to 10 μL of the supernatant for measurement. Then, 10 μL of the sample was injected into the HPLC.

### Creatinine measurement

After a urine sample was diluted with physiological saline at a ratio of 10:1, measurement was conducted using a Hitachi 7180 automatic analyzer (Hitachi High-Technologies, Tokyo, Japan) and creatinine-measuring reagent (Aqua-auto Kainos CRE-II reagent; Kainos, Tokyo, Japan) under the conditions designated by the manufacturer.

### Standard substances

As compounds containing a keto group, 2,3-butanedione, 2-butanone, and 2-nonanone were used. As compounds containing a hydroxy group, ethanol, 1-octanol, 1-propanol, 2-propanol, 2-butanol, and 2-aminoethanol (FUJIFILM Wako Pure Chemical, Osaka, Japan) were used. Compounds with a keto group were dissolved in purified water (milliQ). The compounds containing a hydroxy group were dissolved in benzene. The concentration of all standards was adjusted to 70 μmol/L.

### HPLC measurement conditions

To measure fluorescence-labeled substances, a high-performance liquid chromatograph was used

(Prominence Series, Shimadzu, Kyoto, Japan). As a column, the Wakosil-II 5C18HG (φ3.0 mm × 250 mm; FUJIFILM Wako Pure Chemical) was used. As a guard column, the Shim-pack GWS (G) (4.0 mm × 10 mm; Shimadzu) was used. Measurement conditions for compounds with a keto group included a methanol/water ratio of 40/60 (v/v) as a mobile phase, flow velocity of 0.5 mL/min, excitation wavelength of 373 nm for detection, fluorescence wavelength of 448 nm, column oven temperature of 40°C, and fluorescence-labeled sample volume of 10 μL. Conditions for compounds with a hydroxy group included a methanol/water ratio of 80/20 (v/v) as a mobile phase, flow velocity of 0.5 mL/min, excitation wavelength of 400 nm for detection, fluorescence wavelength of 500 nm, column oven temperature of 40°C, and fluorescence-labeled sample volume of 10 μL.

Creatinine-correcting formula for urinary compounds with keto and hydroxy groups

Using the following formula, the creatinine concentration (nmol/g) was calculated: concentration of urinary compound with a keto group (nmol/L)/concentration of urinary creatinine (mg/dL) × 100 = creatinine concentration (nmol/g).

Samples

Urine samples were collected from 60 heal- thy adults during a health check-up and 42 patients definitively diagnosed with cancer (20 with liver cancer/cholangiocarcinoma, 9 with uterine/ovarian cancer, 5 with colorectal cancer, 3 with pancreatic cancer, 3 with lung cancer, and 3 with prostate cancer). Urine samples from patients with cancer and healthy adults were stored at -80°C and -20°C, respectively. The urine samples were confirmed to be positive for cancer at Chiba University Hospital. The cancer stage was not considered.

Statistical analyses

The non-parametric Mann–Whitney U-test was used to compare the data for healthy adults and patients with cancer. In addition, the Dunn2 test was used for multivariate analyses of various cancer groups in comparison with healthy adults. Sensitivity and specificity were determined by determining the cut-off value for healthy subjects from the ROC curve.

## 3. Results

Elution time on chromatograms

The elution times for compounds with keto groups, i.e., 2-nonanone, 2-butanone, and 2,3-butanedione, were 2.958, 9.715, and 20.289 minutes, respectively (Fig. 1). The elution times for compounds containing hydroxy groups, 2-aminoethanol, 1-propanol, 2-propanol, 2-butanol, and 1-octanol, were 3.121, 3.744, 3.749, 4.079, and 8.973 minutes, respectively. It was possible to isolate 4 components. However, it was not possible to isolate 1-propanol from 2-propanol (Fig. 2).

Comparison of components detected in urine between healthy individuals and patients with cancer

The urinary components of patients with cancer (n = 43) and healthy adults (n = 60) were compared. Two components, 2,3-butanedione and 2-butanone, were detected only in urine samples from patients with cancer. The other components were not significantly different between healthy subjects and patients with cancer. Subsequent analyses focused on 2,3-butanedione and 2-butanone.

Linearity

The upper limits of linearities for 2,3-butanedione and 2-butanone were 2.2 nmol/L and 10.4 μmol/L, respectively, as shown in Fig. 3. The correlation coefficients for the peak area against the concentration for 2,3-butanedione and 2-butanone were 0.999 and 0.999, respectively, indicating good linearity.

Simultaneous reproducibility

The concentrations of standard solutions for 2,3-butanedione and 2-butanone were 0.547 μmol/L and 2.594 μmol/L, respectively. Five measurements were obtained to examine reproducibility. The coefficients of variation were 1.5% and 2.3%, respectively.

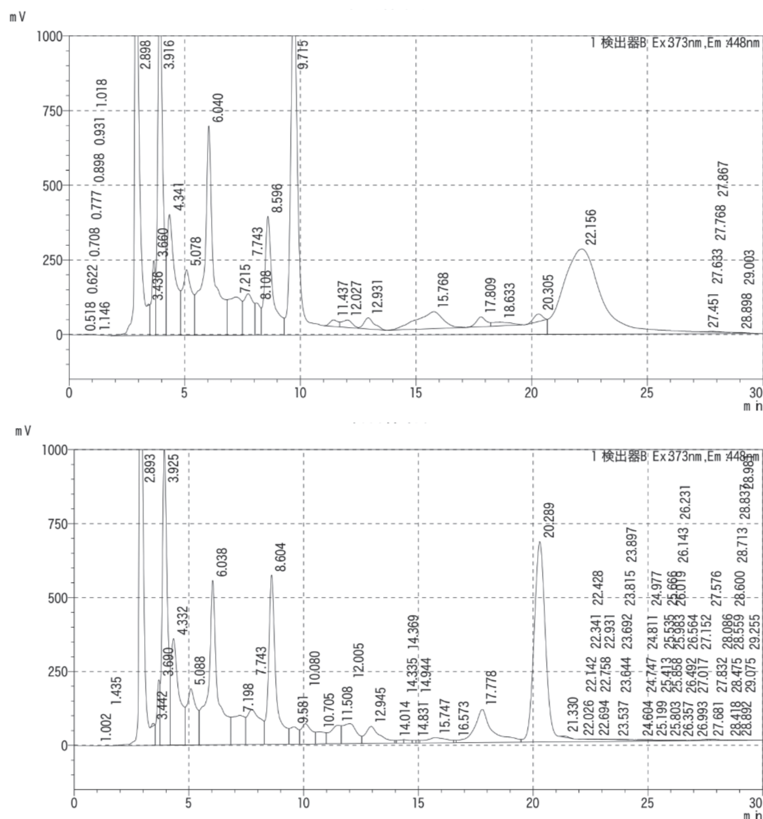


Fig. 1 Chromatogram of compounds with a keto group.
Upper panel, 2-butanone; lower panel, 2,3-butanedione. Elution times for 2-butanone and 2,3-butanedione standard solutions were 9.715 and 20.289 minutes, respectively. Other peaks are related to fluorescent reagents.

Usefulness for all cancer types and specific cancer types

Some cancer patients detected 2-butanone in the urine but were excluded from the study because of the low positive rate.

Accordingly, analyses focused on 2,3-butanedione.

Table 1 summarizes 2,3-butanedione levels in urine samples from patients with cancer (n = 43) and healthy adults (n = 60). Based on the Mann–Whitney U-test, we found a significant difference between these two groups (P = 0.00378) (Fig. 4). Subsequently, values for samples from patients with different cancers were compared with those from healthy adults using a multiple comparison test (Dunn2 test).

The area under the curve (AUC) for all patients with cancer relative to that for healthy adults was 0.67 (Fig. 5). Similarly, the AUC values were 0.70 for patients with liver cancer, 0.83 for colorectal cancer (although the number of patients was small), and 0.73 for liver and colorectal cancer (Fig. 6). The sensitivity and specificity for the comparison between all patients with cancer and healthy adults were 0.63 and 0.6 when the cut-off value was 0.0011 μmol/g creatinine (defined as the intersection of the sensitivity and specificity curves). The sensitivity and specificity for the comparison between patients with liver and colorectal cancer and healthy adults were 0.76 and 0.6, respectively, indicating an improved sensitivity.

## 4. Discussion

We examined the levels of compounds containing keto groups, 2,3-butanedione and 2-butanone, and hydroxy groups, 1-octanol and 2-butanol, in urine samples from patients with cancer and healthy adults. We detected 2,3-butanedione and 2-butanone. In particular, there was a significant

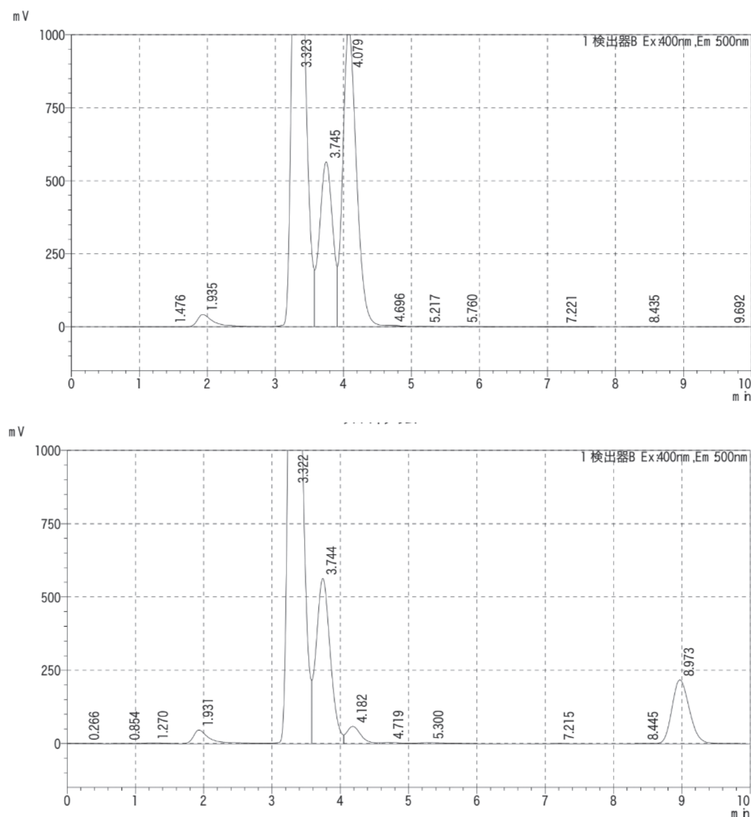


Fig. 2 Chromatograms of compounds with a hydroxy group.
Upper panel, 2-butanol; lower panel, 1-octanol. Elution times for 2-butanol and 1-octanol standard solutions were 4.079 and 8.973 minutes, respectively. Other peaks are related to fluorescent reagents.

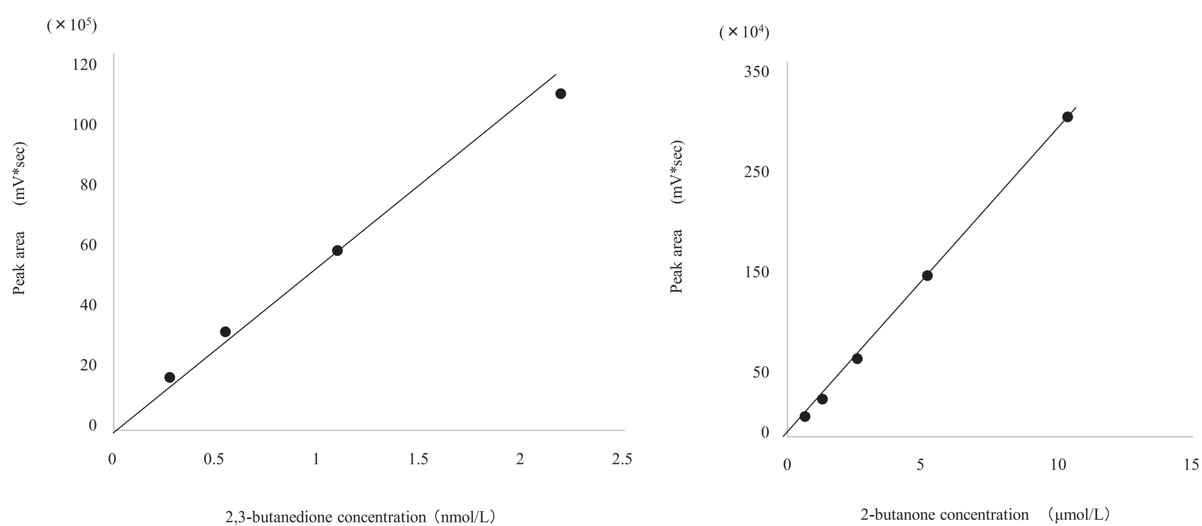


Fig. 3 Linearity of 2,3-butanedione and 2-butanone

Table 1 Comparison of urinary 2.3-butanedione in healthy adults and all patients with cancer as well as patients with liver cancer, colorectal cancer, or liver and colorectal cancer.

| Case | healthy adults | all cancer patients | liver cancer patients | colorectal cancer patients | liver and colorectal cancer patients |
|---|---|---|---|---|---|
| No. of Cases | 60 | 43 | 20 | 5 | 25 |
| Average ± SD (nmol/g・Creatinine) | 1.03±0.730 | 1.69±1.494 | 2.02±1.959 | 1.88±0.450 | 1.99±1.765 |

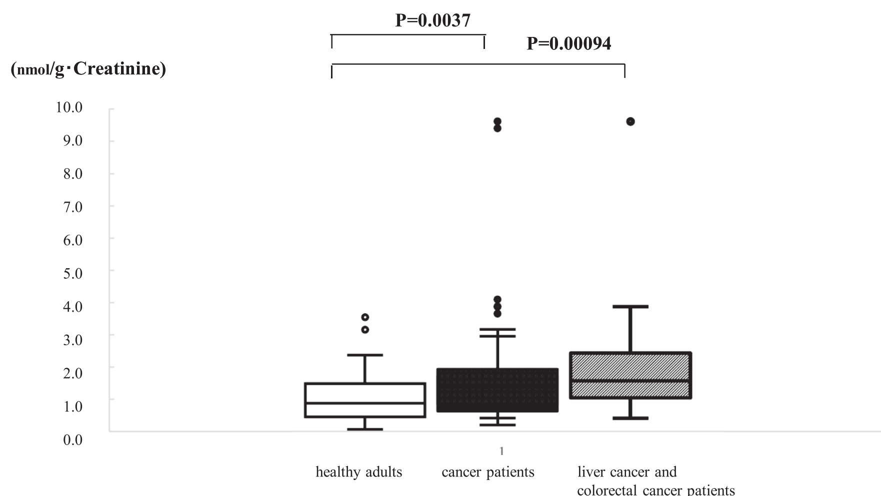


Fig. 4 Box plot of concentrations for healthy adults (n = 60), all patients with cancer (n = 43), and patients with liver cancer and colon cancer (n = 25). Data were analyzed using the Mann–Whitney U test.
Urinary 2,3-butanedione levels in healthy controls (n = 60), all cancer cases (43), and liver and colorectal cancer (25).

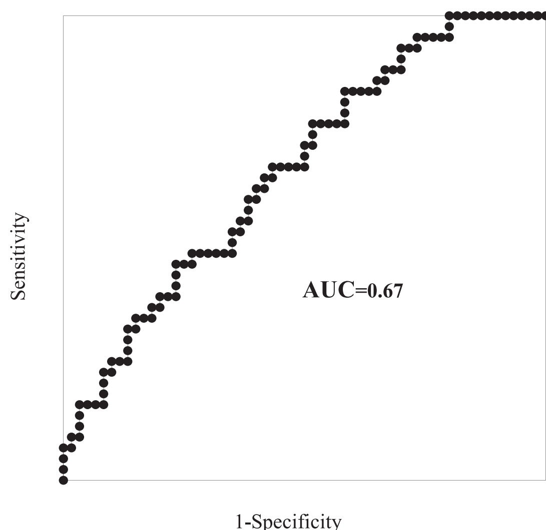


Fig. 5 Receiver operating characteristic curves for 2,3-butanedione for all cancers.
The relative area under the curve, sensitivity, and specificity values for this model for healthy adults and all cancer patients were 0.67.

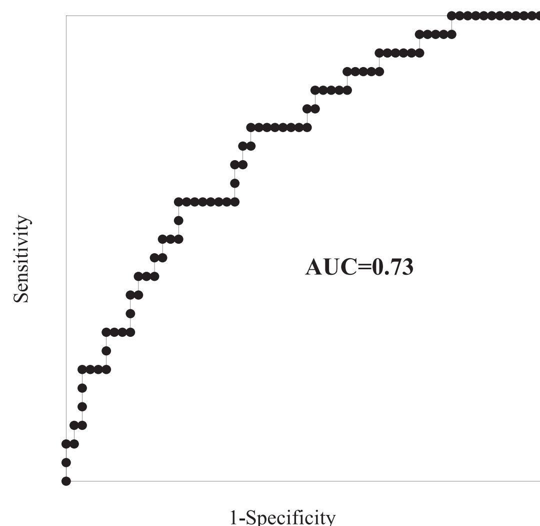


Fig. 6 Receiver operating characteristic curves for 2,3-butanedione of liver and colorectal cancer.
The relative area under the curve, sensitivity, and specificity values for this model for healthy adults and liver and colorectal cancer were 0.76 and 0.62, respectively.

difference in the level of 2,3-butanedione between the patient and healthy groups, suggesting that this parameter is an effective tumor marker for early detection.

Concerning the storage stability of urine, samples were stable at 4°C for 1 week and at -20°C for 2 weeks. In this study, urine samples from patients with cancer were stored at -80°C and those from healthy adults were stored at -20°C. In the future, storage conditions must be further examined. Furthermore, in this study, the level of 2,3-butanedione was slightly elevated in one urine sample from a healthy adult, but this subject had diabetes mellitus. The usefulness of this screening test may be improved by the simultaneous measurement of urinary glucose levels. However, this approach should be confirmed using a larger number of patients. To detect 2,3-butanedione in urine with high sensitivity, sample collection should ideally be performed in the early morning, when the concentration is highest.

The AUC value for colon cancer (0.83) was higher than that for liver cancer (0.70). In two cases classified as diabetic in the healthy group, 2,3-butanedione levels were at the threshold value. By removing these cases, the AUC is further improved. The sensitivity of screening tests based on 2,3-butanedione can be improved by excluding patients with diabetes. Furthermore, the urinary excretion of 2,3-butanedione may be high in patients with liver or colorectal cancer. It is possible that the levels of pyruvate kinase, which functions in the synthesis of pyruvic acid, and lactate dehydrogenase are increased due to concentration-dependent anaerobic metabolism[12]. As an intermediate metabolite, 2,3-butanedione may be synthesized. Regarding enzyme metabolism with yeast or bacteria, acetoacetic acid is synthesized by acetolactate synthase from pyruvic acid and acetaldehyde. A previous study has reported that the oxidative decarboxylation of acetoacetic acid leads to the synthesis of 2,3-butanedione[13]. Acetaldehyde is not synthesized *in vivo*, but it is synthesized by alcohol dehydrogenase through alcohol consumption and converted to acetic acid by acetaldehyde dehydrogenase. However, the concentration of acetaldehyde is elevated in patients with acetaldehyde dehydrogenase deficiencies. In such patients, intestinal microorganisms or yeast may contribute to the synthesis of 2,3-butanedione via acetoacetic acid by acetolactate synthase, utilizing acetaldehyde and pyruvic acid as substrates, and 2,3-butanedione may be excreted in urine through the enteric circulation.

Among various cancers investigated in this study, the urinary level of 2,3-butanedione was highest in patients with liver and colorectal cancer. Several studies have indicated that alcohol consumption is an etiological factor for these cancers[14,15]. Tsuruya et al.[16] examined the gut microbiota of patients with colorectal cancer who consume large amounts of alcohol. Patients with cancer exhibited greater growth of streptococci than that in healthy individuals[17]. This result was consistent with the detection of 2,3-butanedione in urine samples from patients with colorectal cancer in this study.

We only detected 2,3-butanedione in urine samples from patients with liver and colorectal cancer, consistent with these previous findings. However, the metabolic mechanisms must be confirmed.

## 5. Conclusion

The detection of compounds containing a keto group was effective for cancer screening. In particular, 2,3-butanedione was excreted in urine from patients with liver and colorectal cancer, providing a useful method for primary screening.

## Conflicts of interest

The authors have no conflict of interest.

## Ethics

Urine samples from patients diagnosed with cancer between April 2018 and August 2019 that were submitted to the Division of Laboratory Medicine, Chiba University Hospital were used for measurement. When utilizing urine samples from

patients with cancer, the following ethical applications were submitted and approved: “Ethical application/approval No.3472, Graduate School of Medicine, Chiba University, Development of a measurement method for various clinical examinations” and “Ethical application/approval No.: 18-Io-132, International University of Health and Welfare, Development of a screening test with a urinary low-molecular-weight cancer marker.”

The person in charge of applying for obtaining “Ethical application/approval No. 3472” is Kazuyuki Matsushita, director of the clinical laboratory at Chiba University Hospital.

Urine samples from healthy adults were collected during a routine health checkup at Sanritsu Co., Ltd. after receiving approval from the examinees and the company.

This study was performed in accordance with the World Medical Association declaration of Helsinki’s ethical principles for medical research involving human subjects.

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