Acta Medica Okayama

Volume 39, *Issue* 1 1985 *Article* 4

FEBRUARY 1985

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Abstract

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*PMID: 3984779 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **39,** (1), 35-45 (1985)

POLYAMINE LEVELS IN GYNECOLOGIC MALIGNANCIES

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Received July 31, 1984

Abstract. Polyamines are closely related to many aspects of cell growth. Since increased amounts of polyamines in the urine of human cancer patients were reported in 1971, polyamines have been studied from the standpoint of tumor markers. In this study, polyamines in erythrocytes, plasma and urine were determined in 42 controls and 105 patients with gynecologic malignant tumors. The changes in polyamine levels were investigated before and after treatment. With advances in the stage of uterine cervical cancer, the frequency of abnormal levels of polyamines (concentrations greater than two standard deviations above the mean control level) became greater, and reached nearly 80 % in recurrent and ovarian cancer. In the early stage of cancer, the diagnostic value was low. Comparison with carcinoembryonic antigen (CEA) was also performed. The polyamines lack specificity for malignant diseases, but they can be used to some extent as a tumor marker in the gynecologic field.

Key words : polyamine, gynecologic malignancy, high performance liquid chromatography, tumor marker.

Polyamines are aliphatic amines with low molecular weight and are widely distributed in all living cells. Putrescine (put.), spermidine (spd.) and spermine (spm.) form a group of polyamines that play an important physiological role in cell proliferation and synthesis of nucleic acids and proteins. In 1971, Russell and coworkers (1, 2) reported that polyamine levels increased in the urine of cancer patients, and since then, there have been many reports (3-10) on the usefulness of polyamine levels in the diagnosis and management of malignant diseases. It is also known that polyamine concentrations rise in serum and urine of patients with non-malignant disease (11) or pregnancy (12). However, there are few reports on polyamines in the gynecologic field.

Recent improvements in high performance liquid chromatographic techniques have led to the rapid and easy measurement of polyamines. We determined polyamines in erythrocytes, plasma and urine of gynecologic patients, mainly those with uterine cervical cancer, and studied the clinical utility of polyamines.

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MATERIALS AND METHODS

Human subjects. The subjects were 142 patients and five volunteers. The patients were all admitted and treated at Okayama University Medical School Hospital. Thirty-seven control patients, in addition to the five volunteers, had benign diseases such as uterine myoma, ovarian tumor, cervical erosion, uterine malformation and sterility. The sterile patients had secondary sterility due to tubal adhesion. The other 105 patients had gynecologic malignancies such as uterine cervical, endometrial, ovarian and recurrent cancer. Table 1 shows the diagnoses of these subjects. Patients who were pregnant, were taking hormonal medications or had complications such as heart diseases or diabetes mellitus were excluded from the study.

TABLE 1. DIAGNOSES OF 147 SUBJECTS OF POLYAMINE DETERMINATIONS

Controls: 42	No.	Malignancy: 105	No.
Uterine myoma	19	Uterine cervix cancer	82
Uterine adenomyosis	1	CIS	19
Ovarian tumor	5	Stage I	16
Dermoid cyst	3	II	27
Mucinous cyst	1	III	20
Fibroma	1	Recurrence	9
Paraovarian cyst	1	Uterine cervix cancer	6
Cervical erosion	2	Sarcoma of uterus	1
Malformation of uterus	1	Vaginal cancer	1
Sterility	8	Ovarian cancer	1
Volunteers	5	Uterine endometrial cancer	8
		Ovarian cancer	6

Ovarian cancer patients consisted of one in stage II, four in stage III and one in stage IV.
Uterine endometrial cancer patients were all in stage I.

Sample preparations. Blood (2.5 ml) was collected from the antecubital vein with a heparinized syringe from 2 : 00 to 3 : 00 in the afternoon. Some of the collected blood was immediately placed in a polypropylene capillary tube (internal diameter, 0.2 cm; length, 6.8 cm) to obtain the erythrocyte sample, and the remaining blood was centrifuged (3,000 rpm, 5 min.) to obtain the plasma sample.

To prepare erythrocyte samples, blood in the capillary tube was packed with clay and small lead balls and centrifuged (11,000 rpm, 20 min., 4℃). The separated erythrocyte portion in the capillary tube was cut. Erythrocytes transferred to a test tube were disrupted and deproteinized by shaking in a solution containing 1 % Triton X-100, 10 % trichloroacetic acid (TCA) and triethylene tetramine, generously supplied by Dr. Samejima, Tokyo Biochemical Research Institute (2×10^{-5} mol/l) as an internal standard (I. S.). After recentrifugation (3,000 rpm, 5 min.), the supernatant was used for analysis. Erythrocytes were prepared according to Tokunaga *et al.* (13).

One milliliter of plasma was treated with 1.0 ml of 10 % TCA including I. S. (1×10^{-5} mol/l) for 30 min. in an ice bath and then centrifuged (3,000 rpm, 10 min.). The supernatant was decanted into a glass tube. The precipitate was treated with 0.5 ml of 10 % TCA and centrifuged as above. The resultant supernatant was added to the same glass tube. After adding 2.0 ml of 6N HCl, the sample was hydrolyzed for 15 h at 110℃ and evaporated. The

residue was dissolved in 0.2 ml of 0.1 N HCl, and 20 μl of the solution was analyzed.

After 0.5 ml of urine collected for 24 h was hydrolyzed (110℃, 15 h) with 0.5 ml of 6N HCl and 1.0 ml of 10 % TCA including I. S. (4×10^{-5} mol/ml), the mixture was centrifuged (3,000 rpm, 10 min.). Twenty μl of the supernatant was analyzed. Urinary data were expressed as mg/g of creatinine. The preparation of plasma and urine samples was based mainly on the method of Russell *et al.* (12).

Measurements. Our analytical system (14) using high performance liquid chromatography was based on that of Yoshida *et al.* (15). Equipment used included a HLC-805 (Toyo Soda Co. Ltd., Tokyo), a FS-970 LC fluorometer (Kratos Inc.) and a recorder. A TSK-IEX-215 SC column (Toyo Soda) was used for separation, followed by fluorometric detection with O-phthalaldehyde (OPA). Polyamines were quantified by calculating the ratio of each peak height to the height of the internal standard peak (13, 16).

The stock buffer was composed of 686 g of sodium citrate 2 ml of n-caproic acid, 80 ml of 25 % Brij 35 and 230 ml of 20 % hydrochloride in a total of 20 l of deionized water, and its pH was adjusted to 5.28. Buffer 1 was a 1 : 4 dilution of buffer 2. Buffer 2 consisted of 3.21 of stock buffer, 0.8 l of methanol and 468 g of sodium chloride. The pH of both elution buffers was adjusted to 5.28. OPA reagent was prepared according to the method of Benson *et al.* (17). Samples from erythrocytes, urine and plasma were eluted in 15 min., 20 min. and 25 min., respectively, as shown in the chromatograms of Fig. 1.

Carcinoembryonic antigen (CEA) was measured by radio-immunoassay (RIA) using CEA Roche Kits following the Roche procedure (Jack Snyder of Hoffman-La Roche, Inc., Nutley, New Jersey). Blood was collected in EDTA-containing tubes, and the plasma was separated and stored in polypropylene screw-capped tubes at -40℃.

Statistical analysis was performed using Student's t test.

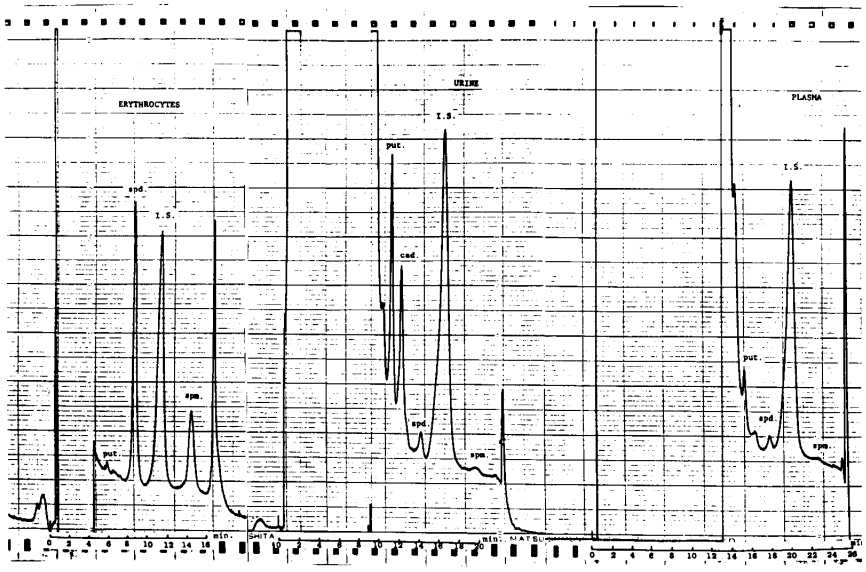

Fig. 1. Examples of chromatograms. put.: putrescine, cad.: cadaverine, spd.: spermidine, spm.: spermine.

RESULTS

Polyamine levels of the controls. The polyamine levels in erythrocytes of 41 controls, divided into four age-groups, were measured (Table 2). Putrescine in erythrocytes was excluded due to low detection levels. Since eight controls aged over 50 were menopausal woman, the control group was classified into non-menopausal and menopausal groups (Table 3). Spermidine and spermine levels in erythrocytes were 9.57 ± 3.46 and 10.52 ± 4.19 (mean ± SD) nmol/ml packed cells, respectively. The non-menopausal and the menopausal levels were 10.15 ± 3.48 and 7.19 ± 2.10 for spermidine and 11.46 ± 4.00 and 6.66 ± 2.35 for spermine. Plasma putrescine was 0.11 ± 0.09 nmol/ml, and spermine was 0.09 ± 0.06. Urinary putrescine was 3.16 ± 1.64 mg/g of creatinine, and spermidine was 2.04 ± 1.21.

The polyamine levels in erythrocytes of the non-menopausal group were significantly higher than those of the menopausal group (spermidine: $p < 0.01$, spermine: $p < 0.001$), but there was no difference in plasma and urinary polyamines.

Polyamine levels of cancer patients. The polyamine levels of cancer patients in erythrocytes, plasma and urine before treatment are shown in Table 4. Each polyamine had a higher mean concentration in patients with stage II and stage III uterine cervical cancer, recurrent cancer and ovarian cancer than in

TABLE 2. POLYAMINE LEVELS* IN ERYTHROCYTES OF CONTROLS BEFORE THERAPY

Age (yr)	No.	Average age (yr)	Spermidine (nmol/ml packed cells)	Spermine (nmol/ml packed cells)
21 – 30	13	26.5 ± 2.7	10.81 ± 4.09	12.37 ± 3.95
31 – 40	6	36.2 ± 2.4	9.75 ± 1.24	11.46 ± 4.34
41 – 50	14	43.9 ± 2.6	9.71 ± 3.42	10.61 ± 3.70
51 –	8	63.1 ± 7.3	7.19 ± 2.10	6.66 ± 2.35

*Mean ± SD.

TABLE 3. POLYAMINE LEVELS* IN ERYTHROCYTES, PLASMA AND URINE OF NON-MENOPAUSAL AND MENOPAUSAL CONTROLS

	Erythrocytes (nmol/ml packed cells)			Plasma (nmol/ml)			Urine (mg/g of creatinine)		
	No.	spd.	spm.	No.	put.	spd.	No.	put.	spd.
A	33	10.15±3.48 (a)	11.46±4.00 (b)	26	0.10±0.10 (c)	0.09±0.06 (c)	34	3.05±1.69 (c)	2.03±1.29 (c)
B	8	7.19±2.10	6.66±2.35	8	0.12±0.07	0.08±0.06	7	3.64±1.20	2.12±0.67
Total	41	9.57±3.46	10.52±4.19	34	0.11±0.09	0.09±0.06	41	3.16±1.64	2.04±1.21

*Mean ± SD.

A: non-menopausal group, B: menopausal group. put.= putrescine, spd.= spermidine, spm.= spermine. a: $p < 0.01$, b: $p < 0.001$, c: Not significant.

TABLE 4. POLYAMINE LEVELS* IN ERYTHROCYTES, PLASMA AND URINE OF CANCER PATIENTS BEFORE THERAPY

	Erythrocytes (nmol/ml packed cells)			Plasma (nmol/ml)			Urine (mg/g of creatinine)		
	No.	spd.	spm.	No.	put.	spd.	No.	put.	spd.
Uterine cervix cancer									
—CIS	19	8.94±2.20	8.63± 2.61	16	0.12±0.10	0.09±0.06	18	2.81± 0.88	1.09±0.81
I	16	9.05±2.51	8.39± 2.66	15	0.09±0.10	0.06±0.02	13	3.74± 2.59	1.85±1.28
II	27	10.83±7.66	13.40±14.13	23	0.10±0.07	0.08±0.04	26	3.69± 1.91	3.31±5.02
III	20	9.79±4.19	12.27± 8.50	17	0.17±0.15	0.12±0.14	19	4.67± 2.73	2.35±1.44
Recurrent cancer	8	13.10±7.30	17.21±15.71	7	0.23±0.18	0.11±0.06	9	5.98± 3.14	4.54±3.00
Uterine endometrial cancer	8	9.01±2.64	8.76± 5.00	8	0.08±0.09	0.10±0.04	8	2.92± 2.06	2.35±1.59
Ovarian cancer	6	11.46±5.34	22.38±15.50	6	0.15±0.15	0.14±0.10	6	10.58±13.86	6.51±8.70

*Mean ± SD. Abbreviation; See Table 3.

TABLE 5. LEVELS OF ABNORMAL POLYAMINES

Erythrocytes (nmol/ml packed cells)		
less than 50 years old	spd. > 17.11	spm. > 19.46
over 50 years old	spd. > 11.39	spm. > 11.36
Plasma (nmol/ml)	put. > 0.29	spd. > 0.21
Urine (mg/g of creatinine)	put. > 6.44	spd. > 4.46

Abbreviation: See Table 3.

normal controls.

In this paper, an abnormal polyamine level is defined as a concentration greater than the mean plus 2 SD of the control polyamine level (SD: standard deviation). As polyamine parameters, we used the following 6 parameters: erythrocyte spermidine and spermine, plasma putrescine and spermidine, and urinary putrescine and spermidine. Erythrocyte putrescine, plasma and urinary spermine were excluded due to low or absent peaks on chromatograms. Only in the case of erythrocyte polyamines, abnormal levels were determined respectively in the non-menopausal and menopausal groups. The mean values + 2 SD of the control polyamine level are shown in Table 5.

We investigated the rate of occurrence of abnormal polyamine levels in cancer patients who had already been diagnosed. When there was at least one abnormal polyamine parameter in one cancer patient, the patient was counted as one positive case. The above rate was used as a diagnostic scale of malignancy. The rate was 55 % for patients with stage III cervical cancer, 78 % for recurrent cases, 83 % for ovarian cancer patients, and less than 50 % for patients with up to stage II cervical cancer. Patients with endometrial cancer were

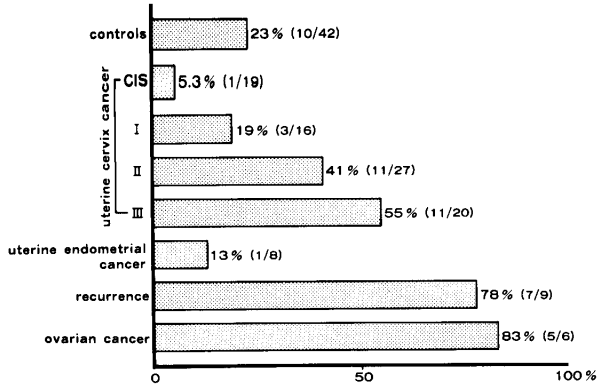

Fig. 2. Retrospective diagnostic rates for malignancy using six polyamine parameters.

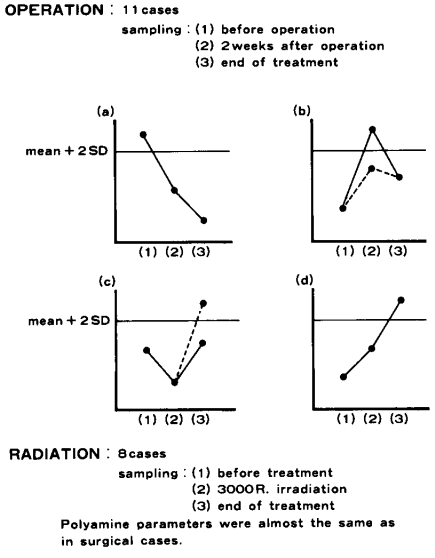

Fig. 3. Changes in polyamine levels before and after treatment.

all in stage I. In controls, the false positive rate was 23 % (Fig. 2).

The rates of total abnormal polyamine levels appearing in the pretreatment period of cancer patients were 50 % in erythrocytes, 33 % in urine and 17 % in plasma.

Changes in the polyamine levels by therapy. We observed changes in the polyamine levels before and after surgical and radiation therapy. The diagnoses of 11 surgical cases were seven of cervical cancer (stage I: 1, stage II: 5, stage III: 1), two of endometrial cancer (stage I) and two of ovarian cancer (stage III). Radical hysterectomies were performed on the cervical cancer patients, and simple hysterectomies and bilateral adnectomies on the other cancer patients. Blood losses in these operations were 600 to 1,200 ml, and transfusions of 500 to 1,000 ml of blood were given ex-

cept for two cases. Assessments were made before the operation, two weeks after the operation, and at the end of treatment. The changes in the six polyamine parameters in these three periods were graphed and then classified into four patterns: (a), (b), (c) and (d) in Fig. 3. Pattern (b) was the most frequent [erythrocytes: spd 7/11 (64 %), spm 7/11 (64 %); plasma: put 4/10 (40 %), spd 5/10 (50 %); urine: put 4/10 (40 %), spd 3/10 (30 %)].

Two cases which had abnormal levels in plasma and urine before the operation had normal levels after the operation. Erythrocyte spermidine and spermine showed similar changes, but urinary and plasma putrescine and spermidine generally showed opposite changes.

The eight patients treated by irradiation were all cervical cancer patients (stage I: 1, stage II: 4, stage III: 3), and their average age was 67. Assessments were carried out before therapy, after administration of 3,000 rads of external irradiation and at the end of therapy. In irradiation cases, the changes in polyamine parameters were almost the same as in surgical cases. However, two out of three cases which showed abnormal levels before therapy had abnormal levels even after therapy. In irradiation cases, the degree of change was less than in surgical cases.

Positive rate by CEA. The positive rate of CEA for the same cancer patients are shown in Fig. 4. There was little difference in the positive rate between CEA and polyamines from stage I to stage III of uterine cervical cancer, but the positive rate of CEA in recurrent and ovarian cancer was lower than that of polyamines. The incidence of positive polyamine cases among positive CEA cases was 55 %, and the incidence of positive CEA in positive polyamine cases was 26 %. The positive rate of α-fetoprotein was 0.8 % in the same subjects.

A case report. The changes in polyamine levels of a patient diagnosed as having stage II cervical cancer are shown in Fig. 5. On admission, the urinary spermidine level was very high, but on discharge, the 6 polyamine levels became normal. However, six months later, upon re-admission for suspicion of recurrence,

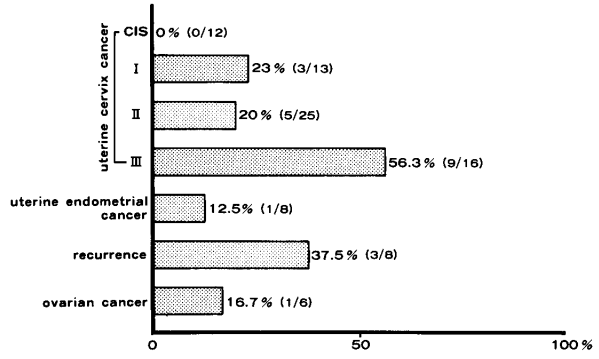

Fig. 4. Retrospective positive rates for malignancy by CEA.

		ope. admission	ope. 18-days after ope.	discharge	re-admission
Erythrocytes	spd.	4.81	14.93†	8.98	5.64
	spm.	13.96	17.80†	11.95	8.09
Plasma	put.	n.d.	0.07	0.15	0.57††
	spd.	0.06	0.12	0.12	0.23††
Urine	put.	6.25	9.33††	2.79	3.86
	spd.	15.25††	1.77	2.28	11.42††

(† > mean + SD, †† > mean + 2SD)

Fig. 5. Changes in polyamine levels during the follow-up of a case. T.M., 48 years old, Uterine Cervix Cancer, stage II (Histology: Papillary adenocarcinoma). spd.: spermidine, spm.: spermine, put.: putrescine.

plasma putrescine and spermidine, and urinary spermidine showed abnormally high levels.

DISCUSSION

Since the initial report by Russell (2) in 1971, there have been many reports indicating increased levels of extracellular polyamines in cancer patients. However, many of these reports are related to advanced cancer, and few of them refer to early cancer or the stage of disease.

Our data are consistent with the results of Cooper *et al.* (18) and Beninatti *et al.* (19) in which age has a great influence on erythrocyte polyamine levels, but has little correlation with plasma and urinary polyamine levels. In this study, we separated polyamines (putrescine, spermidine, spermine) quantitatively from erythrocytes, plasma and urine, and investigated if polyamines can be used as cancer markers. We defined the abnormal polyamine level as a concentration greater than mean plus 2 SD of the control polyamine levels, and studied the frequency (positive rate) of abnormal polyamine levels in cancer patients. The positive rate of polyamines in cancer patients was the highest: 50 % in erythrocytes, 33 % in urine and 17 % in plasma. Takami (20) reported that it was 75.2 % in erythrocytes and 34.3 % in plasma in 215 cancer patients with solid tumors. There are several reports with various positive rates of polyamines in malignant diseases ranging from 5 % in plasma to 100 % in serum and erythrocytes (21). Owing to the difference of diseases and methods of measurements, it is difficult to discuss the significance of these figures.

A correlation of urinary polyamine levels and stage of disease was reported by Durie and Salmon (22). They found statistically significant elevations in the mean urinary putrescine and spermidine levels in multiple myeloma patients with high cell mass ($< 1.2 \times 10^{12}$ cells) as compared to those with low cell mass ($< 0.6 \times 10^{12}$ cells). Nishioka *et al.* (23) reported that patients with metastatic colon carcinoma had significantly more frequent elevation of serum polyamine levels than

patients with no metastasis. However, there is no report in the gynecologic field concerning the polyamine levels and stage of cancer except ours (16). As shown in Figure 2, in stage III uterine cervical cancer, the retrospective diagnostic rate was 55 %. This rate is not very satisfactory, but it does indicate that the frequency of abnormal polyamines increases with the stage of uterine cervical cancer.

When compared with CEA (Fig. 4), there was little difference in the positive rate in uterine cervical cancer, but in recurrent and ovarian cancer, the positive rate of polyamines was much higher than that of CEA. The high positive rate of polyamines in recurrent and ovarian cancer is especially noteworthy. The case presented in Figure 5 demonstrated the usefulness of polyamines as a follow-up diagnostic tool. Measuring polyamines in urine, erythrocytes and plasma, and using other tumor markers such as CEA may enhance the possibility of cancer detection (8).

As many investigators have reported, polyamine levels increase in diseases other than cancer (11) and in pregnancy (12, 14). Our data showed a positive rate of 23 % in control patients, which is very high. Because of this high false positive rate, polyamine determinations will play a minor role in cancer screening (21). In benign diseases or early stages of cancer, only one or two polyamine parameters may show abnormalities, but in advanced cancer, several polyamine parameters tend to become positive (4, 8).

Other investigators (6, 7) found that an increase in urinary spermidine within 72 h of the initiation of chemotherapy significantly correlated with a complete response to therapy. Since there are few cases of single chemotherapy for gynecologic diseases, we observed the changes in isolated polyamines before and after surgery and irradiation. Almost all cases showed abnormal levels before therapy became normal levels after therapy, but no uniform change was seen in cases with normal levels before treatment. We have reported (16) that there are some cases of rapid increase in urinary spermidine after chemotherapy and irradiation. In this study, the same pattern was seen the most frequently in erythrocytes, but the degree of increased levels was lower. The interesting finding was that erythrocytes spermidine and spermine showed similar changes, while urinary and plasma putrescine and spermine generally showed opposite changes.

Marton (9) reported the usefulness of polyamine determinations in one kind of brain tumor. In the gynecologic field, polyamine determinations may be useful in the detection of difficult-to-follow lesions of recurrence and cured advanced cancer. They also can be employed as one of the parameters in the diagnosis and therapeutic evaluation of malignant tumors.

Our analytical methods are simple and easy, but cannot handle a large number of samples. The refinement of immunoassay techniques is eagerly awaited.

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