Metabolomics Study on the Biochemical Profiles of Odor Elements in Urine of Human with Bladder Cancer

Kohei Jobu, a,b Changhai Sun, b,c Saburo Yoshioka, b Junko Yokota, b Masahide Onogawa, b Chiaki Kawada, a Keiji Inoue, d Taro Shuin, d Toshiaki Sendo, e and Mitsuhiro Miyamura a,b

a Department of Pharmacy, Kochi Medical School; b Kochi Medical School Hospital; c Nankoku, Kochi 783–8505, Japan; d Department of Pharmacy, Kochi Medical School Hospital; e Nankoku, Kochi 783–8505, Japan.

It has been reported that dogs are capable of identifying cancer in humans by detecting a specific odor: bladder cancer by detecting urine odor and other cancers by detecting exhaled breath odor. However, no odor recognized by dogs that indicates cancer has been identified. In this study, we examined whether bladder cancer could be detected by gas chromatography-mass spectrometry (GC-MS)-based metabolomics analysis of urine odor. Nine patients with bladder cancer and 7 healthy controls were recruited as participants. Patients collected urine 3 d before and for 3–7 d after surgery. The concentrated urine odor was analyzed by GC-MS and principal component analysis (PCA). Results indicated 12 metabolites of urine odor. Score plots of 7 of the preoperative bladder cancer patients were clearly different from those of controls on the PCA map. The distribution of controls was in the negative domain of principal component (PC) 1, whereas the distribution of preoperative patients was in the positive domain of PC1. Bladder cancer was diagnosed in 5 of the 9 patients on the basis of urinary cytology. The findings indicate the potential to screen bladder cancer by analyzing urine odor. Moreover, diagnosis of bladder cancer on the basis of urine odor might have higher sensitivity than screening by urinary cytology.

Key words metabolomics; urine odor; bladder cancer; GC-MS

The annual incidence of bladder cancer in men and women is showing a gradual tendency to increase.8) Urinary tract epithelial cancer is the main bladder cancer and is conventionally detected by hematuria. Screening examinations include urinary cytology, and imaging by cystoscopy, and definitive pathological diagnosis is made by transurethral bladder biopsy. But they have high invasiveness. The bladder cancer is classified roughly into a permeative cancer of the intramural muscular layer and a superficial (muscular layer non-permeation) bladder cancer. When permeative cancer of bladder was discovered, a patient needs all bladder enucleation which is normal treatment that causes urinary passage change. Decline of the quality of life (QOL) is an important problem for patients. On the other hand, when superficial bladder cancer was discovered, a transurethral resection of bladder tumor (TURBT) is considered first-choice treatment. By receiving TURBT, the patient can still keep the bladder. Survival rates for five years of the patient become more than 95%. The life convalescence of the patient is good, too.7) However, many patients have a relapse of a cancer intravesically after a surgery early. It becomes necessary to perform the enforcement of the bladder cancer screening examinations including a purpose to detect a recurrence frequently. For such a reason, the physical, mental and financial burden for the patient is big, and development of the noninvasive screening is urgent business. Therefore, we decided to develop a new screening system for indentifying urinary tract epithelial cancer by metabolomics analysis of urinary odor.

The identification of bladder cancer from urine odor by dogs was first reported in 2004, and that of breast and lung cancers was reported in 2006.3,4) The identification of cancer from odor is now attracting attention. We believe that the change in urine odor is caused by cancer cell invasion and proliferation. The volatile organic compounds found in the exhaled breath of patients with lung and breast cancers are potential diagnostic markers. These components, such as alkanes, methylated alkanes, aromatic compounds, and benzene derivatives, have been identified by GC-MS.5–8) In both lung and breast cancers, relative concentrations of these exhaled compounds differ between patients and healthy volunteers. However, the odorant that specified cancer has not been identified.

We previously reported that the analysis of urine metabolites from the model animal, after herbal medicine and natural products were given, could be applied to both diagnosis and treatment.9) In this study, we concentrated a urine odor and analyzed it with GC-MS. We examined whether we could identify bladder cancer by GC-MS-based metabolomics analysis.

MATERIALS AND METHODS

Participants Nine patients with bladder cancer and seven controls were recruited to participate in this study. Patients received bladder enucleation or TURBT.

Sample Preparation The patients collected urine 3 d before and for 3–7 d after surgery in the early morning. Urine was kept in a sealed sampling bag until concentrated. We used 20 mL of urine to concentrate urine odor. Urine was kept in a sealed sampling bag until concentration. We used 20 mL of urine to concentrate urine odor by fatty acid demarcation in a NeedlEx® (Shinwa Chemical Industries, Ltd., Kyoto, Japan) extraction needle with a Kitagawa-style collection System (Komyo K.K., Kawasaki, Japan). Urine was kept in a sealed sampling bag until concentration.

Received November 25, 2011; accepted January 26, 2012; published online January 31, 2012
centration of urine odor. Concentration was performed after urine odor was pervasive for 15 min in sampling bag. Urine odor was concentrated as soon as possible after urinary collection. After concentration, samples were stored at 4°C until measured. Procedures were approved by the local Ethics Committee of Kochi Medical Graduate School.

GC-MS Samples were analyzed in a GC-MS (Model QP5050A, GC-MS Shimadzu, Kyoto, Japan) using a fused-silica capillary column (DB-1 column, 60 m×0.32 mm; film thickness, 0.1 µm) in split injection mode (1:15). Oven temperature was initially set at 40°C and was raised to 280°C at a rate of 8.0°C/min after 2 min and maintained for 33 min. Other instrumental parameters were as follows: electron energy, 70 eV, ion source temperature, 250°C, and injector temperature, 250°C. Helium was set as a carrier gas to a column flow rate of 2.4 mL/min. All data were collected in full scan mode (40–500 m/z). Dwell time for each scan was set at 100 ms. The automated mass spectral deconvolution and identification functions of in-house software were first employed to support peak identification and to perform coupled deconvolution of reference mass spectra. In addition, a comparison of the relative retention time and peak area was performed when available.

Analysis. Collection and Preprocessing of Raw Data For each run, GC-MS raw data was stored in the proprietary QGD format and later converted to CDF format by a software utility. Analyses used 12 CDF files, which were each input into Matlab, where the data were organized in a regular mesh with respect to m/z and migration time. The mesh was defined by one vector of m/z and one vector of time values for the consecutive spectra. Data points were stored in a Matlab data file as a list of intensities (s) together with corresponding lists of m/z and time indices (i and j, respectively). In addition, the mesh vectors (masses and spectra) and identification data were stored in the data file.

Next, data were preprocessed run by run to remove background noise and spikes. A data source is regular sampling data collected for 35 min for 1/120 min. At first, we performed noise reduction by neighborhood moving average processing of 3 points. Then, we performed revision disposal. The revision disposal of baselines extracted the baseline values excluding the peak wave pattern. Furthermore, we assumed a wave pattern with a moving average of 60 points within the neighborhood of baseline values and deducted the baseline wave pattern from wave pattern data after noise reduction. Next, the peak wave pattern area was smoothed by differentiation of 14 points of neighborhood for baseline revision wave pattern data to detect the peak and valley positions and demanded area under the curve by using Simpson’s rule for the definite integral. Consequently, the amount of data was substantially reduced. Each data set (for a total of 12 runs) was stored in a single file that enabled Matlab to write and read the results from individual runs. Different data blocks were addressed by paths in a similar manner to ordinary file addressing. Finally, a coarse linear time alignment was performed for all runs within each data set. Starting with the CDF files, the procedure for data collection and preprocessing was performed using in-house developed Matlab routines.

Visualization of Sample Differences by Principal Component Analysis (PCA) PCA the most commonly used algorithm in metabolomics studies, was used to process the GC-MS data in Matlab. Simultaneous comparison of a large number of complex objects was facilitated by reducing the dimension of the data set via two-dimensional (PC1 vs. PC2) mapping procedures. The resulting data were displayed as score plots representing the distribution of samples in the multivariate space. The score plots of the first two principal components enabled visualization of the data and determination of whether any cancer-induced difference in the metabolites of urine odor exists. In addition, to control bias, measurements and analysis were conducted by two people.

Urine Cytophology We classified the results of urine cytology into five classes. Class I is the absence of atypical or abnormal cells. Class II is atypical cytology, but no evidence of malignancy. Class III is cytology suggestive of malignancy, but not conclusive. Class IV is cytology strongly suggestive of malignancy. Class V is cytology conclusive of malignancy.

RESULTS

To verify reproducibility, we assessed intra-day and inter-day precision of the assay by measuring the coefficient of variation (CV) of the urine odor range. The intra-day assay precision was within 8%, and the inter-day assay precision was within 5%. Since both intra-day and inter-day precision were within 10%, we regarded our procedure as reproducible. Twelve peaks in the GC-MS chromatogram were confirmed as endogenous metabolites based on the NIST library, illustrating the metabolic perturbation induced by bladder cancer (Table 1).

Figure 1 shows box plots of the peak areas of the GC-MS total ion chromatograms of urine samples from the controls and patients with bladder cancer before and after surgery. A clear difference exists in urine odor between controls and preoperative patients.

Figure 2 shows a PCA map of urine odor from controls and preoperative and postoperative bladder cancer patients. The contribution ratio of PC1 was 51.68%, PC2 was 31.57%. The accumulated contribution ratio of PC1 and PC2 was 83.25%.

The distribution of controls is in the negative domain of PC1, whereas the distribution of preoperative patients is in the positive domain of PC1.
positive domain of PC1, indicating a clear difference between
groups. On the other hand, the distribution of controls was
closer to that of postoperation than to that of preoperation.

Table 2 shows the results of bladder cancer stage and
the urine cytology. Urine cytology revealed 2 cases each
for Class I, Class III, and Class IV, and 3 cases of Class V.
Consequently, 5 of the 9 patients were diagnosed with bladder
cancer.

DISCUSSION

In cancers such as lung, breast, head-and-neck, and gynecological
tumor, fatty acids emanate as odors from the affected
area or tumor.\(^{10-12}\) In addition, it has been reported that
the urine odor changes in people with maple syrup urine disease
(MSUD), phenylketonuria, methionine malabsorption syn-
drome, or trimethylaminuria. In MSUD, a particular fatty acid
is detectable in urine odor.\(^{13}\) However, there are no reports of
detectable fatty acids in the urine odor of people with urinary
tract diseases. Presumably, identification of disease-specific
odors can lead to disease diagnosis. We considered, therefore,
that we could distinguish fatty acids derived from cancer due
to their change in urine odor and apply such a technique to

Table 2. Diagnosis of Cancer Stage by Urinary Cytology and PCA

<table>
<thead>
<tr>
<th>Stage diagnosis</th>
<th>Urinary cytology</th>
<th>PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 pTis, ly0, v0</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>No. 2 pTa, ly0, v0</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>No. 3 pTa, ly0, v0</td>
<td>III</td>
<td>–</td>
</tr>
<tr>
<td>No. 4 pTis, ly0, v0</td>
<td>III</td>
<td>+</td>
</tr>
<tr>
<td>No. 5 pTa, ly0, v0</td>
<td>IV</td>
<td>+</td>
</tr>
<tr>
<td>No. 6 pT2, ly0, v0</td>
<td>IV</td>
<td>–</td>
</tr>
<tr>
<td>No. 7 pT1, ly1, v0</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>No. 8 pTa, ly0, v0</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>No. 9 pT2, ly0, v1</td>
<td>V</td>
<td>+</td>
</tr>
</tbody>
</table>

In the result of PCA, + showed that PC1 was positive and – showed that PC1
was negative.
the development of a bladder cancer screening method.

Many biomarkers and tests for bladder cancer have been developed. However, none have a sufficient sensitivity for specifically diagnosing bladder cancer. Urinary cytology is noninvasive and is common in a clinical setting. The sensitivity of urine cytology for urothelial cancer is low at around 40%.\(^\text{14,15}\) In this study, 5 of 9 patients were diagnosed with bladder cancer as a result of urinary cytology, a rate comparable with the previous reports. On the other hand, the preoperative score plots of 7 of 9 patients were distinct from the score plots of controls as a result of PCA. This result suggests that bladder cancer can be screened by analyzing urine odor, with a higher sensitivity than urinary cytology. However, two preoperative score plots were notably close to those of control score plots on the PCA map, suggesting that metabolomics screening should be used in conjunction with other biomarkers or tests. Hematuria in postoperative urine could not be confirmed macroscopically. However, the patient receives a loud infestation by a surgical operation. All postoperative score plots did not move around control score plots.

Hematuria is considered as a factor affecting preoperative and postoperative scores. A main ingredient in blood is protein, but we examined fatty acid demarcation because we consider the influence of hematuria to be negligible. Similarly, we think that the influence of urinary protein is also negligible.

In this study, we discovered five substances in urine odor which have the potential to serve as biomarkers for bladder cancer in GC-MS, since 5 of 12 peaks were detectable in bladder cancer patients. This suggests that ethylbenzene, nonanoyl chloride, dodecanal, (Z)-2-nonenal, and 5-dimethyl-3(2H)-isoxazolone can be used as biomarkers in bladder cancer. We considered the medicines that were administered to the patients might be an influence on the urine odor. But the medicines and their metabolic substance had a very small effect on five substances.

Metabolomics is a noninvasive procedure requiring only urine for analysis and is therefore well received by patients. In addition, the development of metabolomics analysis enables screening without the need for expensive imaging procedures. This system will benefit bladder cancer patients and also reduce the burden on healthcare workers. We intend to develop a metabolomics analysis technique for the early detection of bladder cancer and the prevention of postoperative recurrence.

REFERENCES


