Urinary 5-Aminolevulinic Acid Concentrations as a Potential Tumor Marker for Colorectal Cancer Screening and Recurrence

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Abstract. *Background/Aim: Tumor biomarkers, such as carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9), are used to screen and monitor tumor recurrence in patients with colorectal cancer (CRC). 5-Aminolevulinic acid (5-ALA) is used in photodynamic diagnosis and therapy. Porphyrins produced by tumor cells are excreted in the urine after 5-ALA administration. In this study, we evaluated the use of porphyrins as novel tumor markers in urine samples from patients with CRC. Patients and Methods: Porphyrin metabolite concentrations were measured in urine samples of 33 patients with CRC, 16 patients with benign disease and 8 healthy adults, after 5-ALA administration. Results: The porphyrin metabolite concentrations were significantly increased in the CRC group compared to the control group, while in CRC patients, the porphyrin metabolite concentrations in urine were significantly decreased after surgery. Conclusion: These results suggest that the measurement of porphyrin metabolites in urine may potentially serve as a new screening and recurrence marker for CRC.*

Colorectal cancer (CRC) is the third most common malignant neoplasm and the third most common cause of cancer-related death in the world (1). Recently, CRC mortality and morbidity have increased significantly in Japan. According to the vital statistics of 2012 (2), CRC mortality in women ranks highest among all deaths from all malignant neoplasms. In men, CRC ranks third after lung and stomach cancer.

For the early detection of cancer, useful screening methods should be exploited. Currently, several methods for cancer screening are available and tumor markers are clinically used as one of these methods. Tumor markers, such as carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9), are known markers of CRC. However, the sensitivity and specificity of these markers remain low (3-6). Therefore, the importance of developing useful diagnostic methods should be emphasized to improve the clinical outcome of patients with CRC.

5-Aminolevulinic acid (5-ALA) is widely used during cancer treatment for photodynamic therapy (PDT) (2-8) and photodynamic diagnosis (PDD) (9, 10) because tumors selectively accumulate the fluorescent substance protoporphyrin IX, that is produced from 5-ALA. 5-ALA has been used clinically for PDD in neurosurgery (9) and urology (10). Previous studies have reported improved diagnostic power in these fields by administering 5-ALA (11). Recently, we reported the efficacy of 5-ALA for detecting lymph node metastasis in rectal cancer models in mice (12, 13). In addition, we previously reported the diagnostic utility of 5-ALA for peritoneal dissemination and lymph node metastasis in patients with gastric cancer and CRC (14, 15).

After treatment with 5-ALA, tumor cells accumulate uroporphyrinogen (UP) and coproporphyrinogen (CP). These porphyrins can be detected in body fluids following their extracellular secretion from tumor cells (16). In a recent study, porphyrin concentrations in the urine and plasma of tumor-bearing mice were significantly increased compared with normal mice after the administration of 5-ALA in hepatoma cells (17). Another study revealed that urinary

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porphyrin concentrations from patients with bladder cancer were increased compared to healthy adults and that urinary UP I and CP I demonstrated high sensitivity and specificity 8 h after 5-ALA administration (18). However, whether 5-ALA administration is also effective in the diagnosis of CRC is unknown.

Therefore, the measurement of porphyrins in urine after the administration of 5-ALA may serve as a new tumor marker for patients with CRC, making thus porphyrins a potentially new photodynamic screening (PDS) system for CRC. In this study, porphyrin concentrations were measured in urine samples after administration of 5-ALA to investigate the feasibility of this screening system in patients with CRC. To our knowledge, this is the first clinical study of ALA-PDS for patients with CRC.

Patients and Methods

Patients. This study was conducted at the University Hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan, and was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. Between April 2012 and October 2014, 43 preoperative patients with CRC, 20 preoperative benign disease surgery patients (colostomy closure, cholecystectomy and hernia repair) and 8 healthy adults were enrolled in this study and all subjects provided signed informed consent.

Experimental protocol. Urine samples from all subjects were collected before 5-ALA hydrochloride (Cosmo Bio Co., Tokyo, Japan) oral administration, 8 h after administration of 100 mg 5-ALA and 8 h after administration of 300 mg 5-ALA. Excretion in the urine occurs within 24 h after the 100 and 300 mg 5-ALA dose administration. Additionally, urine samples were collected 8 h after the administration of 300 mg 5-ALA in 10 CRC patients four weeks after surgery.

Immediately after collection, urine samples were stored at –82˚C until further processing. During the processing, samples were protected from light exposure.

All patients with CRC were pathologically diagnosed as having colon or rectal cancer through biopsy specimens; all patients with benign disease underwent medical examinations and did not have other cancerous diseases. Of the 43 patients with CRC, one patient was post-chemotherapy; three were double or triple cancer patients; and six were post-endoscopic mucosal resection patients. Excluding those 10 patients, the CRC patients group consisted of 33 patients. The control group consisted of 24 patients: 8 healthy adults and 16 preoperative benign disease surgery patients, excluding 4 patients with high white blood cell or C-reactive protein levels, to reduce the influence of inflammation.

High-performance liquid chromatography (HPLC) analysis of porphyrin metabolites and ALA. The concentrations of porphyrin metabolites and 5-ALA were determined by a HPLC-system. The details of the methods have been described previously (18).

Urine creatinine levels. Creatinine levels were measured using the enzyme-based colorimetric method of the Shikari Kit (Kanto Chemical, Tokyo, Japan).

Statistical analysis. The Mann–Whitney *U*-test was used to compare the differences in porphyrin concentrations between the CRC and control groups, while the Wilcoxon T-test was used to compare the paired samples before and after the operations. A *p*-value of 0.05 was considered significant.

Table I. *Characteristics of the CRC group and the control group (n=57 patients).*

Characteristic	CRC patients group (n=33)		Control group (n=24)	
	No. of patients	%	No. of patients	%
Age (years)				
Median	69		61	
Range	34-84		29-81	
Gender				
Male	20	60.6	17	70.8
Female	13	39.4	7	29.2
Primary tumor site				
Colon	23	69.7		
Rectum	10	30.3		
Venous invasion				
v0	15	45.5		
v1	11	33.3		
v2-3	7	21.2		
Lymphatic invasion				
ly0	17	51.5		
ly1	13	39.4		
ly2-3	3	9.1		
T-stage				
T1	8	24.2		
T2	6	18.2		
T3	17	51.5		
T4	2	6.1		
N-stage				
N0	20	60.6		
N1	10	30.3		
N2	3	9.1		
N3	0	0.0		
M-stage				
M0	31	93.9		
M1	2	6.1		
Stage				
I	12	36.4		
II	8	24.2		
III	11	33.3		
IV	2	6.1		

Results

Samples from the CRC patient group were compared to samples from patients with benign diseases and healthy adults. The patients characteristics (age, sex, primary tumor site, venous invasion, lymphatic invasion, T-stage, N-stage, M-stage, stage of disease and resection status) are described in Table I. The tumor stage was assessed according to the Union of International Control of Cancer (UICC)

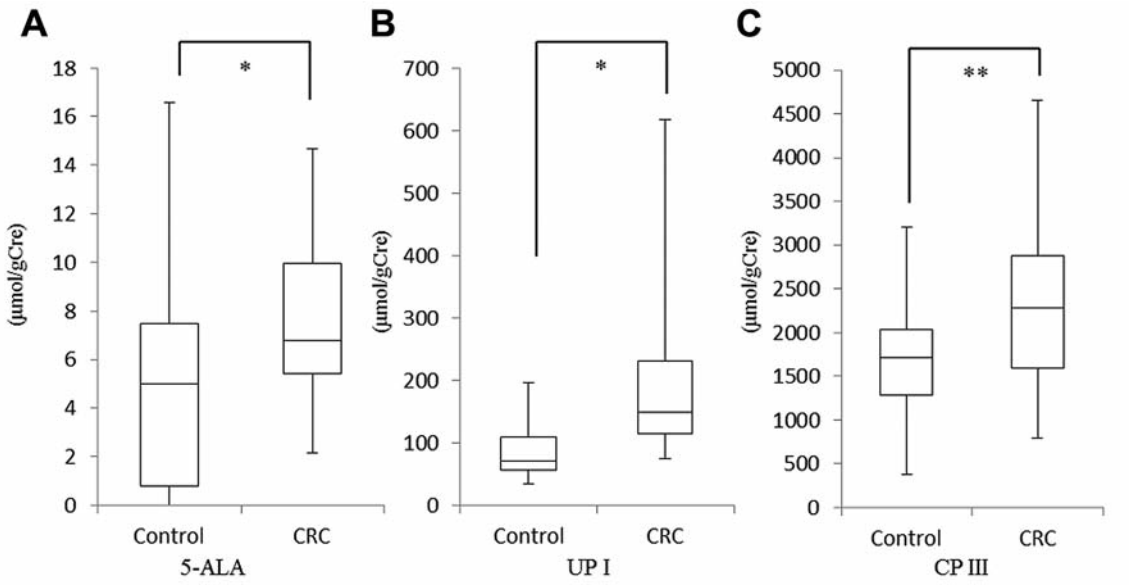

Figure 1. *5-ALA and porphyrin metabolite concentrations in urine samples. A: 5-ALA. B: UP I. C: CP III. *p<0.01, **p<0.05, Mann-Whitney U-test.*

classification (13). The macroscopic and microscopic tumor classification was based on the UICC/TNM (tumor-node-metastasis) staging system (19).

The concentrations of 5-ALA and porphyrin metabolites in urine samples were measured. Prior to 5-ALA administration, 5-ALA concentrations in urine samples of the CRC group were significantly higher than the control group (5-ALA; p=0.006) (Figure 1-a). After administration of 300 mg 5-ALA, UP I and CP III concentrations were significantly higher in the urine samples of the CRC group compared to the control group (UP I, p<0.0001; CP III, p=0.038) (Figure 1-b, c). In addition, UP I urinary concentrations after administration of 100 mg 5-ALA were significantly higher in the CRC group than the control group (p=0.011) (data not shown). No differences in the other measurements were noted (data not shown).

Figure 2 summarizes the urine concentrations of 5-ALA, UP I and CP III according to each stage. In stage I patients without 5-ALA administration, 5-ALA urinary concentrations were significantly higher than the control group. Similarly, in almost all stages, urinary UP I and CP III concentrations after 300 mg 5-ALA administration were also significantly higher than the control group.

Figure 3 presents the receiver operating characteristic (ROC) curves for the primary porphyrins (5-ALA without 5-ALA administration, UP I with 300 mg 5-ALA and CP III with 300 mg 5-ALA). The area under the curve (AUC) values were higher for the primary porphyrins compared with CEA and CA19-9 (5-ALA, AUC=0.67; UP I, AUC=0.84; CP III, AUC=0.65; CEA. AUC=0.64; CA19-9, AUC=0.40).

The porphyrin concentrations in the urine samples were also measured after operation. Figure 4 presents the urinary concentrations of CP I and CP III in the pre- and post-operative samples. The postoperative samples were collected one month after surgery. The CP I and CP III concentrations were significantly decreased after surgery (CP I, p=0.01; CPIII, p=0.05). Furthermore, urinary CP I and CP III concentrations after surgery were decreased to the same level as the control group.

Discussion

Screening methods for CRC, such as fecal occult blood, tumor marker, barium enema and colonoscopy, are widely used in clinical practice. Fecal occult blood and tumor markers are minimally invasive methods. However, the diagnostic ability of these screening methods is low (3-6). Barium enema and colonoscopy have high diagnostic power (14). However, these methods are invasive with a low consultation rate due to a sense of shame associated with these procedures. Therefore, a minimally invasive screening tool that provides an accurate diagnosis is urgently needed having the potential to alter the clinical approach.

The screening method assessing urinary porphyrins as new tumor markers is convenient and non-invasive. In this study, we evaluated the use of porphyrins as a tumor marker for CRC. The concentrations of 5-ALA without 5-ALA administration and UP I and CP III after 300 mg 5-ALA administration in the urine of patients with CRC were

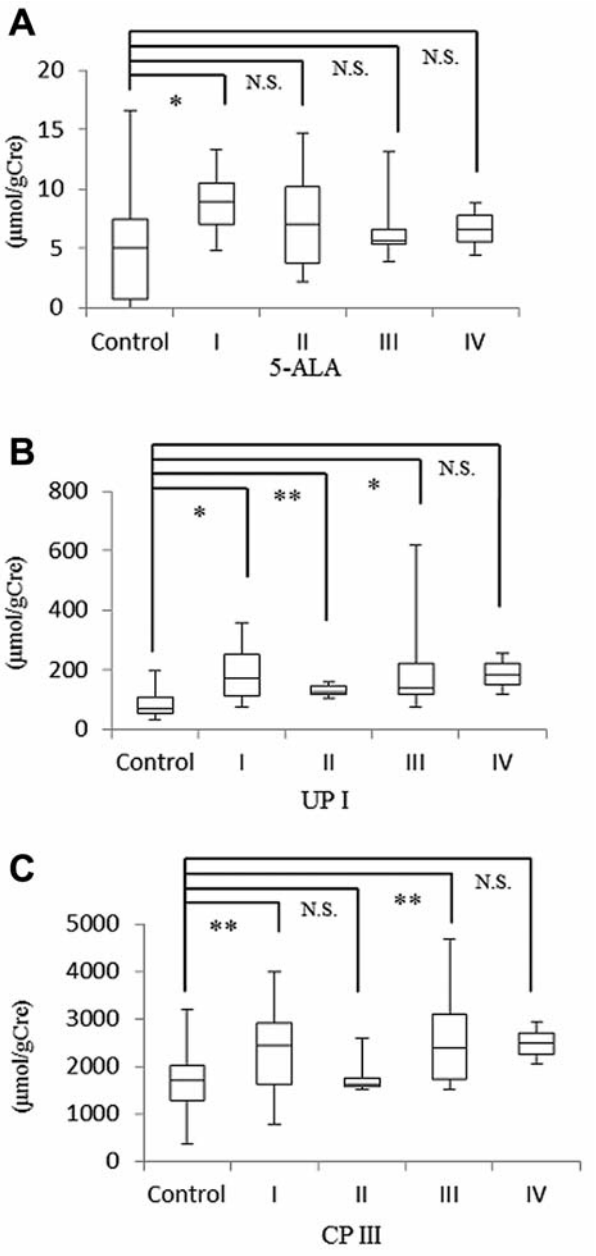

Figure 2. *The urinary concentrations of 5-ALA, UP I and CP III in each stage. A: 5-ALA. B: UP I. C: CP III. *p<0.01, **p<0.05, Mann-Whitney U-test.*

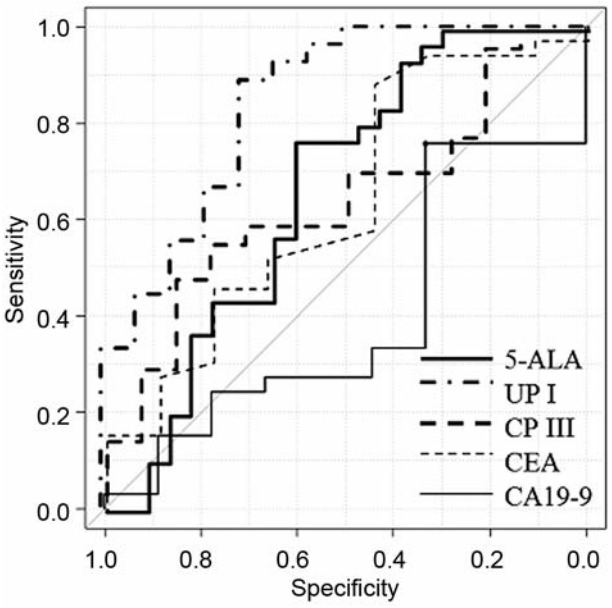

Figure 3. *The receiver operating characteristic curves.*

significantly increased compared to the control group. Furthermore, in almost every stage, the urinary concentration of porphyrins was significantly higher in the CRC group than the control group. These results suggest that urinary porphyrin levels following 5-ALA administration could serve as a useful new tumor marker. In recent studies, it was found that porphyrins accumulated in tumor cells were extracellularly discharged *via* a transporter (17). High urinary 5-ALA concentrations in CRC patients without 5-ALA administration could be explained by the tendency of 5-ALA to accumulate in tumor cells due to anaerobic metabolism. High UP I concentrations after 300 mg 5-ALA administration could be due to the porphyrin metabolic pathway. In the third step of the metabolic pathway, hydroxymethylbilane is generated by the enzyme porphobilinogen deaminase. In the next step, the enzyme uroporphyrinogen III synthase converts hydroxymethylbilane into UP III. Conversely, wihtout the action of uroporphyrinogen III synthase, hydroxymethylbilane is converted into UP I, which is subsequently converted into CP I (20, 21). Uroporphyrinogen III synthase may not be present in tumor cells. AUC values for many urinary porphyrins were considerably higher than the AUC values for CEA or CA19-9. In a previous study, the sensitivity of the preferred CRC screening method ranged from 18 to 74% (fecal occult blood, 18-73%; CEA, 35-74%; CA19-9, 17-37%) (3-6). These results suggest that urinary porphyrins are comparable tumor markers.

Postoperative samples were studied to determine whether the high level of porphyrin metabolites decreased after surgery. The samples were collected a few weeks after operation to reduce the influence of prolonged postoperative inflammation (because porphyrins accumulate at sites of inflammation (22)). Urinary CP I and CP III concentrations were significantly decreased after surgery. This indicates that urinary porphyrins concentrations are a useful screening

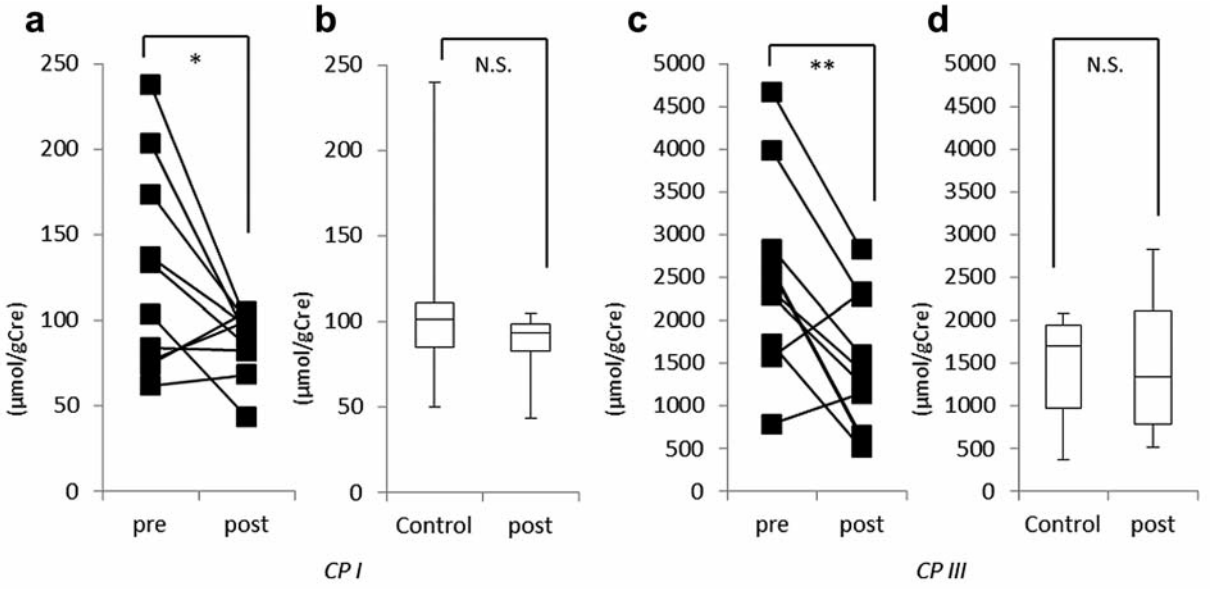

Figure 4. *Pre- and postoperative urinary concentrations. A: CP I, pre- and postoperative concentration in CRC group. B: CP I, concentration in control group and postoperative concentration in CRC group. C: CP III, pre- and postoperative concentration in CRC group. D: CP III, concentration in control group and postoperative concentration in CRC group. *p<0.01, **p<0.05, Mann-Whitney U-test.*

method to detect the postoperative recurrence of CRC. The decreases in urinary CP I and CP III concentrations after surgery may be related to the porphyrin metabolic pathway. However, further research is needed as the molecular mechanism of these phenomena remains unclear.

5-ALA is widely used in cancer therapy, such as PDT and PDD. High doses of 5-ALA can cause side effects, such as nausea, liver dysfunction, nasal congestion and sensitivity to sunlight (23, 24). However, low doses of 5-ALA are comparatively safe since no significant side effects were observed in this study. In addition, low doses of 5-ALA are better from an economic standpoint.

A previous study demonstrated that porphyrin levels increased following 5-ALA administration and remained elevated for up to 12 h (17). Therefore, in this study, urine samples were collected 8 h after 5-ALA administration.

In conclusion, we demonstrated that the measurement of urinary porphyrins after administration of 5-ALA may serve as a new tumor marker for CRC, thus rendering the measurement of porphyrins a potential new PDS system for CRC. Our new screening method is safe, simple and non-invasive. However, our sample size (33 subjects) was small and more patients are needed to confirm this finding.

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