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Metabolic Profiling of Urine Samples from Colorectal Cancer Patients Before and After Surgical Treatments

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Abstract : Metabolites of urine samples from 6 colorectal cancer patients were analyzed by two-dimensional NMR spectroscopy, where the samples were collected before and after the surgical treatments per patient. NMR data were analyzed with the help of the metabolome database and the statistics software. Urine samples before and after the treatments showed significantly different metabolic profiles from each other. We were able to compare 10 different metabolites. Most of the assigned metabolites of every patient showed a tendency of increase after the surgery except for a few cases. The amount of changes in individual metabolites varied significantly from patient to patient, but the combination of such changes could be used to distinguish the condition before the surgery from after, which could be done by PCA analysis. The analysis via ^{1}H-^{13}C HSQC spectra proved to be applicable in assessing and classifying global metabolic profiles of the urines from colorectal cancer patients.

Keywords : NMR, metabolomics, profiling, urine, colorectal cancer, PCA

INTRODUCTION

Colorectal cancer includes the growth of cancer in the colon, rectum and appendix. More than 600,000 people die of this kind of cancer each year worldwide, making it the third leading cause of cancer-related death (World Health Organization, http://www.who.int/mediacentre/factsheets/fs297/en/). People think colorectal cancers arise

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from polyps in the colon. These benign tumors can change to cancer over time. The localized colon cancer is diagnosed mostly through colonoscopy, and the treatment is determined by the stage of the cancer. If the colorectal cancer is detected at early stages, it can be cured. However, if it is found after metastasis, it becomes less likely to be cured. Treatment is usually through surgery, and the chemotherapy follows.

Currently staging and prognostication of colorectal cancer is done through the TNM or Duke system which histologically assesses tumor invasion and the degree of lymph nodal spread.[1] Recently, people started to use genomics, proteomics, or metabolomics approach for further understanding at the molecular level. Of those three "-omics" approach, metabolomics can snapshot metabolic changes that occur in living systems in response to various factors.[2] Metabolomics has already proven its potential in identifying metabolite-based biomarkers in various cancers including ovarian, brain and liver cancers.[3-5] The resonance intensities of taurine, choline-containing compounds and lipid resonances were reported to be significantly increased in malignant colon mucosa via the one-dimensional ^{1}H NMR technique.[6] Altered metabolic profiles would point potential biomarkers for detection, staging, prognostication, and treatment of colorectal cancer.[7]

In this present study, we hypothesized that the metabolic profile of the urine sample would reveal whether the cancerous colorectal tissues were present. To test the hypothesis, we used two-dimensional ^{1}H-^{13}C HSQC technique,[8-10] and profiled 12 samples from 6 patients collected before and after the surgical treatment. This report would demonstrate the application of the two-dimensional NMR technique to metabolic profiling, and this analysis would be extended to the diagnosis of colorectal cancer based not on the unique biomarker but on the global profile of common and abundant metabolites.

EXPERIMENTAL

Materials and Methods

Patient Population : This study involves the use of human urine samples obtained from 6 patients (5 men, 1 woman) with histologically proven colorectal cancers. Two urine

samples were collected from each patient before and after the surgical treatments. Detailed clinical analysis will be published elsewhere (S.H. Kim, unpublished data).

NMR sample preparation

Metabolites from the urines were prepared by a modified version of the hot water extraction method.[11] The tissue sample was incubated at 121 °C for 15 min, and insoluble remnants of tissues were removed by centrifugation at 4000 g for 15 min. A 0.45 μm syringe filter was used to remove fine debris from the supernatant. The resulting clear solution was further filtered through a membrane of molecular weight cutoff of 5000 Da (Vivaspin 20, Sartorius Stedim Biotech, Bohemian, NY, USA). The filtered solution was freeze-dried. The mass of the dried extract was measured, and dissolved in 5 mM HEPES solution in D_2O with 0.2 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), 0.5 mM NaN_3 to the final volume-mass ratio of 17.5 per 1 mg of dried extract. The pH was adjusted to 7.4 with NaOD or DCl (Cambridge Isotope Laboratories, Andover, MA, USA).

NMR Experiments and Data Processing

NMR experiments were performed at 298 K on Varian Inova 500 MHz (Varian, USA). The spectrometer was equipped with a triple-resonance (^1H, ^{13}C, ^{15}N, ^2H lock) probe. Sensitivity enhanced ^1H–^{13}C HSQC spectra were collected with 64 scans, 128 increments (TPPI), and GARP decoupling. The spectral widths were 20 ppm for ^1H and 100 ppm for ^{13}C. The carbon carrier frequency was set at 55 ppm.

All spectra were processed and visualized using nmrPipe[12] and Sparky[13] softwares, respectively. Picked peaks were converted to a proper format for MMCD (Madison Metabolomics Consortium Database, http://mmcd.nmrfam.wisc.edu)[14] to identify the metabolites using FMQ (Fast Metabolite Quantification) module (Ian Lewis, personal communication) written in R, a free statistics software package (http://www.r-project.org). FMQ generated a dataset-specific project file for Sparky, and spectral comparison was facilitated by overlap function. The intensities of resonances of metabolites were measured as the maximum heights inside Sparky. The intensity data were standardized using Microsoft Excel, and the resulting data was analyzed by PCA in R.

RESULTS AND DISCUSSION

Sample Preparation

The urine samples were boiled, and only the soluble metabolites were used for NMR analysis. By boiling the sample, we not only sanitized and sterilized it, but also eliminated any remaining enzyme or protein activities, essentially stopping any further changes of metabolites. We tried to bypass the ultrafiltration step because of its time-consuming nature, but this step was necessary because the background resonances from the larger molecules obscured those from the metabolites. We might have circumvented this problem by employing a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence,[15] but we preferred physically cleaner samples. When we dissolved the freeze-dried urine powder in the HEPES buffer, we observed insoluble materials forming at the bottom of the tube. We used only the fully soluble portion for NMR experiments.

NMR Experiments

Each 2D HSQC experiment took about 6 hours, and we collected 2 spectra per day, which was just due to the limited time and the matter of manual sample change. If the spectrometer had been equipped with an autosampler feature, we could have collected 2 more data during the night time, and the total collection time could have been reduced to one third. The automatic tuning and matching module would play a great supporting role to the autosampler. The cryoprobe would be another critical feature to reduce the data collection time since it is known to produce 3-4 times as large signal-to-noise ratio as the room-temperature probe. With these two equipments, we could have collected all 20 spectra within a day or two. Compared to the traditional profiling method based on one-dimensional NMR data, this two-dimensional NMR experiments take about 10 times as much time, which is why the 1D technique is still the method of choice when a large number of samples need to be analyzed. However, as mentioned in a recent report,[11] as far as the accuracy and robustness are more heavily concerned, the two-dimensional experiments can be considered as a potent alternative. In fact, it showed the quantitative NMR data could be extracted from the 8 minute ^{1}H-^{13}C HSQC experiment. Figure 1 shows one of the spectra collected in this report along with the names of the identified metabolites.

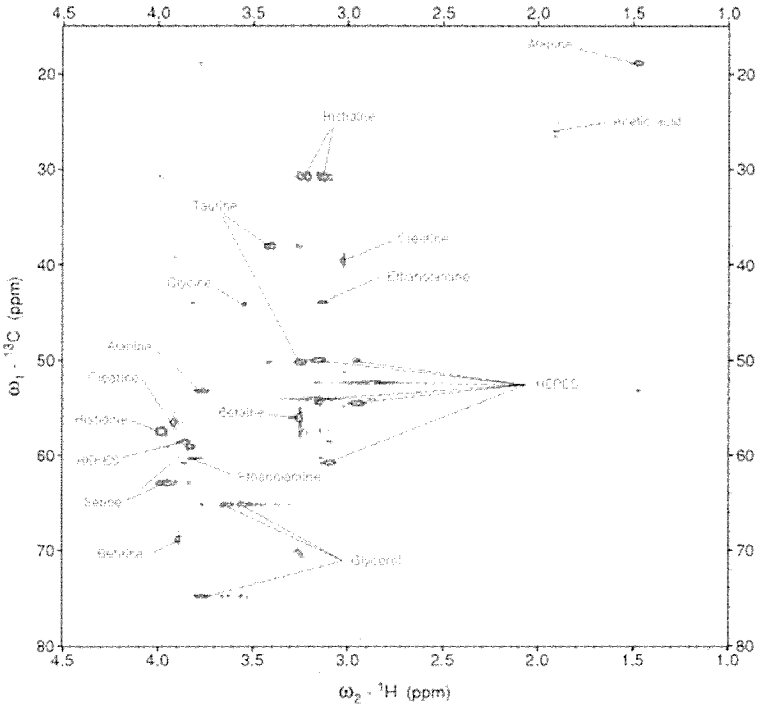

Figure 1. Two-dimensional ^{1}H-^{13}C HSQC spectrum of metabolites in urine samples from colorectal cancer patients. The assigned resonances are labeled with the names of the metabolites.

NMR Data Processing and Analysis

NMR data were processed using nmrPipe software. The processed data was converted to a suitable format, and read into Sparky where the peak list was generated. The peak list was properly formatted by FMQ module and sent to MMCD to identify the metabolites in the samples. The identities of metabolites were confirmed in Sparky using the overlay feature. The softwares used in these procedures were freely available, which was one of the advantages of the metabolite profiling method proposed in this study. In practice, the most important step was referencing the spectra since the slight variation in chemical shift could generate a list of improper metabolites from MMCD. Fortunately, DSS showed up in the spectrum, though with a very low intensity, serving as the reference point. Since we constructed a table of representative resonances of major metabolites,[10] the intensities of those peaks were quickly measured and used for semi-quantitative analysis.

Since each urine sample was unique (one before and the other after the surgery from the same patient, no replicates) and experimental variation existed as always, the intensities of resonances had to be normalized carefully for proper semi-quantitative comparison. The internal standard, HEPES, served as an excellent reference to such a purpose since all NMR samples contained the same 5 mM concentration of HEPES. This is based on the assumption that there is a linear relationship between concentration and resonance intensity. According to the previous study,[10] this assumption should be correct if the concentration was kept lower than 10 mM which could be regarded as an upper bound of the concentration of most metabolites.

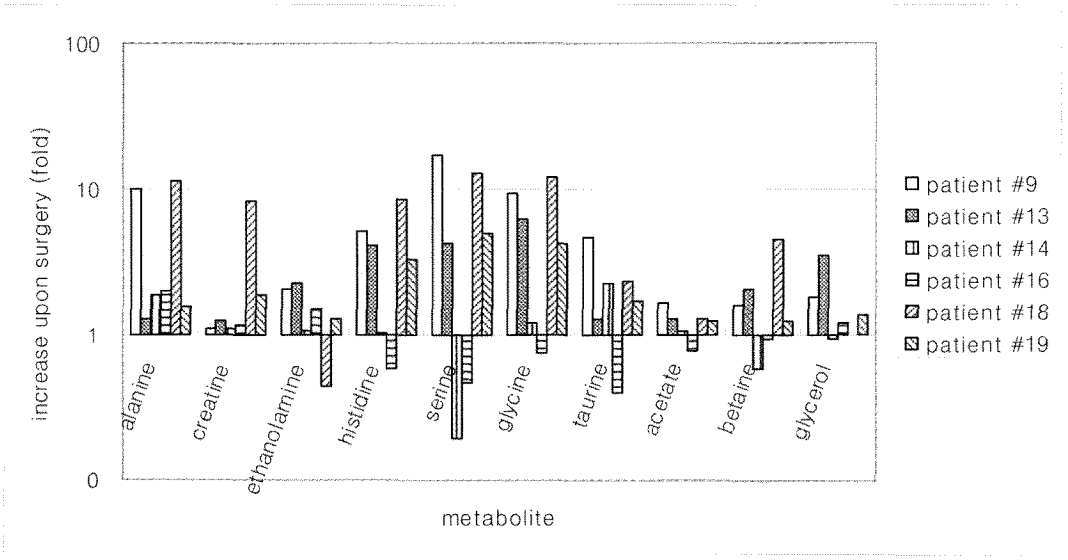

Figure 2. *Increases in metabolite concentrations upon surgical treatments. Note that the y-axis is in* logarithmic scale.

As shown in Fig. 2, we can see the trends of the changes of metabolites upon the removal of the cancer tissues from the patients. Roughly all of the analyzed metabolites were increased after the surgery. Among these, alanine and creatine increased in all cases. According to the previous reports, in the actual colorectal tissues, levels of taurine, glutamate, aspartate, and lactate were elevated while those of myoinositol and glucose were

lowered in the cancerous tissues.[1,3,16] Interestingly, the urinary taurine level rose after the surgery in 4 out of 6 patients. We speculate that the accumulated taurine molecules produced from pancreas until the surgery was performed were excreted from the body although this does not explain why the urine samples from other two patients did not show the same trend.

Table 1. Comparison of metabolite levels in urine samples from before and after surgical treatment.

metabolites	Increase of signal intensities (fold) for partients						average	relative error (%)
	#9	#13	#14	#16	#18	#19		
alanine	9.9	1.3	1.9	2.0	11.2	1.5	4.6	99.5
creatine	1.1	1.2	1.1	1.2	8.1	1.9	2.4	115.1
ethanolamine	2.1	2.3	1.1	1.5	0.4	1.3	1.4	46.3
histidine	5.2	4.1	1.0	0.6	8.7	3.4	3.8	77.2
serine	17.2	4.3	0.2	0.5	13.0	5.0	6.7	103.4
glycine	9.3	6.3	1.2	0.8	12.0	4.2	5.6	79.4
taurine	4.7	1.3	2.3	0.4	2.4	1.7	2.1	68.8
acetate	1.7	1.3	1.1	0.8	1.3	1.3	1.2	24.3
betaine	1.6	2.1	0-.6	0.9	4.5	1.3	1.8	76.7
glycerol	1.8	3.5	0.9	1.2	1.0	1.4	1.6	59.3
average	5.5	2.8	1.1	1.0	6.3	2.3	3.2	75.0

As can be seen in Table 1 and Fig. 2, the amount of changes varied from patient to patient, leading to a large relative variation among the patients. In case of creatine, the

relative variation was over 100 %. This situation is similar to that of our previous study where the actual colorectal tissues were analyzed (Y. K. Chae, unpublished result). To better analyze and diagnose the urine samples from cancer patients, we needed a better way to draw any meaningful information from these widely spread data, and the principle component analysis was performed.

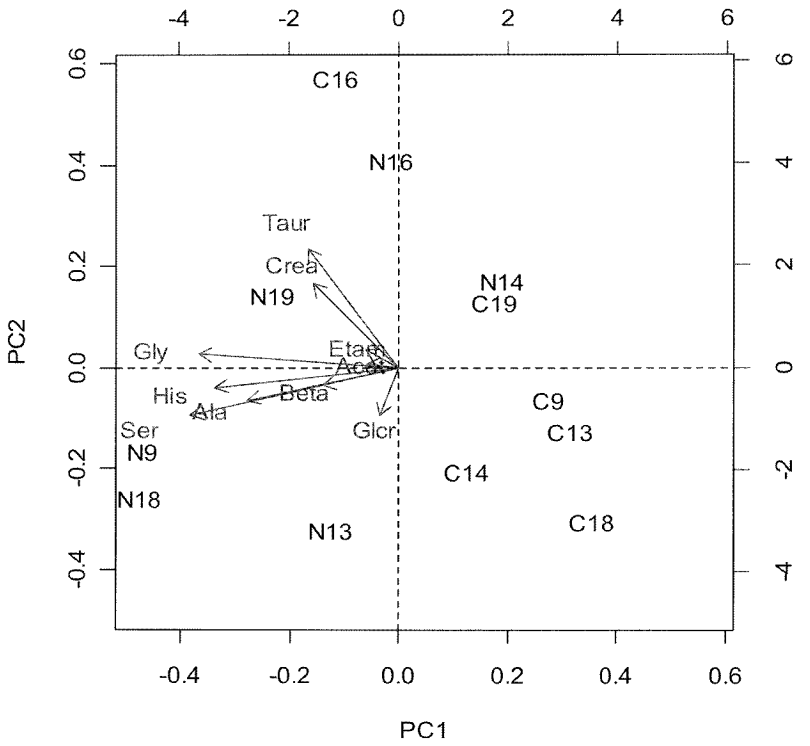

Figure 3. PCA analysis of metabolite concentrations extracted 2D HSQC data. "C" and "N" denote the samples from before and after the surgery, respectively

The script for PCA analysis in R was kindly written and provided by Ian Lewis (Univ. of Wisconsin – Madison, USA). As shown in Fig. 3, normal and cancerous tissues can be roughly divided into 2 regions by the vertical line at PC1=0. The right and left halves of the plot mostly contained urine samples before and after the surgery, respectively. In this

scheme, two patients, #14 and #16, were the outliers. If the levels of metabolites were compared, these two patients did not show much difference before and after the surgery while others did. This is why data points from those patients in the PCA plot. Apparently, these two patients did not share similarities: opposite sex, 18 years of age difference. We need more information from other metabolites to better define and diagnose the colorectal cancer. Currently, such an investigation is under way.

Conclusion

We have shown that 2 dimensional HSQC spectral data could be applied to analyze the metabolites in the urine samples without much difficulty. The PCA result from HSQC data showed two roughly separated clusters corresponding to the patient's status: before and after the surgery. Since the early detection of cancer is of great importance, this kind of analysis would serve such a purpose. This method does not depend on finding a unique biomarker which may exist in a very small quantity, but uses a combination of common and abundant metabolites globally. We prospect that this method can lead to the quick and easy diagnosis of not only the colorectal cancers but also other cancer types.

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