

USEFULNESS OF VEGF, MMP-2, MMP-3 AND TIMP-2 SERUM LEVEL EVALUATION IN PATIENTS WITH ADRENAL TUMOURS

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Objective. To estimate serum levels of vascular endothelial growth factor (VEGF), metalloproteases MMP-2 (gelatinase A), MMP-3 (stromelysin 1) and metalloprotease tissue inhibitors (TIMP-2) in patients with various benign and malignant adrenal tumours before and after surgery, as well as to evaluate if there is a correlation between serum levels of these agents and tumour types.

Methods. Serum levels of VEGF, MMP-2, -3 and TIMP-2 were estimated in 43 patients with adrenal tumour at the admission and, in case of surgery, again one month after surgery. The patients were divided into 6 groups according to the type of the tumour (I – patients with adrenal cortex carcinoma, II – with benign hormonally active adrenocortical adenomas, III – with benign, hormonally inactive adrenocortical adenomas (incidentaloma), IV – with benign, hormonally active pheochromocytomas, V – with hormonally quiescent pheochromocytomas, VI – hormonally inactive adrenal tumours of extraglandular origin. The control group consisted of 10 healthy individuals.

Results. There was no correlation between MMP-2 serum levels and tumour types and no significant difference between MMP-2 level before and after surgery. There were no significant differences between TIMP-2 serum levels in patients with adrenal tumours and the control values. Significant increase of serum MMP-3 level was found in patients with cortex cancer and hormonally active benign adrenocortical tumours. The MMP-3 mean serum level was also significantly higher in patients with malignant incidentalomas than in those with benign ones. In all groups of patients with adrenal tumours the means serum VEGF level was significantly higher than in control patients, and it was also significantly higher in patients with malignant incidentalomas than in those with benign ones. After surgery the VEGF level decreased significantly in patients with extraglandular tumours and cortex cancers who had no recurrence.

Conclusions. Since MMP-3 and VEGF serum levels were found significantly higher in patients with malignant adrenal incidentalomas than in those with benign ones, they might be applied as markers of malignancy of incidentalomas. VEGF and MMP-3 levels decreased after tumour resection in all patients with malignant tumors and increased significantly in patients with recurrence. Therefore, they are supposed to be of prognostic value in these patients.

Key words: Adrenal tumour – Vascular endothelial growth factor – Metalloproteases – Angiogenesis

Tumour growth exceeding the size of 2-3 mm³ is possible only if the sufficient angiogenesis occurs to maintain oxygen and nutrition support. The supply of such tumours by diffusion is insufficient and can lead to regressive processes. The development of new blood vessels (angiogenesis) is stimulated by angio-

genic cytokines, secreted by tumour cells due to their numerous genetic mutations leading to the formation of angiogenic phenotype (BOUCK et al. 1996; D'AMORE AND SHIMA 1996; FALKMAN et al. 1989). The vascular endothelial growth factor (VEGF) is one of the most important proangiogenic cytokines (FERRANE

1996; TOI et al. 1996). It stimulates the proliferation and migration of endothelial cells and takes part in developing neoplasms of the digestive tract (BROWN et al. 1993a; KRAFT et al. 1999), kidney, urine bladder (BROWN et al. 1993b), lung (MATTERN et al. 1993), breast (BROWN et al. 1995), thyroid (Viglietto et al. 1995) and other organs (YAMAMOTO et al. 1996). Angiogenesis takes place exclusively in case of blood vessel basal membrane disruption followed by proliferation and migration of endothelial cells towards the reconstructed connective tissue of extracellular matrix (ECM). The degradation of basal membrane and ECM is crucially dependent on proteolytic enzyme activity with the remarkable role of metalloproteases (MMPs), because these enzymes are exclusively responsible for collagen type IV degradation, which constituted the framework of vascular basal membrane. MMPs are Zn^{2+} ion dependent endopeptidases, produced as inactive proenzymes, are activated at neutral pH and at the presence of Ca^{2+} ions (KOHN et al. 1994; RAY et al. 1994; WOJTOWICZ-PRAGA et al. 1997). Although MMP-2 (gelatinase A) and MMP-3 (stromelysin 1) belong to different MMP groups, they are both responsible for collagen type IV degradation. Their role in developing numerous malignant neoplasms was confirmed (RAY et al. 1994; WOJTOWICZ-PRAGA et al. 1997).

Metalloprotease tissue inhibitors (TIMPs) are specific proteins decreasing MMP activity. They exhibit specific affinity towards MMP-2 and MMP-3, as the inhibitory effect is mediated not only by N-terminating domain (as in case of other MMPs) but also by C-terminating one. (JOHNSON et al. 1994; and WILLENBROCK et al. 1994; WOJTOWICZ-PRAGA et al. 1997; LIOTTA et al. 1991)

It seems that angiogenesis can play a significant role in the growth of adrenal tumours, especially of hormonally active ones, because the cells that produce excessive amount of hormones need sufficient nutrient and oxygen supply. So far, there have been no imaging studies and cytological or biochemical tests that reliably differentiate between benign and malignant adrenal tumours. The diagnosis is especially difficult in patients with hormonally inactive tumours (incidentaloma) (KASPERLIK-ZALUSKA et al. 1996, KOLOMECKI et al. 1999).

The aim of our study is to investigate VEGF, MMP-2, -3 and TIMP-2 serum levels in patients with

various benign and malignant adrenal tumours prior and after surgery, as well as to evaluate if there is a correlation between serum levels of these agents and tumour types, especially in patients with incidentalomas.

Subjects and Methods

VEGF, MMP-2, -3 and TIMP-2 serum levels were evaluated in 43 patients with adrenal tumours treated at the Clinic of Endocrinological and General Surgery between 1997 and 1999. The mean age was 43 years (range, 29-69 yr.). There were 31 women (72.1%) and 12 men (27.9%). Forty one (95.3%) patients underwent adrenalectomy. Two (4.7%) patients were disqualified from surgery due to infiltration of adjacent tissues, including the large blood vessels. Open adrenalectomy from the retroperitoneal approach was performed in all patients. The incision was made under the costal arch without the removal of the twelfth rib. VEGF, MMP-2, -3 and TIMP-2 serum levels were determined in singles at the admission and in case of surgery again one month after the operation. ELISA assay (Amersham Pharmacia Biotech) was applied. The values of VEGF are given in pg/ml and these of MMP-2, MMP-3 and TIMP-2 in ng/ml. The control group comprised 10 healthy individuals (6 women and 4 men, mean age, 39 yr.). Tumour type was determined on the basis of clinical, hormonal and histopathological examination of tumour tissues excised. Ultrasound and computed tomography of the abdomen were performed to visualise the tumours.

Adrenocortical function was estimated by the morning cortisol level, 24-hour free cortisol urine excretion and Liddel's test. The functional state of the adrenal medulla was evaluated with the chromogranine A serum level and 24-hour metoxycatecholamine urine excretion.

Patients were divided into 6 groups according to the type of the tumour. Group I (11 patients) included patients with adrenal cortex carcinoma. Among them, there were 4 patients in whom the symptoms of Cushing's syndrome occurred (confirmed with hormonal tests). Incidentaloma type tumours, without hormonal activity, were found in the remaining 7 patients. Histopathological examination of the tumours resected revealed their malignancy. Nine patients were op-

Table 1
VEGF, MMP-2, -3 and TIMP-2 serum levels in patients prior to surgery

	VEGF			MMP-2			MMP-3			TIMP-2		
	Mean	SD	p	Mean	SD	p	Mean	SD	p	Mean	SD	p
CG	32.3	35.0		1169.0	83.0		15.9	10.4		117.0	34.6	
Group I	304.9	152.3	< 0.01	1049.2	338.7	= 0.29	127.3	81.8	< 0.01	90.3	17.8	= 0.04
Group II	409.2	96.0	< 0.01	1126.7	257.0	> 0.05	106.0	76.2	< 0.01	84.3	37.2	= 0.01
Group III	95.6	38.2	< 0.01	1170.9	218.0	> 0.05	12.2	10.8	> 0.05	113.5	43.6	>0.05
Group IV	368.0	151.0	< 0.01	943.3	512.6	> 0.05	36.5	46.9	> 0.05	92.9	23.0	>0.05
Group V	136.8	81.5	< 0.01	1396.3	143.7	< 0.01	110.8	191.2	> 0.05	99.7	11.5	>0.05
Group VI	154.3	81.6	< 0.01	1060.0	132.8	>0.05	4.8	5.6	= 0.05	105.4	18.9	>0.05

CG – control group

Group I – all patients with adrenocortical cancer (operable and inoperable)

VEGF levels in pg/ml, MMP and TIMP levels in ng/ml

SD – standard deviation

p (accepted significance level) -0.05, between the control group and each group studied

erated on, among them 7 with incidentalomas (tumour diameter range of 4-15 cm) and 2 with Cushing's syndrome (tumour diameters 5 and 16 cm). Two patients with Cushing's syndrome were disqualified from surgery due to numerous metastases to lungs and liver along with infiltration of adjacent tissues, which was detected by US and CT. Among 7 incidentaloma patients operated on, 6 are alive without any signs of recurrence (follow-up period from 8 to 43 months), and 1 patient died because of the recurred tumour (although the operation was radical) 5 months following the operation. One patient with Cushing's syndrome developed recurrence (and hormonal hyperactivity) 2 months following the operation and died 8 months later. In the other patient with Cushing's syndrome, caused by adrenal cortex cancer, no signs of recurrence or hormonal hyperactivity occurred (follow-up period, 16 months). Those patients who were not operated on, died 2 and 3 months after admission.

Group II (6 patients) included patients with benign hormonally active adrenocortical adenomas. Fully symptomatic Cushing's syndrome was diagnosed in 4 of them and Conn's syndrome was found in 2. Adrenalectomy produced a cessation of all clinical symptoms and normalised the hormonal levels.

Group III (11 patients) included patients with benign, hormonally inactive adrenocortical adenoma of the incidentaloma type. No clinical and biochemical signs of hypercortisonism were found. No intra- or postoperative complications occurred.

Group IV (6 patients) included patients with benign, hormonally active adrenomedullary tumours of pheochromocytoma type. Typical symptoms of catecholamine hypersecretion occurred in these patients. Serum CgA level was increased in all patients (mean 670 µg/ml). After preoperative treatment with alpha and beta blockers the patients were operated on. After adrenalectomy blood pressure values, CgA and metoxycatecholamine levels returned to normal ranges.

Group V (4 patients) included patients with hormonally quiescent pheochromocytoma tumours in whom the diagnosis was made after histopathological examination of the lesion. The patients had no symptoms of increase levels of adrenomedullary hormones. Chromogranin A (CgA) levels (up to 18 µg/ml) and 24-hour metoxycatecholamine urine excretions were normal in all patients. Despite the lack of preoperative preparation with alpha and beta-blockers, transient blood pressure elevation up to 170-180 mm/Hg with no further complications occurred in only 2 patients during adrenal preparation.

Group VI (5 patients) included patients with hormonally inactive adrenal tumours of extraglandular origin (non-glandular tumours). Histopathological examination of tumour tissue revealed: myolipoma in 2 patients, fibrolipoma in 2, hamartoma in 1. The intraoperative and postoperative course was uneventful.

The statistical analysis was performed by means of t-Student and chi-square test.

Table 2
VEGF, MMP-2,-3 and TIMP-2 mean serum levels in patients with benign and malignant incidentalomas before surgery

	VEGF	MMP-2	MMP-3	TIMP-2
Malignant tumours	314.5	980.0	134.6	97.5
Benign tumours	118.7	1188.3	30.0	108.7
p	< 0.01	= 0.04	< 0.01	>0.05

VEGF levels in pg/ml, MMP and TIMP levels in ng/ml
 p (accepted significance level) – 0.05, between the groups of patients with malignant and benign tumours

Results

The following mean and standard deviation of the serum levels of the compounds investigated were found in patients from the control group: VEGF 32.3 pg/ml (SD = 35.0), MMP-2 1169.0 ng/ml (SD = 83.0), MMP-3 15.9 ng/ml (SD = 10.4), TIMP-2 117.0 ng/ml (SD = 34.6).

According to the rule of three standard deviations (BLALOCK 1960) the following upper values of normal serum levels were determined: VEGF 137.3 pg/ml, MMP-2 1418.0 ng/ml, MMP-3 47.1 ng/ml, TIMP-2 220.8 ng/ml.

Table 1 shows mean serum levels of the compounds investigated in different groups prior to the operation, their standard deviation and significance levels compared with control values.

In 2 patients with inoperable cortex cancer the following values were determined: VEGF 507.7 and 123.1; MMP-2 500 and 1515, MMP-3 28.8 and 37.6; TIMP-2 90.4 and 58.8. Mean serum levels of VEGF, MMP-2,-3 and TIMP-2 in patients with malignant and benign incidentaloma tumours prior to and after the operation are shown in Table 2. The decrease in the serum levels of the compounds studied was noted in most patients after the tumour removal.

Table 3 shows the mean levels of the VEGF, MMP-2,-3 and TIMP-2 prior to and after the operation.

In patients who developed recurrences and died, VEGF and MMP-3 levels increased or did not change within one month after surgery. In one of these patients (with Cushing's syndrome), the VEGF level increased postoperatively from 193.5 to 411.3, and the MMP-3 level from 264.5 to 747.2. In the other patient (with incidentaloma) the level of the VEGF changed from 342.6 to 307.1, and of the MMP-3 from 198.2 to 204.0. When the levels of these compounds were determined in these patients one month after the operation, there were no clinical symptoms of recurrence which, however, occurred after 4-6 weeks.

Table 4 shows the patients with the elevated VEGF, MMP-2,-3 serum levels before and after the operation. The increased TIMP-2 level occurred only in one patient with hormonally inactive adrenocortical adenoma.

Table 3
VEGF, MMP-2,-3 and TIMP-2 mean serum levels before and after the operation

	VEGF			MMP-2			MMP-3			TIMP-2		
	before	after	p	before	after	p	before	after	p	before	after	p
CG	32.3	35.0		1169.0	83.0		15.9	10.4		117.0	34.6	
Group I*	302.3	187.7	< 0.05	1080.7	979.1	> 0.05	148.3	129.0	> 0.05	93.8	86.1	> 0.05
Group I**	312.4	138.7	= 0.02	1036.6	948.9	> 0.05	124.5	30.0	< 0.01	99.9	89.6	> 0.05
Group II	409.2	150.3	< 0.01	1126.7	1030.8	> 0.05	106.0	55.0	> 0.05	84.3	94.4	> 0.05
Group III	95.9	59.6	< 0.01	1170.9	1153.2	> 0.05	12.2	8.9	> 0.05	113.5	94.7	> 0.05
Group IV	375.2	220.8	> 0.05	943.3	858.3	> 0.05	36.5	17.4	> 0.05	92.9	92.9	> 0.05
Group V	136.8	43.2	> 0.05	1396.0	1106.0	> 0.05	110.8	68.8	> 0.05	99.7	79.7	= 0.02
Group VI	155.5	62.1	= 0.04	1060.0	1008.0	> 0.05	4.8	3.2	> 0.05	105.4	101.4	> 0.05

VEGF levels in pg/ml, MMP and TIMP levels in ng/ml

p (accepted significance level) – 0.05, between the control group and each group studied

Group I* includes all patients with adrenocortical cancers operated on

Group I** includes patients with adrenocortical cancers without recurrence after operative treatment

Table 4
Patients with increased VEGF, MMP-2, and MMP-3 serum levels before and after surgery

	VEGF		MMP-2		MMP-3	
	before	after	before	after	before	after
Group I	7 (77.8%)	5 (55.6%)	1 (11.1%)	0	7 (77.8%)	2 (22.2%)*
Group II	6 (100.0%)	3 (50.0%)*	1 (16.7%)	1 (16.7%)	5 (83.3%)	2 (33.3%)
Group III	4 (36.4%)	0*	1 (9.1%)	0	0	0
Group IV	6 (100.0%)	5 (83.3%)	1 (16.7%)	0	1 (16.7%)	0
Group V	2 (50.0%)	0	2 (50.0%)	0	1 (25.0%)	1 (25.0%)
Group VI	3 (60.0%)	0*	0	0	2 (40.0%)	2 (40.0%)

VEGF levels in pg/ml, MMP levels in ng/ml

Accepted significance level – 0.05, used for the comparison of each marker values before and after surgery

* – the difference is statistically significant

Each marker was assayed in singles.

The decreased MMP-2 level was found in 2 patients of Group I (22.2%). There were patients with incidentaloma tumours (40% of all malignant incidentaloma tumours). The serum levels of the MMP-3 were elevated in 6 (85.7%) patients with adrenocortical cancer of the incidentaloma type. The MMP-3 levels returned to normal ranges after the operation in all patients with adrenocortical cancer who have not developed recurrence up to now, whereas MMP-3 levels increased after surgery in 2 patients in whom recurrence occurred.

Table 5 presents VEGF and MMP-3 mean serum levels prior to and after the operation in patient groups in which the preoperative levels of these compounds were above normal values in over 50 % of patients. Table 6 depicts VEGF, MMP-2,-3 and TIMP-2 levels in patients with adrenal tumours compared to the tumour diameters.

Table 5

VEGF and MMP-3 serum levels before and after surgery (in groups in which the levels of these cytokines exceeded normal values in over 50% of patients)

	VEGF			MMP-3		
	before	after	p	after	Before	p
Group I	390.7	156.9	< 0.01	147.7	23.2	< 0.01
Group II	409.2	150.3	< 0.01	124.5	64.6	> 0.05
Group IV	384.8	233.2	> 0.05	–	–	–

Group I – all patients with adrenocortical cancers operated on
Accepted significance level – 0.05, used for the comparison of each marker values before and after surgery

Discussion

The proangiogenic cytokine, VEGF, not only stimulates the proliferation and migration of endothelial cells, but also activates inactive proMMPs forms to active MMPs and influences the activity of TIMPs. MMPs degrade the vascular basal membrane and ECM proteins and, therefore, enable the migration of endothelial cells and formation of new blood vessels (CHAMBERS et al. 1997; UNEMORI et al. 1992).

The significant role of MMP-2 in the growth of tumours such as colon, breast (ZUCKER et al. 1994), microcellular lung (BROWN et al. 1993c), kidney (KUGLER et al. 1998) and ovarian carcinoma (NAYLOR et al. 1994) was found. The correlation between MMP-

Table 6
VEGF, MMP-2,-3 and TIMP-2 mean levels vs. tumour diameters

	Number of patients	VEGF	MMP-2	MMP-3	TIMP-2
Malignant tumours					
<4 cm	2	265.1	1035	99.9	106.5
4-9 cm	6	305.8	1156	40.5	89.6
>9 cm	3	329.5	910	127.7	81.1
Benign tumours					
<4 cm	18	186.3	970	59.2	106.0
4-9 cm	9	256.9	1227	37.6	103.4
>9 cm	5	283.9	864	14.9	99.6

No statistically significant difference was found in all groups (accepted significance level – 0.05)

2 expression in tumour and its growth rate was revealed. Also, the close relation was detected between the grade of colon tumour growth (in Dukes scale) and percentage of tumour cells expressing MMP-2 (LEVY et al. 1991). It was further suggested that MMP-2 level evaluation in fluid samples may be of prognostic value as well as it may be used as a marker in estimation of response to applied therapy (ZUCKER et al. 1994).

We did not find any statistical correlation between MMP-2 serum levels and the types of tumours. We only noted significantly elevated MMP-2 levels in patients with benign hormonally inactive adrenomedullary tumours, which is difficult to explain. Moreover, there was no significant difference between MMP-2 level prior to and after surgical resection. These data suggest that MMP-2 may not play the significant role in the development of adrenal tumours.

The remarkable ability of MMP-2 activation was noted only in breast cancer cell lines that do not express estrogen receptors. The similar mode of receptor mechanism might occur in adrenal cells.

The significant MMP-3 serum level increase was detected in patients with cortex cancer and hormonally active benign adrenocortical tumours. The MMP-3 mean serum level was also significantly higher in patients with malignant incidentalomas than in those with benign ones. These data suggest that MMP-3 serum level may be used as a marker of malignancy of adrenal incidentaloma tumours.

The MMP-3 mean serum level did not significantly change in patients with cortex cancer after surgery. However, it decreased in all patients after surgical treatment with long-term outcome and increased significantly in patients with recurrence that resulted in their deaths. MMP level increase was noted a month after the operation. No patient developed clinical symptoms of recurrence at that time. These data suggest that MMP-3 level changes after surgical treatment may be of prognostic significance.

The decrease (although not statistically significant due to big standard deviations) of MMP-3 levels was noted in all groups, which can suggest that this protease is a marker of the tumour presence.

MMP-3 is also responsible for collagen type IV degradation and influences tumour growth. This role was confirmed in urothelium cancer recurrence (GOHJI et al. 1996), in prostate (JUNG et al. 1997) and

planoepithelial cancer development and in some non-neoplastic diseases as Crohn disease or ulcerative colon inflammation (BAILEY et al. 1994).

Tissue MMP inhibitors (TIMPs), including TIMP-2, inhibit MMPs activation and active compound formation. It has been revealed that the ratio of MMP-2 and TIMP-1 or TIMP-2 levels is very important for invasion grade of numerous malignancies, such as for example uterine cervix or kidney cancer. Some authors suggest that altered TIMPs expression may play significant role in the growth of numerous neoplasms which may be of prognostic value (KUGLER et al. 1998; NUOVO et al. 1995). We did not find significant differences between TIMP-2 serum levels in our patients with adrenal tumours and the control values, although little decrease (no statistical significance) in TIMP-2 serum level was noted after tumour resection.

The mean serum level of VEGF was significantly higher in all patient groups with adrenal tumours than in control patients. These data suggest that the development of malignant as well as of benign adrenal tumours is dependent on this cytokine. VEGF serum level is also significantly higher in patients with malignant incidentaloma tumours than in those with benign incidentalomas, which suggests that VEGF may be applied as a marker of malignancy of such tumours.

The difference of VEGF levels before and after surgery was not significant in patients with recurrent cortex cancers which probably resulted from the decreased or unaltered postoperative VEGF levels. High VEGF levels, similarly as high MMP-3 levels, preceded the occurrence of clinical symptoms of recurrence. Our data suggest that adrenal tumours secrete VEGF, because the level of this cytokine decreases after tumour removal. Revascularisation of postoperative cicatrix that is dependent on VEGF may partially influence the VEGF serum level (DVORAK 1986). However, if the VEGF serum level was determined over a month after the E-mail: branch@poczta.onet.pl operation, it could not be used as a prognostic factor.

In numerous malignant tumours such as carcinomas of the breast, digestive tract, lung, kidney, ovary, and testis, there was a correlation between the circulating levels of angiogenic cytokines and the degree of tumour malignancy. The level of these cy-

tokines was also of prognostic significance (KRAFT et al. 1999; MAEDA et al. 1990; MATTERN et al. 1996; YAMAMOTO et al. 1996).

In numerous studies the expression of MMPs and VEGF in resected tumour tissue was determined. Many authors, admitting that their serum levels reflect their expression in tumour tissue, measured serum levels of MMPs and VEGF to evaluate their role in neoplastic growth. The determination of angiogenic cytokine levels in the blood may help to assess which of them can be applied as diagnostic and prognostic markers and to evaluate the efficacy of, for example, MMP inhibitors (clinical trials) (BROWN et al. 1993c; GOHJI et al. 1996; KRAFT et al. 1999; WOJTOWICZ-PRAGA et al. 1998; YAMAMOTO et al. 1996; ZUCKER et al. 1993, 1994). Therefore, in our study we attempted to assess the usefulness of MMP-2,-3, TIMP-2 and VEGF serum level measurement for clinical evaluation of patients with different adrenal tumours.

It seems possible that VEGF can modulate the expression of MMPs and TIMPs.

In ovary carcinomas a correlation between VEGF and MMP-2 expression and the grade of tumour malignancy was recorded. A close correlation was also found between the grade of VEGF and MMP-2 expression in ovary carcinoma cells (GARATTI et al. 1999). Our data suggest that the correlation between the levels of VEGF and of MMP-3 may exist, as the increased level of these cytokines was found in the same patients. We did not evaluate the expression of respective genes in tumour cells. Our study is a clinical observation and, therefore, its significance is of the limited value. Only the statistical analysis of the results may suggest a functional correlation between these cytokines. The lack of a correlation between the levels of the cytokines studied and tumour sizes indicates the VEGF, MMP-2,-3, and TIMP-2 levels are dependent on neoplasm activity, and not on a tumour mass.

To summarize, the MMP-3 and VEGF serum levels were significantly higher in patients with malignant adrenal incidentalomas than in those with benign ones, which suggest that these cytokines might be applied as markers of malignancy of incidentalomas. The results of our studies also suggest that the changes of VEGF and MMP-3 level after surgical treatment may be of prognostic value, because they

decrease after tumor resection in all patients with malignant tumors and increase significantly in patients with recurrence.

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