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Separation and Study of the Range of Plasminogen Isoforms in Patients with Prostate Cancer

E. F. Shin[1]*, D. I. Vodolazhsky[1], A. Yu. Golikov[1], T. N. Belova[1], N. V. Boyko[1], D. V. Zimakov[1], E. N. Cherkasova[1], M. I. Kogan[2], M. B. Chibichian[2], S. A. Moshkovskii[3], V. A. Tarasov[1], and D. G. Matishov[1]

[1]*Institute of Arid Zones, Southern Scientific Center of the Russian Academy of Sciences, pr. Chekhova 41, 344006 Rostov-on-Don, Russia; fax: (863) 266-5677; E-mail: johnnyeugenie@gmail.com*
[2]*Rostov State Medical University, Pereulok Nakhichevanskii 29, 344022 Rostov-on-Don, Russia*
[3]*Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, ul. Pogodinskaya 10, 119121 Moscow, Russia*

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Abstract—Using affinity chromatography, two-dimensional electrophoresis, and MALDI-TOF mass spectrometry, plasminogen isoforms were separated and identified in blood plasma. Healthy donors and patients with prostate cancer in various stages of development were included in the studied sample. With the development of prostate cancer, four additional specific plasminogen isoforms are registered in blood plasma; they are characterized by lower molecular weights and higher p*I* values compared to isoforms found in the control group.

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Key words: plasminogen isoforms, two-dimensional electrophoresis, MALDI-TOF-MS, prostate cancer

The study of plasminogen is of interest because it is the precursor of plasmin, a serine protease that is a key factor in the maintenance of normal [1, 2] and pathological processes [3], including carcinogenesis [4, 5], in cells. Functional activity of plasmin is associated with destruction of the extracellular matrix and activation of precursor proteins involved in controlling cell division and embryogenesis. In carcinogenesis, these functions of plasmin directly or indirectly determine the course of invasion [6], angiogenesis [7, 8], and metastasis [9], which are associated with malignant transformation of cells. The activity of plasmin is dependent not only on the characteristics of cell metabolism, which determine the ratio of the activity of plasminogen activators and inhibitors [2], but also the nature of modifications in the amino acid sequence of plasminogen [10, 11]. Plasminogen is a glycoprotein in blood plasma, where it is present in two main forms called plasminogen I and plasminogen II [12]. Plasminogen I is characterized by glycosylation of Trp346 and Asn289, while plasminogen II is glycosylated only at Trp346 [13, 14]. However, based on these two major isoforms of plasminogen, there are subisoforms that differ in the size of the attached sugar moiety. The set of isoforms includes five or six major variants [15].

There are a number of examples that show that the functional activity of plasminogen depends on the degree and type of its glycosylation [15, 16]. In particular, it was found that plasminogen II is more active than plasminogen I [12]. The degree of glycosylation determines not only the level of activity of plasminogen, but also the specificity of this activity. It was found [17] that only highly glycosylated forms of plasminogen bind to the receptors that activate the expression of matrix metalloproteinase-9, which is involved in the destruction of the extracellular matrix. For a number of proteins, it was shown that the development of malignant tumors correlates with an increase in the proportion of *O*-type glycosylation. In particular, there is a plasminogen isoform that is characterized by high levels of *O*-type glycosylation that increases the capacity for invasion of prostate cancer cells *in vitro* [17].

Abbreviations: DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MASCOT score, index of reliability; MS, mass spectrometry; *m/z*, mass/charge ratio; PBS, phosphate buffered saline.

* To whom correspondence should be addressed.

However, the role of posttranslational modifications of plasminogen, in particular glycosylation, in the emergence of somatic abnormalities, especially the development of malignant tumors of various types and localization, is not well understood. The goal of this work was to analyze the spectrum of plasminogen isoforms at different stages of human prostate tumor development.

MATERIALS AND METHODS

Patients. Blood samples were collected from male patients 61 to 70 years of age: nine patients had prostate adenoma, 17 patients had local prostate cancer (stage T_1-T_2), 20 patients had locally advanced prostate cancer (stage T_3-T_4), and 16 were apparently healthy donors (control). All participants of the study were fully informed of its terms and expressed in writing their consent to participate in it.

Preparation of samples. Peripheral blood was sampled by venipuncture using tubes containing EDTA K3 blood stabilizer (Sarstedt, Germany). The blood samples were centrifuged at 10*g* for 15 min at 4°C, then the supernatant (plasma) was separated and stored at −80°C until use.

Affinity chromatography. Plasminogen was preparatively separated by a modification of the method of Deutsch and Mertz [18]. A sample of blood plasma (2 ml) after slow thawing was mixed with 50 mM PBS, pH 7.5, at ratio 1 : 1 (v/v) and applied to a column of lysine-Sepharose (GE Healthcare, Sweden) connected to a BioLogic DuoFlow liquid chromatography system (BioRad, USA). Target proteins were washed from the column at rate 0.3 ml/min with 20 ml of buffer A (50 mM PBS, 0.15 M NaCl, pH 7.5) and 6 ml of buffer B (1 M NaCl, 0.2 M ε-aminocaproic acid, 50 mM PBS, pH 7.5) in isocratic mode, the fraction volume being 2.5 ml. The yield of protein was determined by absorption at 280 nm. The fractions were concentrated and desalted using Amicon Ultra-4 10-kDa filters (Millipore Corporation, USA). Protein was assayed by the Bradford method [19], after which the samples were frozen and stored at −80°C until electrophoresis in polyacrylamide gel.

Deglycosylation. Deglycosylation of chromatographically purified preparation of plasminogen was performed using EDEGLY-1KT reagents (Sigma-Aldrich, USA) according to manufacturer's instructions under denaturing conditions (3 h, 37°C) with the following enzymatic components: glycopeptidase, endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase), α-2(3,6,8,9)-neuraminidase, β-1,4-galactosidase, and β-*N*-acetylglucosaminidase. The resulting isoforms were analyzed using two-dimensional electrophoresis followed by silver nitrate staining.

Two-dimensional electrophoresis. For two-dimensional electrophoresis of the chromatographically isolated proteins, a 16-μg aliquot was applied to the gel. As standards of molecular weight and p*I*, sets of proteins from Bio-Rad were used. The sample was dissolved in buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 2% ampholytes 3-10). The dissolved samples were loaded into a PROTEAN IEF Cell for isoelectrofocusing (BioRad) using 18 cm strips, pH 5-8 (BioRad), in active rehydration mode (50 V, 12 h). A standard protocol of isoelectrofocusing of the proteins on the strips was used: 250 V for 1 h, 5 h with a linear increase in voltage to 3500 V, and 5 h at 3500 V to the total of 60,000 V·h [20]. After isoelectrofocusing the strip was equilibrated for 12 min with 120 mM Tris-HCl, pH 8.8, containing 6 M urea, 2% SDS, 20% glycerol (v/v), and 2% DTT. Then it was equilibrated for 15 min with the same buffer but containing 3.5% acrylamide instead of DTT [21]. Electrophoretic separation in second direction was performed in 12% polyacrylamide gel in Laemmli buffer [22] using a PROTEAN XL Multi-Cells vertical apparatus (Bio-Rad). The gels were fixed, washed, and stained with silver nitrate [23].

Visualization and analysis of gels. The gels were scanned using an ImageQuantTM 300 digital camera (GE Healthcare). The intensities of spots on the electrophoregrams were compared using PDQuest version 7.4.0 (BioRad).

Identification of proteins by MALDI-TOF mass spectrometry. Protein spots of 2-mm diameter were cut from the gel, destained, subjected to trypsin degradation according to Shevchenko et al. [24], and then mixed with matrix material – 2,5-dihydroxybenzoic acid (Sigma) [25]. Mass spectra of tryptic protein hydrolysates were obtained using an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). For each point on the target, 3000 spectra were accumulated in manual mode. The voltage on the ion source and reflector was 19 and 20 kV, respectively. FlexControl version 2.4 (Bruker Daltonics) and FlexAnalysis version 2.4 software (Bruker Daltonics) were used for analysis of the mass spectral data. The proteins were identified using the BioTools version 3.0 program (Bruker Daltonics). The Swiss-Prot database was searched for the mass table of each protein spot using the local version of the Mascot Search program version 2.2.06. The following parameters were used: accuracy of mass, 70 parts per million; NCBI protein database; *Homo sapiens* taxon; one missed cleavage; oxidation of methionine residues; alkylation of cysteine residues in acrylamide.

Results of protein identification were accepted as valid at a significance level of at least 95% and the index of overlapping sequences ⩾50%. In the analysis of peptide patterns (*m/z* peaks), the distinct characteristics of the matrix and the resolution of the instrument and the method in general were taken into the account.

Statistical analysis. Statistically significant differences between the frequency of occurrence of individual

plasminogen isoforms in the control and the experimental samples were determined using the χ^2 method. The distribution of *a posteriori* probabilities of the stage of prostate cancer for the individual patient was evaluated for spectra of plasminogen isoforms using discriminant analysis in the Statistica 7.0 program.

RESULTS AND DISCUSSION

Separation and analysis of plasminogen isoforms. The isoforms of plasminogen in plasma were isolated and identified in three stages. In the first stage, proteins containing lysine-binding domains were isolated from the total pool of blood plasma proteins using affinity chromatography. In the second stage, this fraction was subjected to electrophoretic separation using two-dimensional electrophoresis (Fig. 1). Four groups of proteins similar in molecular weight (M) and pI were seen in the electrophoregram: group 1 (M = 43-66 kDa and pI 4.8-5.1), group 2 (M = 66-76 kDa and pI 5.5-5.8), group 3 (M > 76 kDa and pI 6.0-8.0), and group 4 (M = 43-66 kDa and pI 6.3-8.5). In the third stage, MALDI-TOF mass spectrometry analysis of the proteins from groups 2 and 3 were identified as sets of isoforms of albumin and plasminogen, respectively. It is evident that the third group of proteins is represented by different isoforms of plasminogen with a high level of significance ($p < 0.001$) (Table 1). For protein groups 1 and 4, this analysis was not performed due to insufficient amounts of protein in the samples.

Mass spectrometric analysis was performed for all of the plasminogen isoforms present in the control group and in patients with prostate adenoma and local and locally advanced prostate cancer (Table 1). In all cases we found plasminogen isoforms differing in values of M and pI. Figure 2 shows typical fragments of a gel with sets of plasminogen isoforms at different stages of development of prostate cancer.

Different isoforms of plasminogen in the control group and in groups of patients with adenoma and local and locally advanced prostate cancer were identified using PDQuest. As the degree of tumor development increased, the number of plasminogen isoforms

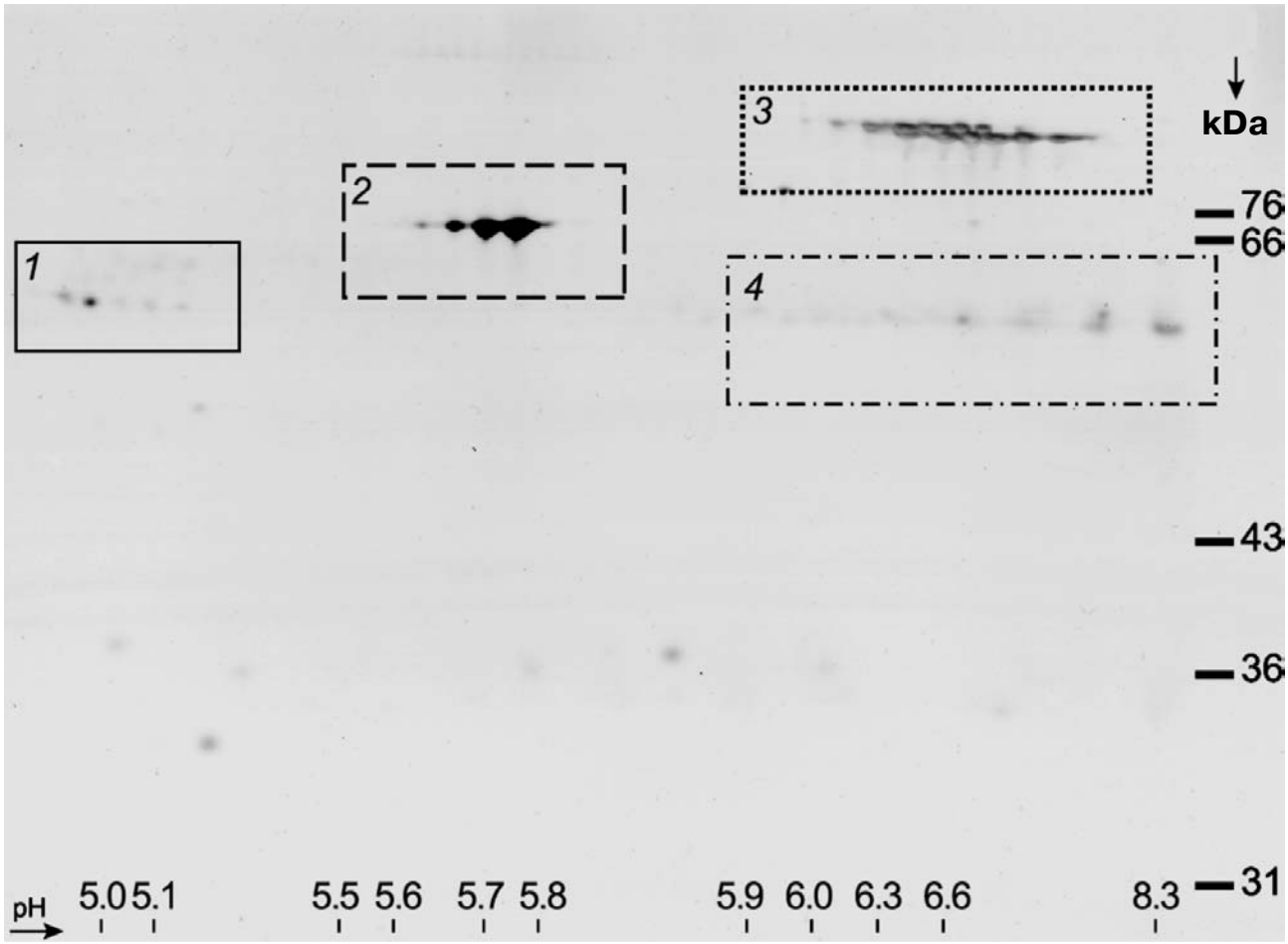

Fig. 1. A sample of the gel fraction after separation of proteins by affinity chromatography.

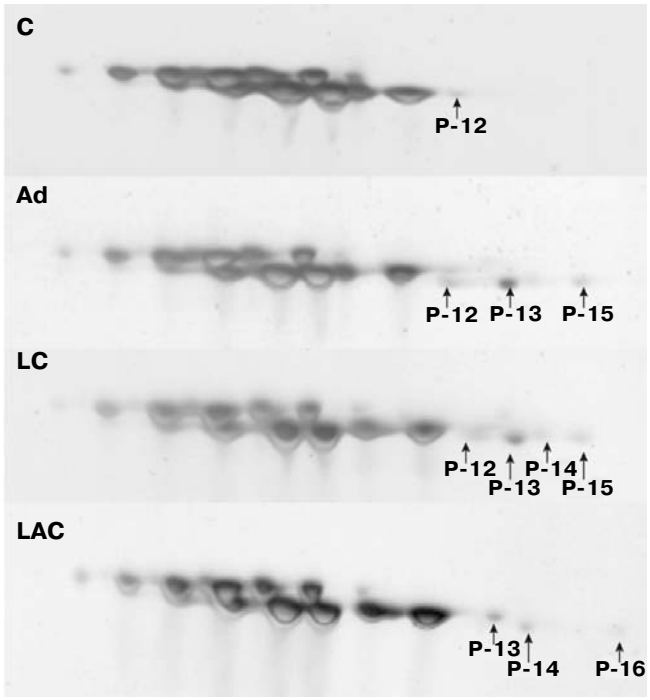

Fig. 2. Spectrum of plasminogen isoforms of control group (C) and patients with prostate adenoma (Ad), local cancer (LC), and locally advanced cancer (LAC). The arrows indicate the variable isoforms of plasminogen.

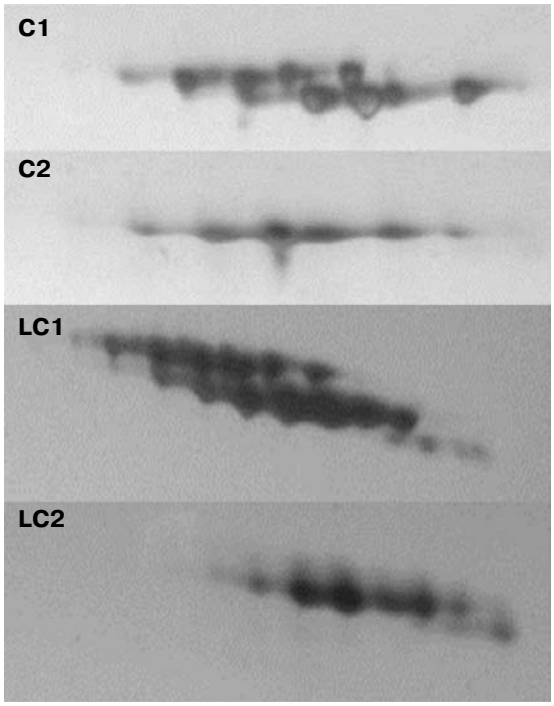

Fig. 3. Spectra of plasminogen isoforms of the control group and of the patient group with local cancer before deglycosylation (C1 and LC1, respectively) and after deglycosylation (C2, LC2, respectively).

increased. In the control group 12 isoforms of plasminogen were identified, in the group with benign tumor 14 isoforms, and in the groups with local and locally advanced cancer there were 15 and 16 isoforms, respectively.

Analysis of the spectra of isoforms of plasminogen in the experimental groups compared with control showed that isoforms P-1 to P-11 were present in all studied cases, regardless of whether they were of the control group or the patient groups. Isoforms from P-12 to P-16, which are characterized by lower M values and higher pI values, were found in the patient groups.

In most cases, the differences in the plasminogen isoforms were associated with differences in the degree and type of glycosylation of amino acid residues of the polypeptide chain. However, the literature describes other types of modifications of the plasminogen molecule, e.g. phosphorylation [10]. In this context, we analyzed the effect of deglycosylation on the spectrum of the plasminogen isoforms.

Figure 3 shows examples of the influence of deglycosylation on the spectrum of emerging plasminogen isoforms in the control group and in the group with local cancer. It is seen that the number of plasminogen isoforms decreases after deglycosylation. The deglycosylation reduces the molecular weight by 4-6 kDa. This means that only some of the plasminogen isoforms are associated with differences in glycosylation. The nature of these isoforms is still unclear.

Dynamics of changes in the spectrum of plasminogen isoforms during the development of prostate tumor. Table 2 shows the results of assessing the significance of differences in the frequency of occurrence of the variable plasminogen isoforms at different stages of tumor development compared with control. It is seen that the transition from normal to adenoma of the prostate is associated with the formation of isoforms P-13 and P-15. This is a statistically significant result for the P-13 isoform. Similar results were obtained for isoforms P-14 and P-16 for the groups of local and locally advanced cancer, respectively. Thus we have shown the existence of specific isoforms of plasminogen whose occurrence is associated with the successive stages of development of prostate tumors in humans.

The presence of specific isoforms of plasminogen, the occurrence of which characterizes the stage of tumor development, allows us to consider the possibility of their use as markers of prostate cancer and its stage of development. To evaluate this possibility, we used discriminant analysis (Table 3). The data on the occurrence of only three isoforms (P-13, P-14, and P-16) was used in the analysis. These isoforms demonstrated a statistically significant difference in the case of the occurrence of prostate adenoma and local and locally advanced

Table 1. Results of identification of plasminogen isoforms using MALDI-TOF mass spectrometry (Swiss-Prot AC P00747)

Isoform	Number of measurements	Overlapping of sequences, %*	MASCOT score**
P-1	62	60.7-64.1	401-423
P-2	62	60.2-68.9	380-458
P-3	62	61.4-62.0	414-425
P-4	62	62.0-65.8	379-396
P-5	62	61.3-63.8	409-419
P-6	62	58.2-61.9	342-364
P-7	62	63.3-70.2	388-502
P-8	62	63.6-66.2	394-448
P-9	62	59.4-65.6	375-401
P-10	62	61.5-65.9	361-414
P-11	62	64.0-72.1	358-488
P-12	29	60.4-62.3	361-398
P-13	26	64.4-67.0	413-435
P-14	22	61.2-64.1	395-416
P-15	21	56.0-66.9	401-424
P-16	12	58.8-60.2	396-414

* The range of variation in the values of the independent measurements.
** Threshold values of MASCOT score for significance level 0.05 and 0.001 are 56 and 73, respectively.

Table 2. Assessment of differences in the incidence of plasminogen isoforms with M = 112 kDa in the experimental groups compared with the control

Isoform	pI	Control		Ad				LC				LAC			
		n		n		χ^2	p	n		χ^2	p	n		χ^2	p
		+	–	+	–			+	–			+	–		
P-12	7.68	7	9	9	0	5.66	0.017	11	6	0.74	0.390	2	18	3.75	0.053
P-13	7.71	0	16	5	4	7.9	0.005	8	9	7.54	0.006	13	7	13.6	0.0002
P-14	7.73	0	16	0	9	–	–	8	9	7.54	0.006	14	6	15.5	0.0001
P-15	7.78	0	16	3	6	3.32	0.068	10	7	10.86	0.001	8	12	6.08	0.014
P-16	7.85	0	16	0	9	–	–	0	17	–	–	12	8	11.8	0.0006

Note: Signs "+" and "–", presence and absence of isoform, respectively; n, number of patients; Ad, prostate adenoma; LC, local prostate cancer; LAC, locally advanced prostate cancer; p, significance level of differences between the experiment and control; χ^2, criterion of statistical significance of differences.

prostate cancer compared with the control group (Table 2).

The distribution of *a posteriori* probabilities reflects prognosis of belonging of a patient with a particular set of plasminogen isoforms to the analyzed stages of tumor development. It can be seen that five of the seven spectra of isoforms are linked to prostate cancer. Switching to the parameters characterizing the prognostic tests give specificity of these spectra equal or close to unity, and this distinguishes them from the conventional prostate-specific antigen test which has low specificity. Sensitivity, which determines the fraction of cases detected for these "oncogenic" sets of plasminogen isoforms is ~0.6. Moreover, high efficiency in the prediction of advanced cancer was shown for three of five sets containing isoform P-16. As a result, 60% of cases of local and advanced prostate cancers can be differentiated with high probability. Advanced cancer can be identified in 70% of cases using the five

Table 3. Predictive efficacy of the spectrum of specific isoforms of plasminogen in the evaluation of prostate tumor stage

Spectrum No.	Isoforms*			Number of cases		Distribution of *a posteriori* probabilities***			
	P-13	P-14	P-16	*n***	share	C	Ad	LC	LAC
Control									
1	–	–	–	16	1	0.58	0.21	0.18	0.03
Adenoma									
2	+	–	–	5	0.55	0.06	0.82	0.11	0.01
1	–	–	–	4	0.45	0.58	0.21	0.18	0.03
Local cancer									
3	+	+	–	6	0.35	0.02	0.06	0.86	0.06
4	–	+	–	2	0.12	0.11	0.01	0.80	0.07
1	–	–	–	7	0.41	0.58	0.21	0.18	0.03
2	+	–	–	2	0.12	0.06	0.82	0.11	0.01
Locally advanced cancer									
5	+	+	+	9	0.45	0.00	0.00	0.01	0.99
6	–	+	+	2	0.1	0.01	0.00	0.00	0.99
7	+	–	+	1	0.05	0.02	0.12	0.00	0.86
4	–	+	–	1	0.05	0.11	0.01	0.80	0.07
3	+	+	–	2	0.10	0.02	0.06	0.86	0.06
1	–	–	–	4	0.20	0.58	0.21	0.18	0.03
2	+	–	–	1	0.05	0.06	0.82	0.11	0.01

* Signs "+" and "–" designate presence and absence of isoform, respectively.
** Number of patients.
*** Probability of belonging of a case to the analyzed categories (C, Ad, LC, LAC) in the presence of the corresponding set of plasminogen isoforms. Cases highlighted in gray were correctly identified taking into account the set of plasminogen isoforms. The spectra of 3 and 4 characterize local cancer, and their presence in patients with advanced cancer is interpreted as incorrectly identified cases.

"oncogenic" sets of plasminogen isoforms. The comparable figure for local cancer was 47%.

Thus, these results suggest that the identified specific isoforms in plasminogen and their sets are possible markers of stages of prostate cancer when used alone or in conjunction with the traditional diagnostic test registering prostate-specific antigen.

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