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Lysosomal exoglycosidases in serum and urine of patients with pancreatic adenocarcinoma

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Abstract: Lysosomal exoglycosidases: N-acetyl-β-D-hexosaminidase (HEX), β-D-galactosidase (GAL), α-L-fucosidase (FUC) and α-D-mannosidase (MAN) modify oligosaccharide chains of glycoconjugates in endoplasmatic reticulum and/or Golgi apparatus and degrade them in lysosomes. In acid environment of lysosome, exoglycosidases degrade oligosaccharide chains of glycoproteins, glycolipids and glycosaminoglycans by eliminating single sugars from the edges of oligosaccharide chains. Neoplasms change biochemical processes in tissues and may significantly change the activity of many enzymes including the activity of lysosomal exoglycosidasses in serum and urine of persons with neoplasmatic diseases. The aim of the present paper was evaluation the activity of HEX, GAL, FUC and MAN in serum and urine of patients with pancreatic adenocarcinoma. Serum and urine samples were collected from 15 patients with adenocarcinoma of the pancreas and 15 healthy persons. The activity of lysosomal exoglycosidases was determined by the method of Marciniak et al. adapted to serum and urine of patients with adenocarcinoma of the pancreas. Our results indicate significant decrease in activity of GAL ($p=0.037$) in serum of patients with pancreatic adenocarcinoma, significant increase in activity of HEX ($p<0.001$) and FUC ($p=0.027$) in serum, and HEX ($p=0.003$) in urine, as well as significant decrease of FUC ($p=0.016$) and MAN ($p=0.029$) in urine o patients with adenocarcinoma of the pancreas, in comparison to the control group. Increase in activity of some lysosomal enzymes in serum and urine of pancreatic adenocarcinoma patients, may indicate on destruction of pancreatic tissue by pancreatic adenocarcinoma. Determination of the HEX, GAL, FUC and MAN in serum and urine may be useful in diagnostics of pancreatic adenocarcinoma.

Key words: N-acetyl-β-D-hexosaminidase; β-D-galactosidase; α-L-fucosidase; α-D-mannosidase; pancreatic adenocarcinoma; tumor markers.

Introduction

Basal lamina is specialized net of proteins and proteoglycans of extracellular matrix (ECM). The basal lamina acts as a selective barrier to the movement of the cell between epithelia of blood vessels and neighboring tissue, as a filter for molecules, and acts as an anchor for normal cells. The basal lamina may induce differentiation and restrains migration of the normal cells [1-3]. Ability of the cancerous cells to degradation of proteins and proteoglycan components of basal lamina, extracellular matrics and stroma of adenocarcinoma is connected with possibility of metastasis. To metastasize, cancer cell must penetrate a blood or a lymphatic vessel by crossing the basal lamina and endothelial lining of the vessel so as to enter the blood or lymph vessel, exit from the vessel elsewhere in the body, and then grow in the new site, forming the micromethastasis. Micrometastases must produce cells that survive and proliferate in new environment [2,3]. In development of neoplasm and its metastasis important role play structures of saccharide chains of glycoconjugates and enzymes that take a part in their synthesis and degradation *i.e.* glycosyltransferases and glycosidases [4,5]. Exoglycosidases facilitate degradation of proteins, by degradation of the oligosaccharide chains of glycoproteins covering protein cores. Lyso-

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Table 1. HEX, GAL, FUC and MAN (within-run) activity in blood serum and urine of 6 healthy people

	pKat/mL serum						pKat/mL urine					
Patients	1	2	3	4	5	6	1	2	3	4	5	6
Enzyme	HEX [11]						HEX [11]					
Mean	358.73	327.51	501.71	428.76	412.35	543.61	82.01	131.97	115.80	158.67	124.61	93.76
SD	14.87	12.37	32.43	22.14	22.49	31.27	5.22	7.12	7.68	8.77	9,71	8.72
CV	4.15	3.78	6.46	5.16	5.45	5.75	6.37	5.40	6.63	5.53	7,79	9.30
Mean CV±SD	5.125±1.004						6.837±1.484					
Enzyme	GAL						GAL					
Mean	108.02	95.98	101.27	108.46	113.57	71.48	60.39	59.64	71.98	56.68	60.80	111.09
SD	8.95	7.70	7.72	8.09	8.79	7.47	4.00	2.26	4.05	4.65	3.92	7.45
CV	8.28	8.02	7.63	7.46	7.74	10.45	6.63	3.80	5.63	8.20	6.45	6.70
Mean CV±SD	8.262±1.110						6.234±1.455					
Enzyme	FUC						FUC					
Mean	115.75	119.18	101.63	95.26	108.71	98.89	55.75	70.62	72.29	57.23	64.04	72.23
SD	11.08	12.15	11.82	9.17	10.70	10.43	4.22	2.81	5.37	5.01	3.57	5.32
CV	9.57	10.19	11.63	9.62	9.84	10.56	7.57	3.98	7.43	8.76	5.58	7.37
Mean CV±SD	8.792±0.668						6.780±1.710					
Enzyme	MAN						MAN					
Mean	113.70	122.10	116.21	112.33	123.25	112.94	65.77	55.82	73.22	56.87	62.68	71.47
SD	10.08	11.18	10.74	13.44	9.45	10.14	5.77	5.48	5.01	4.00	2.91	4.32
CV	8.86	9.16	9.24	11.97	7.67	8.98	8.78	9.81	6.84	7.03	4.65	6.05
Mean CV±SD	9.312±1.421						7.193±1.860					

somal exoglycosidases: N-acetyl-β-D-hexosaminidase (HEX – E.C. 3.2.1.30), β-D-galactosidase (GAL – E.C. 3.2.1.23), α-L-fucosidase (FUC – E.C. 3.2.1.51) and α-D-mannosidase (MAN – E.C. 3.2.1.24) in acid environment of lysosome release single sugars from the edges of oligosaccharide chains of glycoconjugates [6].

The aim of our research was determination the activity of lysosomal exoglycosidases: HEX, GAL, FUC and MAN in serum and urine as a potential markers of pancreatic adenocarcinoma.

Materials and methods

Patients. The urine and blood samples were obtained from 15 patients (7 males and 8 females) aged from 44 to 74 years (mean 59±9.29 years), who were diagnosed histopathologically as bearing pancreatic adenocarcinoma (G 2). All patients were operated in 1st Department of General Surgery and Endocrinology of the Medical University of Białystok. Before the examination, patients were subjected neither to chemotherapy, nor radiotherapy. The control group included 15 healthy people (6 females and 9 males) aged from 22 to 62 years (mean 35±10.45 years).

The clinical exclusion criteria from the study were: diabetes, rheumatoid arthritis, glomerular nephritis, alcohol abuse.

Sample material. The blood samples were obtained from the elbow vein before operation and then they were clotted. The urine samples were taken from the middle stream of the morning portion. The urine and blood were centrifuged at 4,000 × g for 10 min. The supernatant fluids were portioned and stored at the temperature of -80°C.

Glycosidases activity assays. HEX, GAL, FUC and MAN activity were determined by Marciniak's *et al.* method [7] modified by Szajda *et al.*, [8,9] for determination of exoglycosidases in serum and urine. To 10 µL of appropriately diluted urine and serum were added: 40 µL of 0.1M phosphate-citrate buffer at pH 4.7 for HEX and pH 4.3 for remaining exoglycosidases, and 30 µL of 20 mM substrate solution of p-Nitrophenyl-N-acetyl-ß-D-glucosaminide, for determination of HEX; p-Nitrophenyl-β-D-galactopyranoside for GAL, p-Nitrophenyl-α-L-fucopyranoside for FUC and p-Nitrophenyl-α-D-mannopyranoside for MAN determination (Sigma, St. Louis, MO, USA), in 0.1 M of phosphate-citrate buffer at pH 4.7 for HEX and 4.3 for the remaining exoglycosidases. The mixtures were incubated for 60 min at a temperature of 37°C. The reactions were stopped by adding 200 µL of 0.2M borate buffer at pH 9.8. The activity of lysosomal exoglycosidases corresponding to the amounts of released p-nitrophenol, were measured in 405 nm, using the microplates reader EL_X800^{TM} and computer program KC junior (Bio-Tek Instruments, Winooski, VT, USA). The concentration of the activity of lysosomal exoglycosidases in serum and urine was expressed in pKat/mL.

Quality control the determinations of HEX, GAL, FUC and MAN. Reproducibility tests (precision in the series; within-run and day-to-day) of the HEX, GAL, FUC and MAN determinations were performed in serum and urine of 6 control persons according to the standard procedures (Table 1, 2) [10].

Table 2. HEX, GAL, FUC and MAN (day-to-day, between-day) activity in blood serum and urine of 6 healthy people

	pKat/mL serum						pKat/mL urine					
Patients	1	2	3	4	5	6	1	2	3	4	5	6
Enzyme	HEX						HEX					
Mean	303.93	368.746	412.03	371.72	310.88	273.66	139.54	153.39	143.82	156.91	144.78	181.60
SD	33.42	46.11	42.50	44.02	38.91	31.64	18.05	21.81	23.04	29.77	19.16	25.75
CV	11.00	12.01	10.32	11.84	12.52	11.56	12.94	14.22	16.02	18.97	13.23	14.18
Mean CV±SD	11.622±0.864						14.927±2.256					
Enzyme	GAL						GAL					
Mean		124.68	95.45	105.74	105.43	92.53	45.90	49.77	70.21	55.53	58.06	93.48
SD	13.32	17.76	11.95	12.01	12.13	14.95	7.22	8.08	10.61	8.23	9.40	15.17
CV	12.64	14.24	12.52	11.35	11.50	16.15	15.72	16.23	15.11	14.82	16.20	16.23
Mean CV±SD	13.069±1.831						15.719±0.620					
Enzyme	FUC						FUC					
Mean	127.14	122.53	126.99	126.92	99.08	106.58	50.57	48.50	70.47	58.68	60.93	72.60
SD	13.21	15.01	12.67	14.99	13.93	11.89	4.91	7.73	9.70	5.42	6.72	8.41
CV	10.39	12.25	9.98	11.81	14.06	11.15	9.70	15.94	13.77	9.23	11.03	11.59
Mean CV±SD	11.606±1.469						11.877±2.553					
Enzyme	MAN						MAN					
Mean	105.54	102.21	98.45	102.81	101.92	109.81	52.11	65.72	74.76	70.22	64.29	76.80
SD	14.92	13.49	14.52	18.43	18.87	18.34	9.05	10.20	10.71	11.90	10.04	11.47
CV	14.13	13.20	14.74	17.93	18.52	16.69	17.37	15.51	14.33	16.95	15.62	14.94
Mean CV±SD	15.870±2.160						15.785±1.165					

Ethical issues. The study was approved by the Bioethical Committee of Bialystok Medical University who gave permission numbers R-I-003/153/2005 and R-I-003/300/2006 for our examinations.

Statistical analysis. The data are presented as means±standard deviation (SD). Statistical analysis was performed using the SPSS® statistical package (SPSS Inc., Chicago, IL, USA). The data were verified for normal distribution using the Kolmogorov-Smirnov test, and their distributions were found to be normal, the Student's-t test for unpaired observations was used to determine the significance of differences, with $p<0.05$ was considered statistically significant. The sensitivity and specificity of the method were calculated used NCSS statistical package.

Results

Coeficients of variations (CV) calculated on the basis of determinations performed in the series (within-run) of serum and urine of 6 control persons were in the case of HEX: 5.125±1.004 for serum and 6.837±1.484 for urine [11]. Coefficients of variations calculated on the basis of determinations performed in the series (within-run) of serum and urine of 6 control persons were in the case of: GAL 8.262±1.110 for serum and 6.234±1.455 for urine, in the case of FUC: 8.792±0.668 for serum and 6.780±1.710 for urine and for MAN: 9.312±1.421 for serum and 7.193±1.860 for urine (Table 1).

Coefficients of variations for determinations between series (day-to-day, between-day) based on measurements performed in serum and urine obtained from 6 healthy persons were in the case of HEX: 11.622±0.864 for serum and 14.927±2.256 for urine, GAL: 13.069±1.831 for serum and 15.719±0.620 for urine, FUC: 11.606±1.469 for serum and 11.877±2.553 for urine, and MAN: 15.870±2.160 for serum and 15.785±1.165 for urine (Table 2).

We found significant decrease in activity of GAL ($p=0.037$), significant increase in activity of HEX ($p<0.001$) and FUC ($p=0.027$) in serum of patients with pancreatic cancer and significant increase in activity of HEX ($p=0.003$) in urine, as well as significant decrease in urinary activity of FUC ($p=0.016$) and MAN ($p=0.029$) of patients with pancreatic adenocarcinoma. We did not observed significant differences in activities of MAN in serum and GAL in urine of pancreas adenocarcinoma patients in comparison to the control (Table 3, Fig. 1, 2).

In examining the power of the test we found huge effect of HEX in the serum (effect size=2.41) and very large effect for urine (effect size=1.28), large effect of GAL (effect size=0.80) and FUC (effect size=0.85) in serum and FUC (effect size=0.96) and MAN (effect

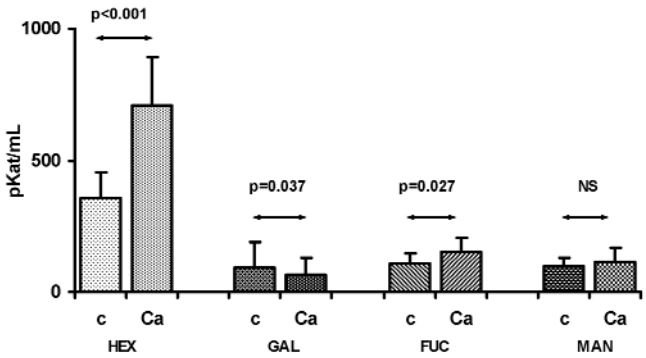

Fig. 1. Concentrations of the activities of HEX, GAL, FUC and MAN in blood serum of patients with adenocarcinoma of the pancreas. c: control group; Ca: pancreatic adenocarcinoma.

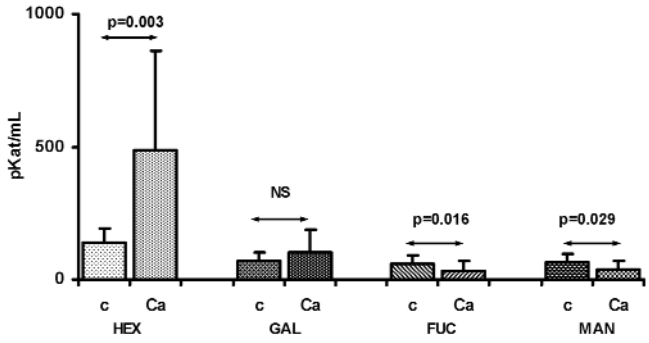

Fig. 2. Concentrations of the activities of HEX, GAL, FUC and MAN in urine of patients with adenocarcinoma of the pancreas. c: control group; Ca: pancreatic adenocarcinoma.

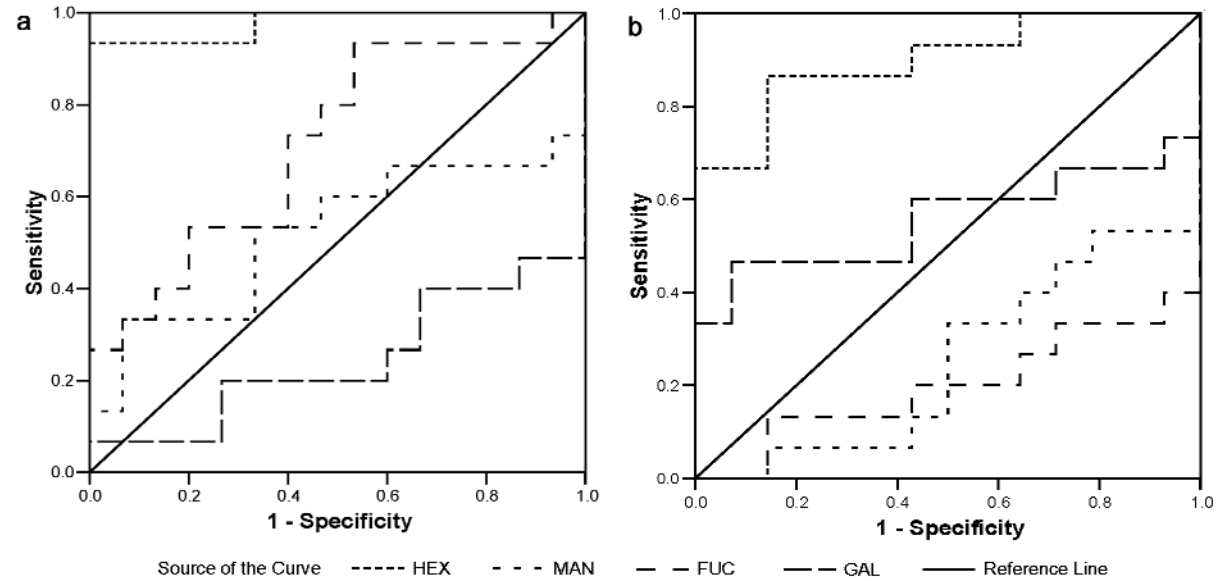

Fig. 3. Sensitivity and specificity of determination of HEX, GAL, FUC and MAN activity in (a) blood serum and (b) urine of patients with adenocarcinoma of the pancreas.

size=0.86) in the urine, a medium effect for GAL (effect size=0.52) in the urine and small effect for MAN (effect size=0.31) in serum.

These results proved a high diagnostic value (AUC: 0.97778 and 0.71556; p[AUC=0.5]: 0.0001 and 0.0251) for the activity of HEX and FUC in serum, respectively as well as (AUC: 0.9000; p[AUC=0.5]: 0.0001) for HEX respectively in the urine of patients with pancreatic adenocarcinoma, in comparison with the activity in healthy people (Table 4).

The concentration of HEX activity in serum of patients with pancreatic cancer has 93.33% sensitivity and 100% specificity at the value of cut-off > 494.42 pKat/mL, GAL has 40.00% sensitivity and 33.33% specificity for the value of cut-off >70.76 pKat/mL, FUC has 73.33% sensitivity and 60.00% specificity for the value of cut-off >113.62 pKat/mL, and MAN has 53.33% sensitivity and 66.67% specificity for the value of cut-off >106.83 pKat/mL (Table 5, Fig. 3a). The concentration of HEX activity in urine of patients with pancreatic cancer has 86.67% sensitivity and 85.71% specificity for the value of cut-off >168.63 pKat/mL, GAL has 60.00% sensitivity and 57.14% specificity for the value of cut-off >71.11 pKat/mL, FUC has 33.33% sensitivity and 28.57% specificity for the value of cut-off >45.40 pKat/mL, and MAN has 53.33% sensitivity and 21.43% specificity for the value of cut-off >39.68 pKat/mL (Table 5, Fig. 3b).

Discussion

Currently, for diagnostics and monitoring of pancreatic adenocarcinoma are used USG and computer tomography as well as protein markers associated

Table 3. Activities of HEX, GAL, FUC and MAN in serum and urine of patients with pancreatic adenocarcinoma

Exoglycosidase		HEX	GAL	FUC	MAN
Blood serum	N	[pKat/mL] *p*	[pKat/mL] *p*	[pKat/mL] *p*	[pKat/mL] *p*
Pancreatic adenocarcinoma patients Control group	15 15	710.76±183.61 <0.001 357.02±96.14	64.87±41.81 =0.037 93.95±30.04	150.36±55.58 =0.027 110.39±36.07	113.38±55.45 NS 99.70±28.41
Urine		[pKat/mL]	[pKat/mL]	[pKat/mL]	[pKat/mL]
Pancreatic adenocarcinoma patients Control group	15 15	432.97±316.30 =0.003 136.62±58.21	106.07±94.20 NS 69.01±31.64	34.58±32.42 =0,016 64.69±30.36	39.49±28.51 =0.029 64.69±30.36

Table 4. Diagnostic value the concentration of HEX, GAL, FUC and MAN activity in blood serum and urine of patients with adenocarcinoma of the pancreas

	N	n(-)	n(+)	AUC	SE	95% C.I. (AUC)	*p* (AUC=0.5)
Blood serum [pKat/mL]							
HEX	30	15	15	0.97778	0.02376	(0.829-0.997)	0.0001
MAN	30	15	15	0.52000	0.11536	(0.260-0.709)	0.8624
FUC	30	15	15	0.71556	0.09627	(0.471-0.858)	0.0251
GAL	30	15	15	0.24444	0.09480	(0.052-0.420)	0.0070
Urine [pKat/mL]							
HEX	30	15	15	0.90000	0.05775	(0.705-0.969)	0.0001
MAN	30	15	15	0.25238	0.09258	(0.064-0.423)	0.0075
FUC	30	15	15	0.20000	0.08826	(0.022-0.365)	0.0007
GAL	30	15	15	0.55714	0.11950	(0.281-0.748)	0.6325

n – number of cases included in the analysis; n(-) – number of negative cases; n(+) – number of positive cases; AUC – area under curve; SE – standard error for assessment of AUC; 95%C.I. (AUC) – 95% confidence interval of determined AUC; interval that with 95% confidence contains the real value of AUC for the population; p(AUC=0.5) – p-value of the test checks a diagnostic value of the method. If AUC varies significantly from 0.5, it means that the method differentiates significantly the study cases into positive and negative.

Table 5. Sensitivity and specificity of determination of HEX, GAL, FUC and MAN activity in blood serum and urine of patients with adenocarcinoma of the pancreas

	Blood serum				Urine			
	concentration of activity (pKat/mL)				concentration of activity (pKat/mL)			
	HEX	GAL	FUC	MAN	HEX	GAL	FUC	MAN
Cut-off	>494.42	>70.76	>113.62	>106.83	>168.63	>71.11	>45.40	>39.68
Sensitivity	93.33%	40.00%	73.33%	53.33%	86.67%	60.00%	33.33%	53.33%
Specifity	100.0%	33.33%	60.00%	66.67%	85.71%	57.14%	28.57%	21.43%

with pancreatic adenocarcinoma: carcinoembrionic antigen (CEA) and carcinous antigen of alimentary tract (CA 19.19) [12,13].

Serum concentration of CEA significantly increases in colon adenocarcinoma as well as adenocarcinomas of: pancreas, stomach, lungs, reproductive organs

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and urinary bladder. Significant increase in serum concentrations of CEA occurs in nonepithelial neoplasms (neuroblastomas, sarcomas, lymphomas) and in non neoplastic diseases as: hepatitis and hepatic cirrhosis, chronic pancreatitis, chronic gastric and duodenal ulcer disease, ulcerative colitis as well as in some physiological states *e.g.* pregnancy. CEA has limited diagnostic sensitivity and specificity, which made difficult use of CEA in diagnostics of defined neoplasm and use in screening tests [14].

Ca 19-9 antigen now used in diagnosis of pancreatic adenocarcinoma, initially was determined in diagnostics of colon cancer [15]. Presently it is generally understood that concentration of Ca 19-9 increases in cases of carcinomas of alimentary tract, particularly pancreas and gallbladder and in inflammatory diseases of alimentary tract, liver and pancreas. [14,15].

Healthy cells and tissues demonstrate stable enzymatic activity. In pathological states activity of one or several enzymes change. Neoplastic cells produce and secrete to extracellular space normal and pathological components of connective tissue, and among them glycosaminoglycans, proteoglycans and collagens. Neoplastic cells produce different hydrolytical enzymes, and among them lysosomal exoglycosidases which are able to degradation of connective tissue barriers [3,16].

In our previous investigations we analyzed mostly usefulness of N-acetyl-β-hexosaminidase (HEX) and its isoenzymes A and B, the most active of lysosomal exoglycosidases, in detection and monitoring of neoplasmatic diseases. Determination the activity of HEX, HEX A and B in serum may be useful in differential diagnosis cancers of thyroid, renal and pancreatic adenocarcinoma, because in serum of thyroid increases only HEX A activity [17]; HEX, HEX A i HEX B significantly increase in real cancer [18,19], instaed in panreatic adenocarcinoma significantly increase HEX and HEX A [20]. We found high diagnostic value of determination the activity of HEX, HEX A and HEX B in serum and urine of patients with colon cancer [11].

Determination the activity of HEX A and HEX B in cancerous renal tissue by colormetric and electrophocusing methods, gave similar results [19]. As colorimetric method is simpler, easier and cheaper, therefore we recommend it for determinations multiple samples.

Colon cancer is one of the main death causes, because of late cancer diagnosis. In our opinion determination the activities of N-acetyl-β-D-hexosaminidase (HEX), β-galactosidase (GAL), α-mannosidase (MAN) and α-fucosidase (FUC) in serum and urine alongside of routinelly determined cancer markers may create substantial progress in diagnostic of pancreatic cancer. Our results (Table 3, Figs 1, 2), indicate on significant differences in activity of lysosomal exoglycosidases in serum and urine of patients with pancreatic adenocarcinoma in comparison to patients with colon cancer [9]. Our preliminary results indicate on possibility of differential diagnosis pancreatic and colon cancers, by determination of lysosomal exoglycosidases. In serum of patients with pancreatic adenocarcinoma, activity of GAL significantly decreases whereas there is lack of differences in GAL activity in urine (Table 3, Fig. 1). On the other hand in serum and urine of patients with colon cancer activity of GAL significantly increases in comparison to control [9]. There was significant decrease in activity of FUC and MAN in urine of patients with pancreatic adenocarcinoma (Table 3, Fig. 2) and no changes in colon carcinoma, in comparison to control, which may help in differential diagnosis of pancreatic and colon carcinomas [9]. We observed significant increase in activity of FUC ($p<0.0001$) in serum of patients with colon cancer [9], but in serum of patients with pancreatic cancer, significance was at the level of $p=0.0269$ (Table 3, Fig. 1).

In a summary it may be stated, that our results suggest possibility of easy and cheap determinations the activity of lysosomal exoglycosidases in serum and urine for diagnostics of pancreatic cancer. Our investigations suggest possibility of use of profile of lysosomal exoglycosidases in differential diagnosis of pancreatic adenocrcinoma with other neoplasms.

Conclusions

1. Pancreatic adenocarcinoma significantly changes activity of lysosomal exoglycosidases in serum and urine.
2. Determination the activity of lysosomal exoglycosidases in serum and urine may be suitable for differential diagnosis of pancreatic cancer.
3. Simplicity and low costs of exoglycosidases determinations in serum and urine imply their use in screening of cancers.

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