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IMMUNOHISTOCHEMICAL ANALYSIS OF BETA-DEFENSIN-2 EXPRESSION IN HUMAN LUNG TUMORS

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***Aim*: The present research was directed on analysis of the expression patterns of human beta-defensin-2 (hBD-2) in human lung tumors. *Materials and Methods*: Specimens of surgically resected human lung tumors (n = 31) of different histological type (1 case of small cell lung cancer, and 30 cases of non-small cell lung cancer (1 case of clear cell carcinoma, 9 cases of squamous cell carcinoma (SCC), and 20 cases of adenocarcinoma (AC)) were analyzed for expression of hBD-2 with the use of immunohistochemical analysis. *Results*: Immunohistochemical analysis has revealed that all lung tumor samples independently on their histological type express hBD-2 peptide, however at different levels (from < 5% to 100% cells). According to our observations, low-differentiated AC differs from moderately differentiated AC by significantly lower hBD-2 expression levels ($p < 0.05$). No correlation between hBD-2 expression patterns and PCNA or Bcl-2 expression has been found. *Conclusion*: Human beta-defensin-2 expression levels may depend on differentiation grade of lung adenocarcinoma.**

***Key Words*: human beta-defensin-2, human lung tumors, lung squamous cell carcinoma, adenocarcinoma, expression.**

Lung cancer is a leading cause of cancer-related death worldwide. That's why numerous studies are directed on estimation of etiology of this disease, search for tumor markers, and development of new strategies for lung cancer treatment. It is well recognized now that the etiology of lung cancer is closely related to smoking habits and is often associated with chronic pulmonary inflammation and underlying immune dysfunction. However, little is known yet about involvement of innate immunity molecules, in particular, defensins, in lung tumorigenesis.

Defensins — cationic antimicrobial peptides — are important components of mucosal immunity which two major functions are thought to be direct antimicrobial action and modulation of innate and adaptive immunity in response to pathogens. According to accumulated evidences, expression of some alpha- or beta-defensins may be altered or deregulated in different tumor types, and these antimicrobials could play a complex and poorly understood role in cancer pathogenesis either promoting or suppressing tumor cell growth [1, 2].

In normalcy, defensins participate in antimicrobial protection of respiratory tract along with other defense molecules [3]. Mailfunction or altered expression of these peptide antibiotics has been well documented in a number of chronic lung pathologies, in particular, cystic fibrosis, reactive airway disease, tuberculosis and many other lung infections [4]. There are some data evidencing on reduced levels of beta-defensin-2 (hBD-2) in sputum and pharyngeal washes of smokers versus nonsmokers with acute pneumonia [5] what points on possible smoking-dependent down-regulation of this peptide in airway epithelium. At the same time, up-to-date there are scarce data for defensin expression patterns in lung cancer. In our recent pilot study [6] we have recorded an altered expression of hBD-1-4 mRNAs in lung cancer samples versus normal lung tissue.

In this regard we aimed to analyze further the expression patterns of hBD-2 in human lung tumor samples using immunohistochemical approach.

MATERIALS AND METHODS

In the study, 31 samples of surgically resected human lung tumors of different histological type were studied. The tissue samples were obtained during the surgical treatment of lung cancer patients cured in the Thoracic Department of National Cancer Institute (Kyiv, Ukraine) in 2001–2008 (Head of the Dept., prof. V.L.Ganul). Immediately after surgical removal, tissue samples were placed in liquid nitrogen and stored at -70 °C until use. The patients did not receive chemo- or radiotherapy prior to the surgery. All patients provided an informed written consent to perform the study, and the present research was approved by Ethic Board of the Institute. Histological type and differentiation grade of lung tumors has been estimated by clinical pathologists (National Cancer Institute, Dept. Pathol. Anat. Dr. E.N. Kovalchuk and Dr. I.N. Troitskaya). From 31 lung tumor samples, 1 tumor was diagnosed as small-cell lung cancer (SCLC), and 30 — as non-small cell lung cancer (NSCLC). The last group of tumors included 1 case of clear cell large cell lung cancer, 9 cases of squamous cell carcinoma (SCC), and 20 cases of adenocarcinoma (AC). The clinico-pathological characteristics of lung cancer cases are presented in Table 1.

Immunohistochemical analysis. Tumor tissue samples were fixed in 4% formaldehyde for 24 h at room temperature, then dehydrated in 50%, 70%, 80%, 90%, 96% spirits, treated with chloroform, saturated with paraffin at 56 °C for 30 min and placed in paraffin

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Abbreviations used: AC – adenocarcinoma; hBD-2 – human beta-defensin-2; HD – high differentiation; LD – low differentiation; MD – moderate differentiation; NSCLC –non-small cell lung cancer; SCC – squamous cell carcinoma; SCLC – small-cell lung cancer.

blocks. 5 μm tissue slides were prepared with the use of microtom REICHERT-JUNG Mod. 1140/Autocut.

Immunohistochemical analysis of protein expression has been performed on paraffin slides with the use of a number of primary antibodies listed below, EnVision System and DAB reagent (DAKO, Denmark). The slides were twice deparaffinized with xylol (by 20 min), twice washed with 96% ethanol by 10 min, and washed with distilled water. The slides were placed in citrate buffer for 30 min and incubated in water bath at 95 °C. To block endogenous peroxidase activity, the slides were treated with Peroxidase-Blocking Reagent (DAKO, Denmark) according to instructions of the manufacturer.

Incubation with primary antibodies (rat polyclonal anti-hBD-2-Abs (Santa-Cruz, USA) 1 : 100; mouse monoclonal anti-PCNA-Abs (Dako, Denmark) — 1 : 200; mouse monoclonal anti-Bcl-2 (Dako, Denmark) — 1 : 200) was carried out for 1 h at room temperature in humidified atmosphere. To detect binding of primary antibodies, the slides were incubated with visualization system EnVision System (DAKO, Denmark) for 1 h at room temperature in humidified atmosphere. Then peroxidase activity was developed using diaminobenzidine solution (DAB, DakoCytomation, Denmark). Cell nuclei were stained with Meyer's hematoxylin. As negative control, the slides treated with normal mouse serum followed by DAB, were used. Microscopic examination was done with the use of Carl Zeiss Jena microscope (Germany).

The level of protein expression was evaluated by the percent of positive cells or/and by intensity of immunostaining with the use of H-score (counted as follows: H-score = 1 x W + 2 x M + 3 x S, where W, M, S are the percents of cells with weak, moderate and strong staining intensity respectively).

Statistical analysis. The statistical significance of the differences between the values (percent of hBD-2-positive cells, expressed as M±m) was assessed by the Student's *t*-test. Values $p < 0.05$ were considered statistically significant.

RESULTS

In our research we have studied expression of hBD-2 in 31 lung tumor tissue samples from the same patients. In order to find possible association of hBD-2 expression with the processes of lung cell proliferation or apoptosis, expression of proliferation cell nuclear antigen (PCNA) and antiapoptotic Bcl-2 protein has been studied as well.

Lung tumor samples were represented by histologically heterogenous group composed from 1 sample of SCLC and 30 samples of NSCLC; the latest group was composed from 1 case of clear cell large cell, 9 cases of SCC (8 low differentiatied (LD) tumors, 1 moderately differentiated (MD) tumors), and 20 cases of AC (6 LD AC, 6 MD AC, 2 of MD/LD AC, 1 case of mixed AC + SCC type, 1 case of papillary cancer, 2 cases of bronchoalveolar highly differentiatied (HD) cancer, and 2 cases of AC with the regions of clear cell carcinoma) (Table 1).

Table 1. The data for lung cancer patients and lung tumor samples used in the study

N	Case	Gender	Age	TNM classification	Histological type	Differentiation grade	Additional information
1	A	M	52	$T_2N_0M_0$	Small cell lung cancer		PL
2	B	M	-	$T_2N_0M_0$	Clear cell large cell lung cancer		PL
3	C1	M	62	$T_2N_0M_0$	SCC	LD	CL
4	C2	M	72	$T_2N_2M_1$	SCC	LD	PL
5	C3	M	68	$T_1N_0M_0$	SCC	LD	PL
6	C4	M	58	$T_2N_2M_0$	SCC	LD	PL
7	C5	M	68	$T_2N_0M_0$	SCC	LD	PL
8	C6	M	78	$T_2N_0M_0$	SCC	LD	PL
9	C7	M	57	$T_2N_1M_0$	SCC	LD	PL
10	C8	M	57	$T_2N_0M_0$	SCC	LD	CL
11	C10	M	50	$T_2N_1M_0$	SCC	MD	PL
12	D1	F	67	$T_2N_0M_0$	AC	LD	PL
13	D2	M	72	$T_3N_2M_0$	AC	MD	PL
14	D3	M	57	$T_2N_0M_0$	Papillary AC with mucus production	LD	PL
15	D4	M	59	$T_2N_0M_0$	AC	MD	PL
16	D6	M	54	$T_2N_1M_0$	AC	MD	PL
17	D7	F	54	$T_1N_0M_0$	AC	LD	PL
18	D8	M	55	$T_2N_0M_0$	AC	LD	PL
19	D9	M	56	$T_2N_2M_0$	AC	LD	PL
20	D10	M	41	$T_1N_0M_0$	AC	LD	PL
21	D11	M	76	$T_2N_1M_0$	AC with regions of solid clear cell cancer	LD	PL
22	D12	F	63	$T_2N_1M_0$	AC	MD/LD	PL
23	D13	M	44	$T_2N_1M_0$	AC	MD/LD	PL
24	D14	M	40	$T_2N_1M_0$	AC	MD	PL
25	D15	M	65	$T_2N_1M_0$	Mixed type (AC + SCC)		PL
26	D16	M	54	$T_2N_2M_0$	AC	MD	PL
27	D17	F	64	$T_2N_2M_0$	AC	MD	PL
28	D18	M	69	$T_3N_0M_0$	AC	LD	PL
29	D19	F	63	$T_1N_0M_0$	Bronchoalveolar cancer	HD	PL
30	D20	F	60	$T_1N_0M_0$	Bronchoalveolar cancer	HD	PL
31	D21	M	63	$T_2N_2M_0$	AC with regions of solid clear cell cancer	LD	PL

Notes: tumor differentiation: LD – low-differentiated; MD – moderately-differentiated; HD – highly-differentiated; SCC – squamous cell carcinoma; AC – adenocarcinoma; PL – peripheral localization, CL – central localization.

The study has been performed on paraffin embedded tissue blocks of the lung tumors with the use of immunohistochemical approach (Table 2, Fig. 1). Immunohistochemical analysis has revealed that the studied SCC cases of peripheral localization are characterized by moderate to very-high expression of hBD-2 (6 from 7 cases), while 2 cases of SCCs localized in bronchus express low levels of hBD-2 protein (< 10%). High percent of cells expressing PCNA or/and Bcl-2 proteins was detected just in 1/3 of SCC cases.

Immunohistochemical analysis of hBD-2 expression in AC samples has shown that AC of LD grade differs from AC of MD grade by lower hBD-2 expression ($p < 0.05$) (Table 2, Fig. 1). PCNA and Bcl-2 expression levels differ between the AC samples in a wide range (however, both markers were negative in HD bronchoalveolar cancer). There was observed no significant correlation between expression levels of mentioned above markers.

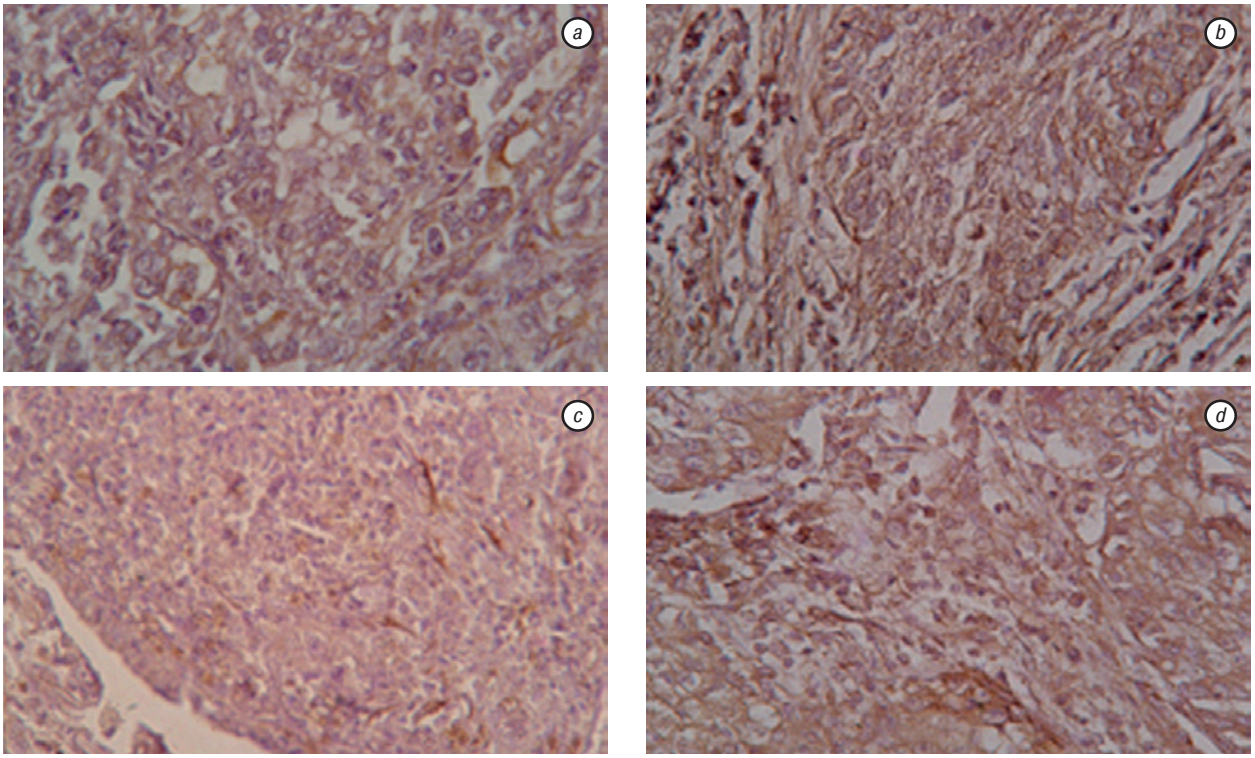

Fig. 1. Immunohistochemical analysis of hBD-2 expression in lung squamous cell carcinoma (cases C4 (*a*), C5 (*b*)) and lung adenocarcinoma (cases D18 (*c*), D21(*d*))

Table 2. Immunohistochemical analysis of hBD-2, PCNA, Bcl-2 expression in human lung tumor samples

№	Case	Expression level			
		hBD-2		PCNA	Bcl-2
		% positive cells	H-scoring	% positive cells	% positive cells
		Small cell lung cancer			
1	A	< 5%	5	90%	80%
		Non-small cell lung cancer: Clear cell large cell lung cancer			
2	B	15%	15	95%	50%
		Squamous cell carcinoma (LD)			
3	C1	10%	10	15%	30%
4	C2	20%	25	20%	7%
5	C3	58%	130	32%	25%
6	C4	45%	85	55%	60%
7	C5	100%	241	64%	60%
8	C6	50%	118	-	6%
9	C7	100%	200	0.5%	5%
10	C8	10%	15	20%	10%
		Squamous cell carcinoma (MD)			
11	C10	40%	65	50%	10%
		Adenocarcinoma (LD)			
12	D1	< 5%	10	50%	25%
13	D7	30%	56	80%	40%
14	D8	20%	29	40%	40%
15	D9	< 5%	3	35%	35%
16	D10	15%	30	20%	40%
17	D18	40%	172	35%	20%
		Adenocarcinoma (MD)			
18	D2	23%	137	60%	60%
19	D4	< 5%	3	ND	ND
20	D6	50%	100	60%	60%
21	D14	98%	219	-	25%
22	D16	90%	196	-	70%
23	D17	98%	138	-	10%
		Adenocarcinoma (MD/LD)			
24	D12	50%	110	10%	< 5%
25	D13	50%	142	50%	50%
		AC with regions of solid clear cell cancer			
26	D11	35%	64	30%	0.5%
27	D21	100%	150	40%	40%
		Bronchoalveolar cancer (HD)			
28	D19	45%	78	-	3%
29	D20	81%	216	1%	-
		Mixed type (AC + SCC)			
30	D15	100%	111	25%	10%
		Papillary AC			
31	D3	10%	81	30%	15%

According to the data of IHC analysis, beta-defensin-2 is distributed in tumor cell cytoplasm as well as in perinuclear space (Fig. 2).

DISCUSSION

Involvement of human antimicrobial peptides, in particular, defensins in antimicrobial protection of airways has been in spite of interest of many scientific groups. The patterns of hBD-2 expression in surface epithelial cells of human lung and bronchus has been firstly described in 1998 in the detailed research of Bals et al [7] who have showed hBD-2 up-regulation in conditions of chronic inflammation. In a short time the molecular mechanisms of hBD-2 induction in respiratory hTBE cells via NF-kB activation with involvement of CD14 and TLR has been reported in the study of Beker et al [8]. Since then, mailfunction or altered levels of defensins have been reported in several pulmonary disorders, in particular, pulmonary fibrosis, panbronchiolitis, alveolar proteinosis, acute respiratory distress syndrome, lung transplantation, etc [9]; at present time the exact role of defensins, multifunctional molecules with direct antimicrobial activity and immunomodulatory properties, in lung disorders is not fully understood and remains largely unstudied in lung cancer.

To our knowledge, our study is among the first ones where beta-defensin-2 expression has been recorded in human lung tumor samples with the use of immunohistochemical analysis. It has been revealed that all lung tumor samples independently on their histological type express hBD-2 peptide, however at different levels (from < 5% to 100% cells). In the majority of cases hBD-2 expression is moderate, in 4 cases very low (< 5% cells), and nearly in 1/3 of cases it is very high (close to 100% of cells, or > 200 by H-score). These data are

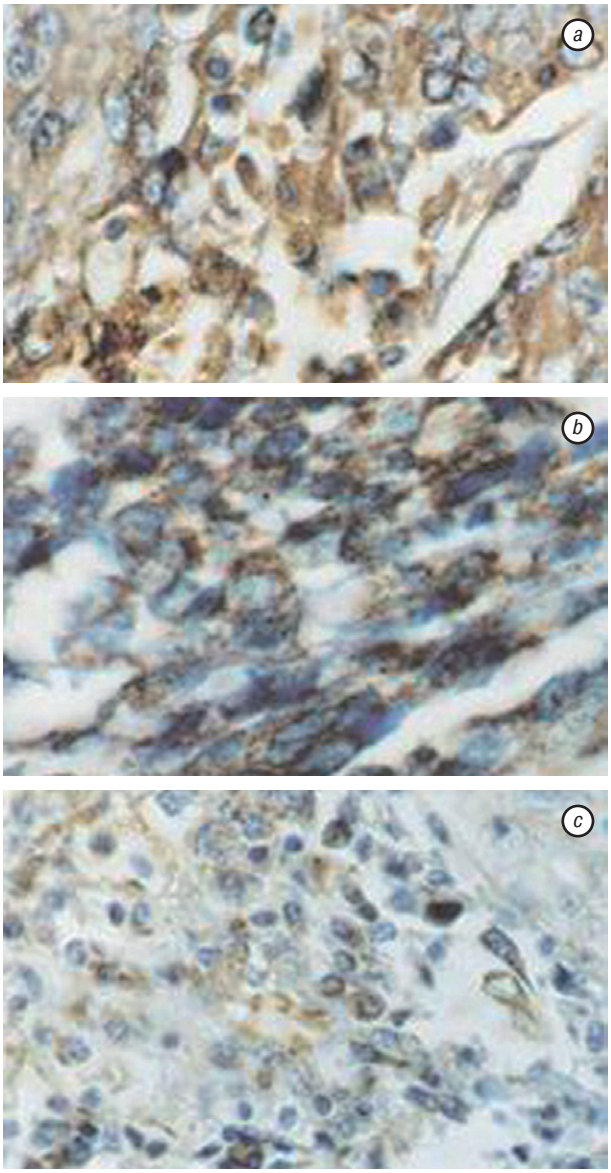

Fig. 2. Immunohistochemical analysis of hBD-2 expression in lung squamous cell carcinoma (case C7 (*a*)) and lung adenocarcinoma (cases D14 (*b*), D16 (*c*))

in some agreement with observations of Arimura *et al*. [10] who have recorded significantly elevated concentrations of hBD-1 and high concentrations of hBD-2 in blood serum of lung cancer patients compared to patients with pneumonia and healthy donors, and the data of our earlier pilot study that has documented the fact that an up-regulation of hBD-1 and hBD-2 mRNAs is a frequent event in both lung SCC and AC compared to normal lung tissue samples [6]. Unfortunately, as far as the data on the history of the patients studied in present study (concominant lung pathologies, smoking habits) were unavailable, there are no grounds to speculate on possible causes of elevated expression of the defensin in lung tumor tissues.

The hBD-2 expression levels in these samples seem to be not correlated with expression of proliferation marker PCNA and antiapoptotic Bcl-2 protein. Interestingly, we have detected also that in lung adenocarcinoma hBD-2 expression depend on tumor differentiation grade — low differentiated tumors differ from moderately differentiated ones by significantly lower percent of hBD-2 expressing cells ($p < 0.05$).

Functional significance of human defensin-2 up-regulation in lung tumor cells and possible relation between its expression and tumor differentiation grade remain to be studied in further researches.

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CASE REPORT

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TWO CASES WITH ATYPICAL METASTASIS IN COLORECTAL CANCER: SPLENIC AND RENAL METASTASIS

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Atypical metastasis, such as splenic and renal metastasis is rare in colorectal cancer. There have been case reports of colorectal cancer patients with isolated splenic metastasis, even after years of surgery in the literature. ***Aim:*** **To report two colorectal cancer cases with atypical metastasis.** ***Results:*** **The first patient was a 58-year old man who had isolated splenic metastasis after 20 months of surgery. The other one was a 51-year old male patient with both lung and renal metastasis at rectal cancer diagnosis. Splenic and renal metastases have been histopathologically documented in both of them. The first patient was given chemotherapy after splenectomy. The other one had also multiple lung metastases besides renal metastasis. He received palliative chemotherapy.**

Key Words**: atypical metastasis, isolated splenic metastasis, renal metastasis.**

Isolated splenic metastasis is rare in colorectal cancer. Malign melanoma, breast and ovarian cancers are the most common solid tumors in which splenic metastasis occurs [1]. Isolated splenic metastasis may be synchronous or metachronous. The rates of isolated splenic metastasis are reported as 4.4% for colon cancer and 1.6% for rectum cancer. It is generally diagnosed while evaluating asymptomatic cancer patients with increasing levels of carcinoembryonic antigen (CEA). In this report, our aim is to report two cases with atypical metastasis.

Case 1. A 58-year old male patient was admitted to the hospital with ileus a year ago. Hemicolectomy was performed after the diagnosis of colon carcinoma. Histopathology revealed colon adenocarcinoma without lymph node involvement (stage III). He was given adjuvant 5-flurouracil (5-FU)-based chemotherapy. He had a CEA level elevation [4.62 ng/mL (N: 0–3.4)] in blood serum fifteen months after hemicolectomy without any evidence of local or distant metastasis on evaluation. However, a splenic mass was diagnosed 5 months later. Splenectomy was performed, and histopathology revealed the metastasis of colon adenocarcinoma in the spleen. A 3.5 x 3 x 2.5 cm size tumor was located just beneath the capsule of the spleen (Fig. 1, *a, b*). Chemotherapy which consisted of 5-FU, leucovorine (LV) and oxaliplatin (FOLFOX-4) was given. He had a second relapse in the splenic localization (5.5 x 3 cm) after 11 months of first relapse. An increased uptake of 18-fluorodeoxyglucose (18-FDG) was observed only in this lesion with standardized uptake value (SUVmax) of 11.7 on 18-FDG-positron emission computerized tomography (18-FDG-PET-CT) (Fig. 2). Palliative chemotherapy including 5-FU, LV, irinotecan (FOLFIRI) and bevacizumab was started. The patient has no evidence of progression for 7 months after the second relapse.

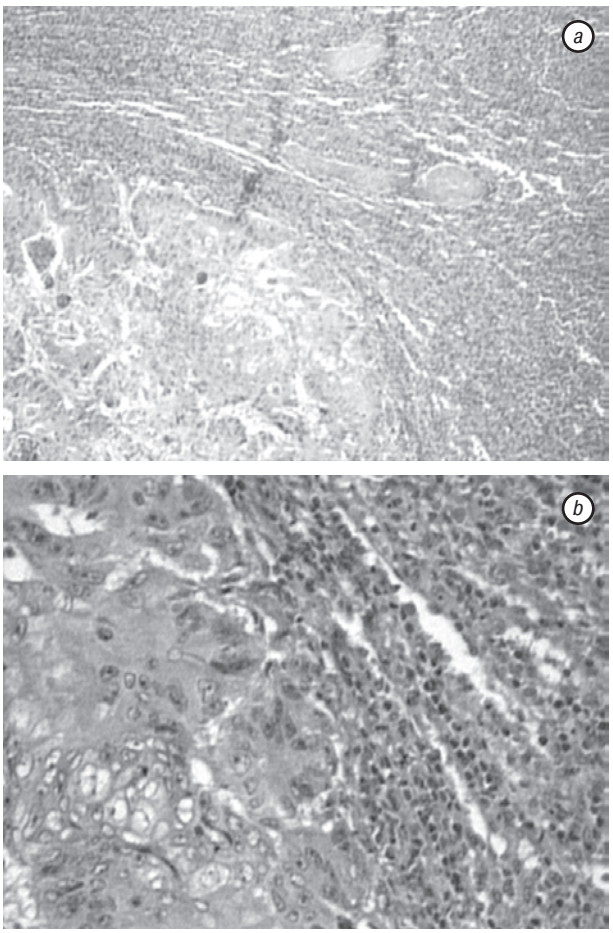

Fig. 1. Focal necrosis and atypical prismatic tumor cells with abortive organization, hematoxylin and eosin staining — 10X (*a*), 40X (*b*)

Case 2. A 51-year old male patient was admitted with 2 months of constipation and hematochezia. He was diagnosed as having rectal adenocarcinoma with bilateral lung metastasis and a solitary nodular lesion (1.8 x 1.5 cm) in the upper pole of right kidney. Renal mass SUVmax was 13.1 on 18-FDG-PET-CT (Fig. 3). Renal aspiration biopsy was performed to determine

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Abbreviations used: 5-FU – 5-flurouracil; 18-FDG – 18-fluorodeoxyglucose; CEA – carcinoembryonic antigen; CT – computerized tomography; PET-CT – positron emission computerized tomography.