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Development of Reagent for Cancer Diagnosis by Urine Color Reaction (I)-Comparative analysis of cancer and non-cancer urine by NMR, HPLC and Gift reagent

Manki Park, Jeongseon Yang, Miyung Lee, Yongki Kim,* Namhee Weon* and Youngdo Kim**

College of Pharmacy, Seoul National University, Seoul 151-742
**The Clinical Pathology Department of Korea University Hospital, Seoul 152-051, and*
***Central Research Center of Samil Pharm. Co. Ltd., B11-6, Ansan 425-090, Korea*
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Abstract □ Urine measurements by NMR were made for 25 persons including cancer and non-cancer patients. The aromatic proton signals of NMR were observed much more often in cancer patients' urine than non-cancer patients' one. To compare the amount of the phenolic compounds excreted in urine between cancer and non-cancer patient, urine analysis by HPLC with UV detector was performed. Total peak area and major peak areas of cancer patients' urine were much greater than those of non-cancer patients' one. To check the phenolic compound excreted in urine, a new jellied reagent named Gift reagent which was based on Millon's reagent, was developed for urine color reaction. When the reagent was tested, the sensitivity and specificity for urine samples of 69 persons including cancer and non-cancer patients were measured by 85.3% and 91.4%, respectively, indicating that the Gift reagent afford a possibility of cancer diagnosis.

Key Words □ cancer diagnosis, urine color reaction, Gift reagent, urine NMR measurement, urine HPLC analysis.

Increasing interest in cancer diagnosis has led to an attempt to find reagents for cancer diagnosis by urine color reaction. In 1987, Russel *et al.*[1] manifested that a relatively large amount of polyamines was detected for cancer patient's urine than non-cancer patients's urine. In 1985, Endo and Hujino[2] suggested the carcinoembryonic antigen in blood to be a cancer marker. Though the CEA method had very low sensitivity (normal, 5.6%; benign tumor, 24%; malignant tomor, 34%), it was specific for a special malignant tumor (hepatoma, 87%). Also, BFP (basic feroprotein) method, enzyme-immunoassay (EIA) method, and hemagglutination technique[3] were developed for cancer diagnosis by analyzing the blood of specific cancer patients.

In 1983, Kim *et al.*[4] introduced that the NMR measurement of cancer urine provided a possible method for differential diagnosis between cancer and non-cancer patients. They suggested that there was a cancer urine NMR index regarded as a cancer marker which had a common scope of 3.00 ± 0.09 to 3.09 ± 0.06 ppm uniquely appearing in the cancer patients' urine. They postulated that the signal was responsible for tyrosine-like substances excreted in the cancer urine.

We have made an attempt to check the aromatic region as an urine index rather than the complicated aliphatic region. Thus, urine NMR analysis was undertaken for 25 persons including cancer patients, non-cancer patients, and normal healthy persons. Compared with non-cancer patients and normal healthy persons, most of cancer patients' urine showed some peaks in aromatic region.

To measure the amount of the phenolic compounds excreted in urine, urine analysis by HPLC with UV detector had been performed. A jellied reagent, named Gift reagent,[5] which was based on Millon's reagent was developed in order to find a reagent for detecting the aromatic compounds excreted in urine. Original Millon's reagent reacts with monohydroxyphenols which are open on at

least one ortho position to give red color, although the chemistry of the color formation was not clear. The sensitivity and specificity of Gift reagent were determined for the urine samples of 69 persons including cancer and non-cancer patients.

EXPERIMENTAL METHODS

Samples

All the 24-hr urine samples of cancer patients and non-cancer patients were collected from the Clinical Pathology Department of Korea University Hospital in Seoul before chemotherapy. The urine samples of normal healthy persons were provided by volunteers. The samples were kept in a commercialized freezer before experiments.

Instruments and reagents

Brucker 80 MHz ^{1}H-FT NMR and Pye-Unicam HPLC with UV detector were used for urine analysis. Pye-Unicam PU 4810 computing integrator (Philips Co.) was used for integration. Deuterium oxide (99.8 atom% D, gold label) and DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) were purchased from Sigma Chemical Co. The Gift reagent was provided from the Central Research Center of Samil Pharm. Co. Ltd., Korea. Five catecholamine derivatives (norepinephrine, epinephrine, DOPA, methylDOPA, dopamine) and tyrosine were purchased from Sigma Chemical Co. for the standards of HPLC.

Table I. NMR data of urine

No	Diagnosis	Gift Reagent	Chemical shift (δ ppm)				
			Aliphatic region			Aromatic region	
1	Stomach cancer with bilirubinuria	+	2.819	2.919	3.015	7.37	7.48
2	Colon cancer	+	2.813	2.910	3.008	7.09	7.39
3	Colon cancer	+	2.677	2.909	3.005	7.23	7.38
4	Carinoma Peritonei	+	—	—	3.055	—	—
246	Pancreatic head cancer	+	2.816	2.912	3.064	—	7.52
249	Stomach cancer	+	2.832	2.934	3.029	7.51	7.65
215	Acute pyelonephritis	−	2.828	2.948	3.082	—	7.56
200	Lt. check abscess	−	—	—	3.025	—	—
221	Fuo R/O Leptospirosis	−	2.828	2.930	3.047	—	—
229	Acute Appendicitis	−	2.824	2.920	3.035	—	—
233	Deep avulsion	−	—	—	3.030	—	—
234	unknown	−	-	—	3.038	7.20	7.40
235	Acute cytosis	−	—	—	3.056	7.20	7.40
256	Acute pyelonephritis	−	2.815	2.826	3.045	—	—
260	Degenerative Spondylosis	−	2.818	2.020	3.058	—	7.61
214	Fibular neck Fx	−	2.868	2.940	3.067	7.42	7.65
N-1	Normal person	−	2.805	2.914	3.013	−	−
N-2	"	−	2.799	2.900	3.018	—	—
N-3	"	−	2.816	2.910	3.033	—	—
N-4	"	−	2.820	2.907	3.022	—	—
N-5	"	−	2.835	2.917	3.080	—	—
N-6	"	−	—	—	3.091	—	—
N-7	"	−	—	—	3.060	—	—
N-8	"	−	—	—	3.051	—	—
N-9	"	−	2.833	2.934	3.079	7.20	7.40

Table II. Total peak area of urine HPLC analysis

No	Diagnosis	major peaks			Total peak areas
		t_r[a]	Area[b]	The sum of peak areas	
1	Stomach cancer	3.28	3,892		
	with	6.51	201,440	209,110	323,438
	bilirubinuria	7.55	3,778		
2	Colon cancer	3.3	774		
		4.77	1,360	119,329	147,558
		6.43	113,778		
		7.71	3,447		
3	Colon cancer	3.31	1,541		
		4.75	1,305	115,468	149,032
		6.35	112,622		
4	Carcinoma Peritonei	4.30	419		
		4.56	2,256	92,986	112,389
		6.33	88,938		
		7.26	1,373		
215	Acute Pylonephritis	3.33	153		
		4.78	685	101,243	115,214
		6.43	100,405		
220	Lt. check abscess	3.28	1,521		
		4.78	1,540	75,878	90,104
		6.27	72,817		
221	Fuo R/O Leptospirosis	3.31	597		
		4.78	874	80,799	94,841
		6.33	79,328		
N-1	Normal	3.20	320		
		4.58	2,018	70,752	81,332
		6.65	68,116		
		7.80	298		
N-2	Normal	3.32	196		
		4.80	2,234	74,179	91,713
		6.38	71,749		
N-3	Normal	3.21	330		
		4.50	1,691	80,788	98,275
		6.60	78,246		
		7.60	521		

[a] t_r(min); 3.20(NE), 4.68(Ep, DOPA), 6.62(Tyr), 7.61(M-DOPA, DA)
[b] Area; relative peak area for PT value 1000 of Pye-Unicam PU 4810 Computing integrator

NMR measurement

Two m*l* of the samples were taken in vials and evaporated by freeze dryer at -70 °C so as not to be decomposed or deformed by heat. Freeze-dried urine samples were dissolved with 0.5m*l* of D_2O and DSS was used as a internal reference standard. Each of NMR chart was obtained at the number of five hundred scans accumulated for large S/N ratio.

Urine HPLC analysis

A mixture of 0.17M acetic acid and 0.2M sodium acetate (92:8 by volume) was used for

Table III. The results diagnosed between cancer and noncancer patients by the Gift reagent

No	age	sex	diagnosis	type*	Gift reagent	No	age	sex	diagnosis	type*	Gift reagent
1	43	F	breast ca	M	+	36	61	M	pneumonia	N	–
2	49	M	laryngeal ca	M	+	37	48	F	GB stone	N	–
3	50	M	stamoch ca	M	+	38	36	M	DM	N	–
4	63	F	lung squam. ca	M	+	39	44	M	pyelonephritis	N	–
5	47	M	lectal ca	M	+	40	30	M	TB, RA, sjogren	N	+
6	57	M	transi, cell ca	M	+	41	18	M	enchondroma	M	–
7	63	M	hepatocell ca	M	+	42	21	M	pleurisy TD	N	–
8	69	M	stomach ca	M	+	43	29	F	pregnancy	N	–
9	69	F	esophageal ca	M	+	44	53	F	hashimoto	N	–
10	51	M	stomach ca	M	+	45	39	F	ueiomyoma	N	+
11	53	F	rectal ca	M	+	46	54	F	unknown, CYTO	N	–
12	46	M	lung ca	M	+	47	42	M	pleurisy, TB	N	–
13	45	M	laryngeal ca	M	+	48	64	M	appendicitis	N	–
14	60	M	GB ca	M	+	49	53	F	insert. obstuct	N	–
15	64	M	stomach ca	M	–	50	16	F	bronchial cleft	N	–
16	17	F	ALL	M	+	51	6	F	lipomatosis	N	–
17	64	F	bronchogenic ca	M	–	52	65	M	CHR gastritis	N	–
18	59	M	hepatocell ca	M	+	53	61	F	CHR bronchitis	N	–
19	48	M	stomach ca	M	+	54	64	F	renal cyst	N	+
20	49	M	periampul ca	M	+	55	26	F	nonsp colitis	N	–
21	36	F	cervical ca	M	+	56	37	F	placenta previa	N	–
22	62	M	stinacg ca	M	+	57	36	F	leimyoma	N	–
23	63	M	bronchogenic ca	M	+	58	55	M	pleural TB	N	–
24	13	M	ALL	M	+	59	70	M	CHR PN	N	–
25	67	M	colon ca	M	+	60	43	F	adenomyosis	N	–
26	79	M	bronchogenic ca	M	+	61	37	M	bronchiectasis	N	–
27	70	F	lung ca	M	+	62	26	M	anal fistula	N	–
28	80	F	stomach ca	M	–	63	74	M	hemoptysis	N	–
29	32	M	stomach ca	M	+	64		F	hydroureter	N	–
30	57	M	stomach ca	M	+	65		M	gastritis	N	–
31	50	F	mullerian ca	M	+	66	67	F	GB	N	–
32	64	M	stomach ca	M	+	67	24	M	pleura TB	N	–
33	70	M	stomach ca	M	–	68	28	F	L/N TB	N	–
34	35	F	Inv. ductal ca	M	–	69	62	F	acute cytosis	N	–
35	72	M	leiomyosarcoma	N	–						

*M; Malignant, N; Normal

mobile phase. All solutes were filtered with 45μm milipore filter before they were used for HPLC. All urine samples were filtered with alumina and 45μm milipore filter before analysis. UV at 280nm was chosen as detector range for all phenolic compounds in urine. Pye-Unicam PU4810 computing integrator was used for calculating peak area and PT value was settled at 1000 for optimum calculation of peak area. Five catecholamine metabolites and tyrosine were used for internal standard.

Urine color reaction by the Gift reagent:

The Gift reagent has been submitted to Korea Patent Bureau. One gram of the reagent was added to 1m/ of urine sample in a test tube and the urine color reaction was observed immediately.

RESULT AND DISCUSSION

Table I showed the urine NMR data measured for 25 persons including cancer, non-cancer patients and normal healthy persons. Compared with urine of non-cancer patients and normal healthy persons, most of cancer patients' urine exhibited the peaks in aromatic region. However, it must be mentioned that the peaks of aromatic region were occasionally found even in the non-cancer patient's urine and normal urine.

Table II showed the data of urine HPLC analysis of cancer and non-cancer patients. The peak patterns of the three groups were not very much different from each other, but the sum of areas of the major peaks and that of total peaks were much larger in cancer patients' urine than non-cancer patients' and normal healthy persons' one. Each peak having the same retention time as standard peak may represent the standard peak itself, or the structurally related compound to the standard.

Original Millon's reagent is known to react with monohydroxy phenols which are open on at least one ortho position to give red color. It was nearly impossible that all compounds in urine giving positive reaction with Millon's reagent were separated and identified. It was practical to find out a difference in total peak area between cancer patients' urine and non-cancer patients' and normal healthy persons' urine rather than to elaborately analyze the composition of urine. The major peaks' areas and the total peaks' areas of cancer patients' urine were turned out to be much greater than those of non-cancer patients' urine or the normal healthy persons' urine.

To confirm the phenolic compounds excreted in cancer patients' urine, we have determined the sensitivity and specificity of the Gift reagent. Table III showed the results diagnosed among cancer, non-cancer patients and normal healthy persons. From the data of urine color reactions in Table III, the sensitivity and specificity of the Gift reagent were obtained as follows:

Sensitivity = 29/34 = 85.3%

(The possibility of determining cancer to be cancer)

Specificity = 32/35 = 91.4%

(The possibility of determing non-cancer to be non-cancer)

In conclusion, although the above NMR and HPLC data were obtained by one trial, the result suggests that the developed reagent can be used for cancer diagnosis.

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