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EVALUATION OF THE PATTERN OF HUMAN SERUM GLYCOPROTEINS IN PROSTATE CANCER

PROCENA PROFILA HUMANIH SERUMSKIH GLIKOPROTEINA KOD KANCERA PROSTATE

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Summary: Glycoprotein profiling at the level of cells, tissues and biological fluids is aimed at discovering new cancer biomarkers and also at finding specific cancer-related structural alterations of known tumor markers. In this study we comparatively evaluated the glycoprotein patterns of human prostate cancer (PCa)- and normal human sera regarding sialylation and fucosylation as structural characteristics relevant for cancer progression. Glycoproteins were isolated using affinity chromatography on Sambucus nigra agglutinin- and Lens culinaris agglutinin-columns and subsequently characterized by SDS-PAGE and on-chip normal phase-surface capture combined with surface-enhanced laser/desorption ionization time of flight mass spectrometry. Comparative analysis of the glycoproteins purified from healthy and PCa sera indicated differences and redundancy of the isolated molecules in terms of the microheterogeneity of counterpart glycans, the relative abundance and the presence/absence of particular molecular species. In PCa there was a general increase in sialylation and decrease in fucosylation of human serum glycans compared to normal sera. Taken together, the results obtained indicated that an affinity-approach based on the use of lectins of narrow specificity reduced the complexity of the examined samples and at this discovery-phase of our study pointed to specific glyco-changes that may be relevant for improving the monitoring of PCa progression.

Keywords: fucosylation, glycoproteins, human serum, prostate cancer, sialylation

Kratak sadržaj: Glikoproteomska istraživanja na nivou ćelija, tkiva i bioloških tečnosti imaju za cilj otkrivanje novih biomarkera kao i strukturnih promena već poznatih biomarkera, koje su specifične za kancer. U ovom radu je izvršena komparativna evaluacija glikoproteinskog profila normalnog humanog seruma i seruma pacijenata sa kancerom prostate (PCa), u smislu sijalinizacije i fukozilacije, kao strukturnih promena relevantnih za progresiju kancera. Odgovarajući glikoproteini su izolovani lektinskom afinitetnom hromatografijom na kolonama sa imobilisanim Sambucus nigra aglutininom i Lens culinaris aglutininom i okarakterisani tehnikama SDS-PAGE i vezivanja za proteinski čip sa normalnom fazom u kombinaciji sa SELDI-TOF masenom spektrometrijom. Komparativna analiza humanih serumskih glikoproteina izolovanih iz pulova normalnih seruma i PCa-seruma je ukazala na brojnost izolovanih molekula i na razliku u smislu njihovog pojedinačnog prisustva, mikroheterogenosti i relativne zastupljenosti. Generalno, uočeno je povećanje sijalinizacije i smanjenje fukozilacije kod PCa. Rezultati dobijeni u ovom radu ukazuju na to da pristup u kome se u inicijalnoj fazi ispitivanja koriste lektini uske specifičnosti smanjuje kompleksnost uzorka, kao i na specifične promene glikoproteinskog profila, koje mogu biti relevantne za poboljšanje praćenja progresije PCa.

Ključne reči: fukozilacija, glikoproteini, humani serum, kancer prostate, sijalinizacija

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List of abbreviations: Gal – galactose; GalNAc – N-acetyl galactosamine; LCA – Lens culinaris agglutinin; MALDI-MS – matrix-assisted laser desorption/ionisation mass spectrometry; NP20 – normal phase proteinchip; PCa – prostate cancer; PSA – prostate specific antigen; SELDI-TOF MS – surface enhanced laser desorption/ionisation mass spectrometry; SNA – Sambucus nigra agglutinin; SPA – sinapinic acid

Introduction

Early detection of cancer and the monitoring of its progression and metastasis are of special biomedical importance (1). Among the experimental approaches used to deal with these issues, glycoprotein profiling at the level of cells, tissues and various biological fluids, including serum, is attracting growing attention (2). This relies on the fact that complex regulatory mechanisms in the biosynthesis of glycans are characteristically changed in all types of human cancer, resulting in the expression and secretion of aberrantly or incompletely glycosylated molecules or the appearance of completely new structures associated with both N- and O-linked glycans (3, 4). Thus, estimation of the serum concentration of glycoprotein tumor markers is widely used in clinical practice. The introduction of serum glycan profiling is aimed at the discovery of new biomarkers and also at finding specific cancer-related structural alterations of known tumor markers. This is expected to be more relevant for distinguishing between normal and pathological conditions than overall concentrations (2).

Thus, in prostate cancer, determination of prostate-specific antigen (PSA) and the free/total antigen ratio suffers from low sensitivity and specificity (5, 6). Improving the diagnostic potential by glycan profiling has been addressed in different basic and clinical examinations and special attention was paid to the N-linked oligosaccharide chain of PSA (7, 8–10). The results obtained indicated lower overall glycosylation in PCa derived PSA, compared to normal PSA, as well as increased branching of N-glycans, and decreases in sialic acid, fucose and GalNAc content (7, 11, 12). In addition to PSA, other molecular markers of PCa are also glycosylated, but there are no systematic data on any structural changes in cancer (13). Serum glycan profiling has been achieved using high-throughput quantitative methods, such as MALDI-MS (14, 15).

As glycoproteins form a major part of the human serum proteome, their analysis is very complex and requires a combination of different experimental methods for the screening, selection, enrichment and purification or identification of differentially expressed components. In this experimental study we have evaluated comparatively the glycoprotein patterns of human prostate cancer (PCa)- and normal human sera using a combination of lectin-affinity chromatography and electrophoretic and on-chip normal phase surface profiling. The plant lectins, Sambucus nigra agglutinin I, specific for sialic acid α2,6 linked to Gal/GalNAc, and Lens culinaris agglutinin, specific for α1,6 core fucose, were used as affinity ligands in order to gain insight into the terminal sugar residues and core fucosylation as possible clinically relevant indicators. At the screening/discovery-phase of this study, the results obtained for PCa sera indicated distinct groups of glycoproteins exhibiting differences in abundance and/or microheterogeneity of their N-glycans in comparison to those present in normal sera.

Material and Methods

Reagents

Affinity columns with immobilized plant lectins: Sambucus nigra agglutinin I (SNA I) and Lens culinaris agglutinin (LCA) were from Vector Laboratories (Burlingame, USA). Acrylamide, N, N'-bis-acrylamide and mannose (Man) were from Sigma (St. Louis, USA). Molecular mass electrophoresis markers Roti mark and Roti Black silver staining kit were purchased from Carl Roth GmbH&Co. (KG, Germany). ProteinChip NP20 (normal phase) array, sinapinic acid (SPA) and ProteinChip All-in-one Protein Standards II were from BioRad (Hercules, CA, USA). All other chemicals were reagent grade.

Serum samples

This study was carried out on archive specimens of sera from PCa patients and healthy individuals seen at INEP-Zemun, Serbia. PCa sera had PSA concentrations in the range of 115–986 μg/L, while PSA levels in sera from healthy individuals were <0.1–0.6 μg/L. Each group of sera was divided in two (n = 12 for PCa sera and n = 17 for healthy sera), randomly, with no respect to age and patient treatment and four pools were formed (pools I and II of healthy sera and pool III and IV of PCa sera). The pools were used immediately or stored in aliquots at −20 °C until processed.

Lectin-affinity chromatography

Columns of the following immobilized plant lectins were employed for affinity chromatography of pooled human sera: SNA I (Sambucus nigra agglutinin I; bed volume – 5 mL) and LCA (Lens culinaris agglutinin; bed volume – 3 mL). A common chromatographic scheme was applied to all columns according to the manufacturer's instructions. Sera (2 mL) were loaded on each column and, after 3 h incubation at room temperature (RT), fractions (1 mL) were collected. The unbound and the retarded fractions were eluted with binding buffer (0.05 mol/L PBS pH 7.2 for SNA I and 0.1 mol/L acetate buffer pH 6.0, supplemented with 100 mmol/L $CaCl_2$, $MgCl_2$ and $MnCl_2$ for LCA). The bound fractions were specifically eluted by the addition of competitive sugars: 0.1 mol/L lactose (for SNA) and 0.1 mol/L mannose (for LCA). Finally, the tightly bound fractions were eluted with low pH buffers: 0.3 mol/L lactose in 0.2 mol/L acetic acid (for SNA) and 0.2 mol/L acetic acid (for LCA). Elution was monitored by measuring the optical density at 280 nm of each fraction. For

each column, fractions eluted with competitive sugar were pooled as were the fractions eluted with low pH buffers. Each pool was concentrated by ultracentrifugation and stored in aliquots at −20 °C until further analysis.

SDS-PAGE

SDS-PAGE was performed on 10% separating gel and 3.75% stacking gel under reducing conditions (16). Proteins were stained using the Roti Black silver staining kit according to the manufacturer's instructions. The gel was calibrated with Roti-Mark molecular mass markers.

Profiling on normal phase, NP20, protein chip array

A 5 μL aliquot of proteins isolated on lectin-affinity columns was applied on an NP20 ProteinChip array spot and allowed to air-dry at RT. The spots were then washed twice with 5 μL 0.01 mol/L HEPES pH 7.5 followed by 5 μL of deionized water. After complete drying, 1 μL of 50% sinapinic acid (in 50/49.9/0.1% acetonitrile/dH_20/trifluoroacetic acid) was added to each spot, dried and then reapplied. All probes were done in duplicate.

Protein chip arrays were analyzed by surface enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) using the ProteinChip Reader, Series 4000, Personal edition (BioRad, USA). All spectra were acquired in 25 kV positive ion acquisition mode, a mass range of 2500–250000 kDa and with 255 laser shots/spot of 6000 nJ laser energy. Mass calibration was performed with the ProteinChip All-in-one Protein Standards II. All spectra were analyzed using CipherGenExpress Software 3.0.

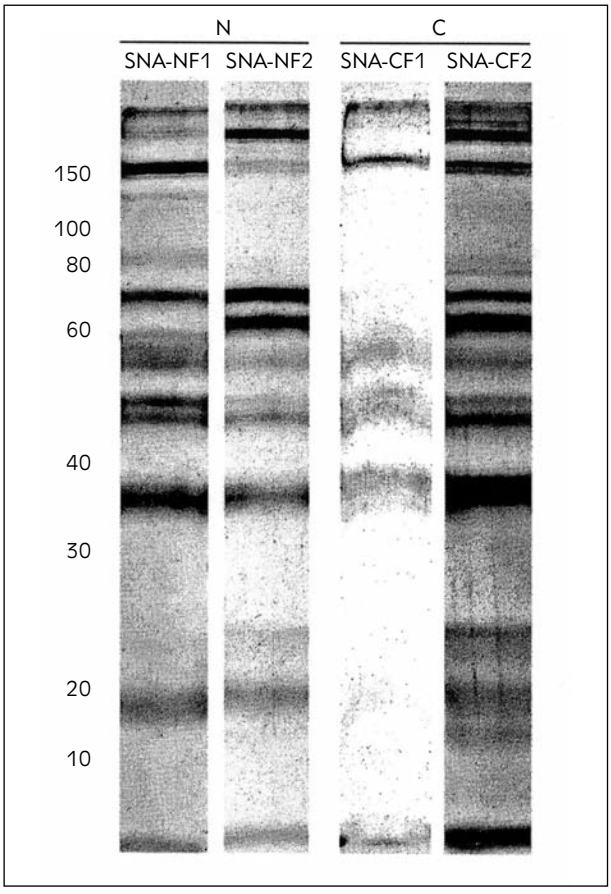

Figure 1 SDS-PAGE of human serum glycoproteins affinity-purified on an SNA-Sepharose 4B column.

Pooled human normal sera (N) and PCa-sera (C) were applied to the SNA-Sepharose 4B column, and the bound fraction was eluted with a) 0.1 mol/L lactose (fractions SNA-NF1 and SNA-CF1) and b) 0.3 mol/L lactose in 0.2 mol/L acetic acid (fractions SNA-NF2 and SNA-CF2). The isolated preparations were subjected to SDS-PAGE on 10% gel under reducing conditions. The proteins were stained with silver. The numbers indicate the molecular mass (kDa) of standard proteins used for gel calibration.

Results

Microheterogeneity of SNA-reactive human serum glycoproteins

Electrophoretic patterns of SNA-reactive glycoproteins isolated by affinity chromatography from pooled human normal (healthy)- and PCa-sera are shown in *Figure 1*. Under the experimental conditions used, SNA-bound molecules were separated into a sugar-elutable fraction designated as SNA-NF1 and a low pH buffer elutable-fraction designated as SNA-NF2. On SDS-PAGE, similar electrophoretic patterns of SNA-NF1 and SNA-NF2 were observed in the range of 15 kD – 220 kD. This indicated microheterogeneity of particular molecular mass species in respect to sialic acid content (level of sialylation).

In contrast, SNA-reactive glycoproteins isolated from PCa-sera were much more abundant in the low pH buffer-elutable fraction, SNA-CF2, than in the sugar-elutable fraction, SNA-CF1. Their patterns mainly corresponded to those seen with normal sera, suggesting increased sialylation of counterpart molecules as the main change, but the appearance of additional distinct molecular mass species in the regions of 15 kD – 30 kD and > 150 kD was also observed.

Microheterogeneity of LCA-reactive human serum glycoproteins

Electrophoretic patterns of LCA-reactive glycoproteins isolated by affinity chromatography from pooled human normal- and PCa-sera are shown in *Figure 2*. Normal sera LCA-bound molecules in the sugar-elutable fraction, LCA-NF1, gave a similar electrophoretic pattern to the low pH buffer-elutable

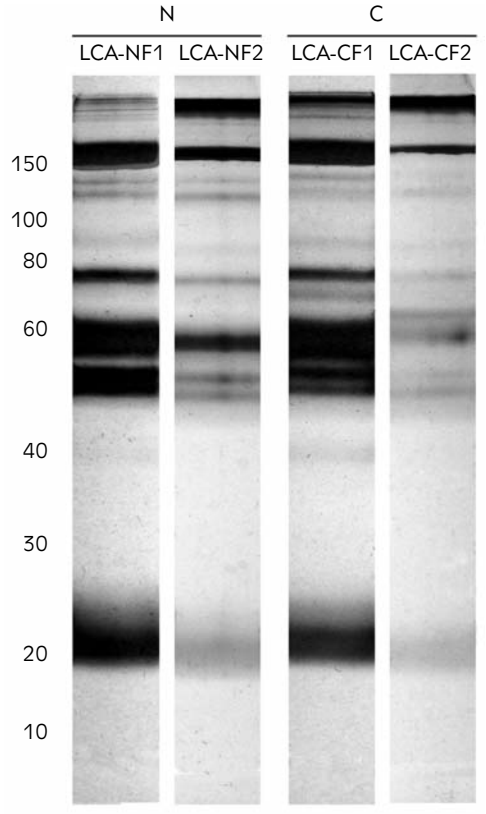

Figure 2 SDS-PAGE of human serum glycoproteins affinity-purified on an LCA-Sepharose 4B column.

Pooled human normal sera (N) and PCa-sera (C) were applied to the LCA-Sepharose 4B column, and the bound fraction eluted with a) 0.1 mol/L mannose (fractions LCA-NF1 and LCA-CF1) and b) 0.2 mol/L acetic acid (fractions LCA-NF2 and LCA-CF2). The isolated preparations were subjected to SDS-PAGE on a 10% gradient gel under reducing conditions. The proteins were stained with silver. The numbers indicate the molecular mass (kDa) of standard proteins used for gel calibration.

fraction, LCA-NF2. The main difference was seen in the relative abundance of molecular species in the range 20 kD to 80 kD, which was greater in LCA-NF1. Compared to this, LCA-reactive glycoproteins from PCa-sera were even more abundant in the sugar-elutable fraction, LCA-CF1, than in the low pH buffer elutable fraction, LCA-CF2, suggesting a decrease in core fucosylation especially associated with molecular mass species in the same range as that observed for normal-sera isolated glycoproteins.

NP20 profiling of affinity purified SNA- and LCA-human serum glycoproteins

The isolated glycoprotein fractions from normal- and PCa-sera were additionally characterized on normal phase protein chip arrays combined with SELDI-TOF detection. The corresponding spectra in the range of selected molecular masses of interest are presented in *Figures 3* and *4*.

Generally, the patterns obtained by mass spectrometry could be correlated with the corresponding electrophoretic patterns. However, mass spectrometry is more sensitive for the detection of low abundant species and resolution of species with close molecular masses, resulting in more comprehensive profiles. Thus, the main differences found were more abundant 40–150 kD SNA-reactive molecular species and more abundant 50–150 kD LCA-reactive molecular species in PCa-sera than in normal sera.

Discussion

Comparative analysis of affinity-purified human serum glycoproteins from healthy and PCa sera indicated differences and redundancy of isolated molecules in terms of: 1) microheterogeneity of counterpart glycans, 2) their relative abundance and 3) the presence/absence of particular molecular species.

Microheterogeneity regarding the level of sialylation (sialic acid content) resulted in separation into two distinct equally abundant fractions in healthy sera. This was found to change in PCa, where the increase of SNA-CF2 indicated a marked increase in sialylation. Concerning fucosylated glycoproteins, intrinsic microheterogeneity at the level of the same molecular species was inverse when compared to sialylation i.e. the total amount of fucosylated species was significantly decreased in both the sugar- and low pH buffer-elutable fractions, especially in the former.

Thus, efficient enrichment of particular glycosylated molecules and visualization of the corresponding molecular mass patterns were achieved by the methods employed. Besides the changes in microheterogeneity, the electrophoretic patterns also revealed the presence/absence of particular species in the distinct molecular mass ranges associated with both SNA- and LCA-reactive glycoproteins. They were assessed by NP20 profiling based on mass spectrometry detection, which enabled the resolution of subtle differences in diversity/abundance between the two groups of samples. Molecular species in the range 40–150 kD were observed as potentially relevant for differentiating between PCa and normal sera.

Taken together, the results obtained indicate that an affinity-approach based on the use of lectins of narrow specificity reduces the complexity of samples. At this discovery-phase of our study they pointed to glyco-changes that may be relevant for monitoring PCa progression. This justifies directing further work towards verification of the presented data on a much higher number of individual samples in order to evaluate their potential as biomarkers.

The documented changes in sialylation and fucosylation at the time of advanced PCa are in

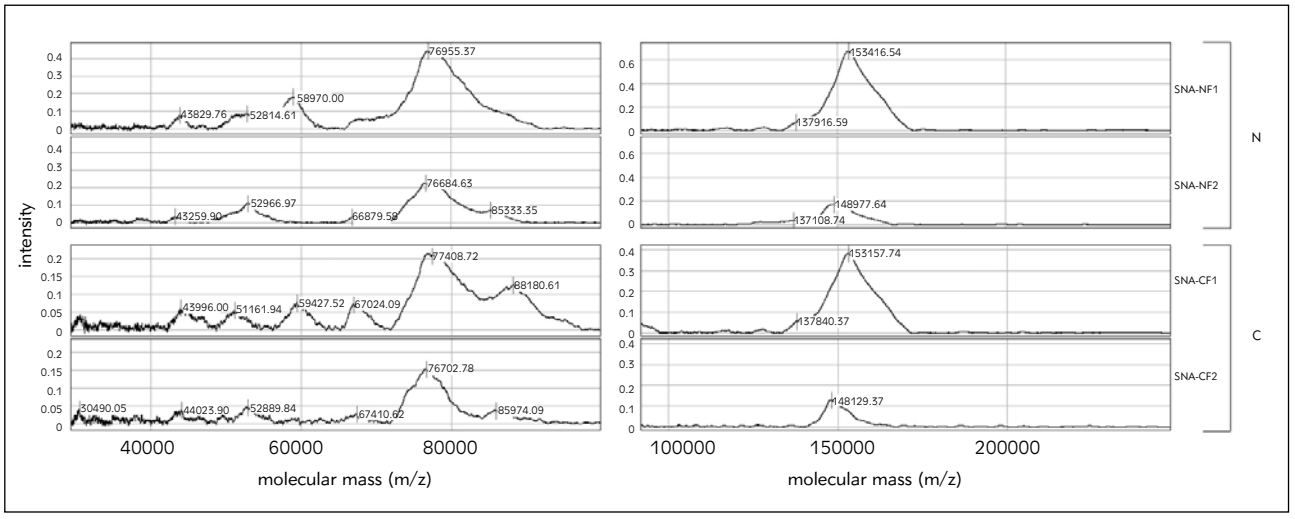

Figure 3 Normal phase (NP20) protein chip profiling of affinity-purified SNA-binding from human sera.

Affinity-purified glycoproteins from normal (N) and PCa human sera (C) eluted from the SNA-column with competitive sugar (SNA-NF1 and SNA-CF1) and with low pH buffer (SNA-NF2 and SNA-CF2) were applied to a normal phase (NP20) protein chip array and processed as indicated in Material and Methods. A surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrum was acquired in ion positive mode at 25 kV and analyzed using CiphergenExpress Software 3.0. Calibration was performed with ProteinChip All-in-one Protein Standards II. Molecular mass ranges: A) 30–100 kD, B) 100–250 kD.

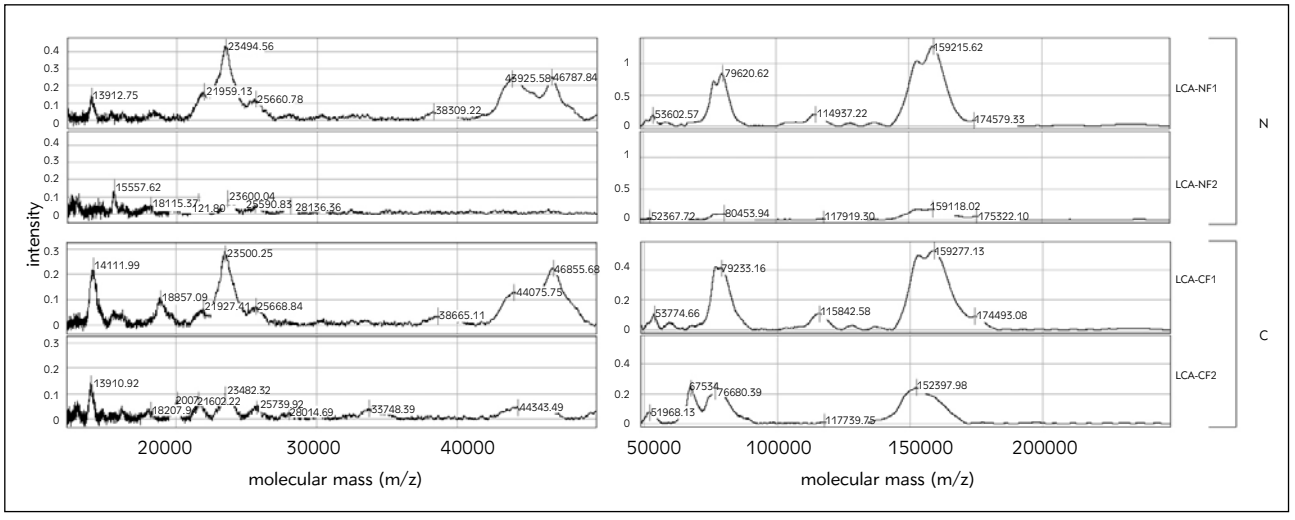

Figure 4 Normal phase (NP20) protein chip profiling of LCA-binding glycoproteins from human sera.

Affinity-purified glycoproteins from normal (N) and PCa human sera (C) eluted from LCA-column with competitive sugar (LCA-NF1 and LCA-CF1) and with low pH buffer (LCA-NF2 and LCA-CF2) were applied to a normal phase (NP20) protein chip array and processed as indicated in Material and Methods. A surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrum was acquired in ion positive mode at 25 kV and analyzed using CiphergenExpress Software 3.0. Calibration was performed with ProteinChip All-in-one Protein Standards II. Molecular mass ranges: A) 10–50 kD, B) 50–250 kD.

agreement with the general changes observed in other types of reproductive tract cancers (3, 17). They also point to possible changes in the activity/expression of enzymes involved in glycosylation processes, both glycosyltransferases and glycosidases (18, 19). In general, the glycophenotype of PCa is, among other changes, associated with an increase of sialic acid in cancerous tissue compared to normal tissue and may differentiate clearly between tumor cell sublines (20, 21). In addition, it was found that human prostatic carcinoma cell lines differ in the expression of fucosyltransferases and accordingly in the presence of fucosylated epitopes, such as ABO and Lewis antigens (17, 22, 23). At the level of specified PCa markers examined so far, changes in sialylation and fucosylation were observed in prostate-specific antigen as well as in fibronectin (24, 25).

Generally, distinct tissue components are surrounded by an increased concentration of serum

proteins which also passively filter into tissue fluids (26). Thus, besides tumor marker potential, human serum glycoproteins could possibly affect cell metastatic spread, which is a complex multi-step process related to tumor attachment, survival in the circulation, adhesion and invasion. Starting from the results obtained, it seems rational to explore human serum glycoproteins further, as a biological system for the definition of tools and targets for PCa cancer management from both clinical and basic aspects.

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