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ORIGINAL ARTICLE

Serum Levels of Glycoproteins are Elevated in Patients with Ovarian Cancer

Vikram Thakkar · Purvi Patel · Neelam Prajapati · Ranjit Kaur · Mukesh Nandave

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Abstract Identification of reliable biomarkers for detection and staging of cancer and monitoring the outcome of anticancer therapy has been considered to be of high importance. We aimed to estimate the levels of serum glycoproteins, protein bound-hexose, protein bound hexosamine, protein bound fucose, protein bound sialic acid and protein bound carbohydrate in 32 ovarian cancer patients and compared them with the levels that found in 25 normal subjects. As compared to the normal subjects, all the four fractions of glycoproteins level were significantly elevated in ovarian cancer patients ($p < 0.05$). Chemotherapy in these patients significantly decreased the levels of serum glycoproteins ($p < 0.05$). Thus, high levels of serum glycoproteins in ovarian cancer patients could be due to abnormal protein glycosylation indicating malignant transformation of the cells.

Keywords Glycoproteins · Sialic acid · Ovarian cancer · Serum hexose · Protein bound hexosamine · Protein bound carbohydrate

V. Thakkar · R. Kaur
Pharmacy Department, M. S. University of Baroda, Vadodara, India

P. Patel · N. Prajapati
Department of Obstetrics and Gynecology, S.S.G. Hospital, Baroda, India

M. Nandave (✉)
Department of Pharmacology, SPP School of Pharmacy and Technology Management, SVKM's NMIMS, Vile Parle (w), Mumbai 400056, India
e-mail: mukeshnandave@gmail.com

Introduction

In recent years, the importance of cancer biomarkers has increased tremendously as they help in early detection and staging cancer, and in monitoring the efficacy of anticancer treatment. Cell membrane constituents are considered to play a prominent role in neoplastic diseases. Biomarkers of malignancy consists of a broad range of biochemical entities that include nucleic acids [1, 2], proteins [3], sugars [4], lipids [5], and small metabolites [6], cytogenetic and cytokinetic parameters [7] as well as whole tumor cells found in the body fluid [8].

Ovarian cancer has been one of the most lethal gynecological cancers in women for many years. Currently, serum CA125 (cancer antigen 125 or carbohydrate antigen 125) is the most widely used biomarker for ovarian cancer detection. As CA-125 levels may be elevated in a person without ovarian cancer, its specificity and sensitivity with respect to diagnosis of ovarian cancer is really limited. Apart from ovarian cancer patients, CA-125 levels may also be elevated in other cancers that include breast, fallopian tube, endometrial, lung, and gastrointestinal cancer [9]. Therefore, for confirmed diagnosis of ovarian cancer, there is a pressing need for other supportive biomarkers, which must be highly sensitive and equally specific.

Glycoproteins are defined as proteins with a carbohydrate moiety covalently attached to their peptide portion. The glycoproteins as a group have multiple and complex functions and are found as enzymes, hormones, blood group substances and as constituents of extracellular membranes. These are organic compounds, composed of protein as well as carbohydrate monosaccharides, usually hexose, hexosamine, fucose and sialic acid, joined together covalently linked to polypeptide chain. The level of different types of serum glycoproteins are maintained within a

narrow range in health [10], but is elevated in many pathological conditions viz. tuberculosis [11], autoimmune disease [12], cardiovascular disease [13, 14], diabetes mellitus [15], cancer of cervix [16], uterus [17] and breasts [18], and arthritis [19]. Thus, it is of great interest to identify and validate serum glycoproteins whose change in levels may provide new insight into cancer progression and may have utility as potential markers for cancer diagnosis.

In this study, we have estimated and compared the levels of the fractions of serum glycoproteins i.e. protein bound hexose (PBH), protein bound hexosamine (PBHex), protein bound fucose (PBF), protein bound sialic acid (PBS) and protein bound carbohydrate (PBC) in 32 ovarian cancer patients with that of 25 healthy subjects. These glycoprotein biomarkers may prove to be useful for the detection of ovarian cancer.

Materials and Methods

Study Subjects

After ethical clearance was obtained, the study was conducted on female patients visited to Out-patient department (OPD) of Department of Obstetrics and Gynecology, S.S.G. Hospital, Baroda, India. The study was carried out on 57 subjects comprising 32 ovarian cancer patients (age ranges from 45 to 75 years) and 25 normal healthy female volunteers (age ranges from 20 to 70 years). The diagnosis of ovarian cancer patients was done by standard clinical criteria, which was confirmed by using the structured clinical interview. Healthy normal subjects were selected on the basis of good health as evidenced by their medical history, complete physical examination and routine laboratory tests. All the subjects were instructed to abstain from alcoholic products/beverages throughout the study period. Subjects were also instructed not to take any over the counter (OTC) medications until the completion of the study.

Sample Collection

Blood samples (3 ml each) were collected only once from healthy subjects and twice from ovarian cancer patients, once before the beginning of chemotherapy and then, after receiving at least three chemotherapy cycles. After collection, the blood samples were centrifuged to separate serum. All the serum samples were stored at −70 °C until analysis. The samples were collected, handled, shipped, stored, and managed according to standard operating procedures (SOP) as specified in the protocol document.

Sample Analysis

Biochemical analysis was performed on serum samples for estimation of PBH, PBHex, PBF, PBS and PBC. Values were calculated and expressed in terms of milligram percentage (mg%). All the reagents used were of analytical grade and the analytical procedures were standardized for reproducible and feasible results.

Chemicals

Standards of Galactose-Mannose, Sialic acid (*N*-acetyl neuraminic acid), and Glucosamine were procured from HiMedia Laboratories, Mumbai while that of methyl pentose was purchased from Sigma Aldrich Chemicals Pvt Ltd., Powai, Mumbai.

Protein Bound Hexose

In this method, the hexose moiety of glycoprotein conjugates precipitated by ethanol at room temperature is determined by the orcinol reaction at 540 nm [20].

Protein Bound Hexosamine

Serum proteins are precipitated by ethanol and then hexosamine is liberated from the glycoproteins in the serum sample by acid hydrolysis. Acetylation in alkaline medium cyclizes the hexosamine to pyrrole derivative that couple with paradimethyl amino bezaldehyde forming a colored complex, whose concentration determined photometrically at 530 nm [21].

Protein Bound Fucose

Dische et al. [22] have described methods for estimation of protein bound fucose, which lead them to develop the method for determination of methyl pentose in serum. The reported method involves heating of the serum sample with sulfuric acid for 3–10 min followed by the addition of cysteine. Satisfactory specificity for methyl pentose has been achieved by determining the optical densities at two wavelengths so as to eliminate any interference caused due to presence of other sugars. Application of this procedure to serum glycoproteins has further supported the conclusion that reaction substance is methyl pentose. Rhamnose and fucose gave identical optical densities under the condition of the determination and can be used as standards. However, since only fucose has been demonstrated in the serum glycoproteins or in related mucoid, it seems reasonable to report methyl pentose value as fucose. Methyl pentose value is determined by measurement of optical density at wavelength 396 and 430 nm [22].

Table 1 Mean levels of serum glycoproteins PBH, PBHex, PBF, PBS and PBC in normal subjects and in patients of ovarian cancer before and after chemotherapy

Group	PBH mg%	PBHex mg%	PBF mg%	PBS mg%	PBC mg%
Normal subjects (N = 25)	113.2 ± 12.69 (86.00–131.06)	89.43 ± 16.63 (72.06–114.76)	8.44 ± 1.45 (4.97–10.54)	65.41 ± 9.23 (55.46–77.00)	276.5 ± 39.23 (239.62–299.11)
Patients of ovarian cancer before chemotherapy (N = 32)	228.7# ± 17.3 (180.63–260.48)	195.5# ± 15.3 (173.9–215.23)	22.52# ± 2.36 (14.63–33.25)	144.9# ± 13.3 (126.45–165.83)	591.6# ± 32.87 (508.06–653.16)
Patients of ovarian cancer after chemotherapy (N = 32)	146.3* ± 12.6 (123.69–169.31)	130.0* ± 9.26 (115.83–145.64)	15.08* ± 1.29 (7.38–23.48)	103.2* ± 9.63 (86.29–120.97)	395.8* ± 22.63 (355.63–427.53)

Values are expressed as mean ± SEM

Values in parentheses represent range

# $p < 0.05$ (Normal subjects versus patients of ovarian cancer before chemotherapy)

* $p < 0.05$ (Patients of ovarian cancer before chemotherapy versus patients of ovarian cancer after chemotherapy)

Protein Bound Sialic Acid

In this method, a red brown colour is produced when the serum is treated with tryptophan in the presence of strong perchloric acid. Absorption spectra were recorded at 500 nm [23].

Statistical Analysis

Data was expressed as mean ± SEM. The data so obtained was analyzed to obtain appropriate conclusions. Student's 't' test was employed to find out the statistical significance. Receiver operating characteristic (ROC) curve was generated, and areas under the curve (AUC) were computed to provide a basis for comparison of each of the markers to discriminate between ovarian cancer patients and healthy subjects. ROC curve analyses were performed using SPSS statistics software (Version 17.0).

Results

The mean levels of serum glycoproteins PBH, PBHex, PBF, PBS and PBC in normal subjects and in patients of ovarian cancer before and after chemotherapy are shown in Table 1.

In the present study, the level of PBH in healthy subjects was 113.2 ± 12.69 mg% which we found elevated significantly ($p < 0.05$) to 228.7 ± 17.3 mg% in patients of ovarian cancer. After chemotherapy, the levels of PBH were declined significantly ($p < 0.05$) to 146.3 ± 12.6 mg%.

Moreover, compared to the healthy subjects (89.43 ± 16.63 mg%), the level of PBHex was significantly ($p < 0.05$) increased to 195.5 ± 15.3 mg% which was found to decrease to 130.0 ± 9.26 mg% after chemotherapy. The difference in the level of PBHex in ovarian cancer patients before and after the chemotherapy was statistically significant ($p < 0.05$).

Similarly, the level of serum glycoprotein PBF (22.52 ± 2.36 mg%) was significantly elevated in ovarian cancer patients when compared to that of healthy subjects (8.44 ± 1.45 mg%). Among all the serum glycoproteins, the concentration of PBF was increased most remarkably ($p < 0.05$) in ovarian cancer patients. However, anticancer chemotherapy to these patients caused significant decrease in levels of PBF (15.08 ± 1.29 mg%).

In view of the possible link between serum glycoproteins and malignancy in the general population, we also investigated whether serum sialic concentrations are elevated in ovarian cancer patients compared with normal healthy subjects or not. In the present study, we found that the total serum sialic acid levels were significantly

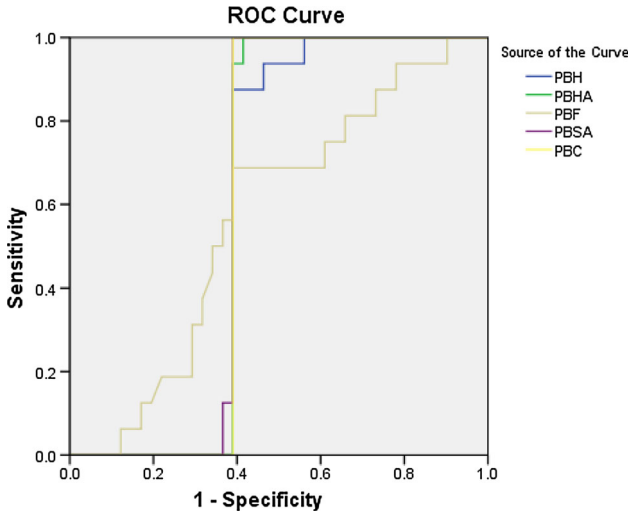

Fig. 1 ROC curves assessing overall diagnostic abilities of serum glycoprotein markers. Diagonal segments are produced by ties

($p < 0.05$) elevated in ovarian cancer patients (144.9 ± 13.3 mg%) compared to normal subjects (65.41 ± 9.23 mg%). After chemotherapy, level of serum sialic acid was remarkably ($p < 0.05$) reduced to 103.2 ± 9.63 mg%.

Protein-bound carbohydrates consider the level of all four fractions i.e. hexoses, hexosamines, fucose and sialic acids. As the present study found the elevated levels of all the four fractions of serum glycoproteins i.e. PBH, PBHex, PBF, and PBS, we ultimately found that the levels of total PBC were elevated in the ovarian cancer patients as compared to healthy subjects.

Figure 1 shows receiver operator characteristic (ROC) curves for each of the five markers; PBSA displayed the highest overall ability to discriminate between ovarian cancer patients and healthy subjects, with an area under the curve (AUC) of 0.613, as compared with an AUC of 0.610 for PBC, 0.608 for PBHA, 0.595 for PBH and 0.569 for PBF. AUC estimates and corresponding 95 % confidence intervals are shown in Table 2.

Discussions

The present study reported the elevated levels of serum glycoproteins i.e. PBH, PBHex, PBF, PBS, and PBC in ovarian cancer patients when compared to that of normal subjects. Also, we first time reported here that anticancer chemotherapy decreases the elevated levels of all the four fractions of serum glycoproteins.

Many recent studies reported higher values of serum glycoproteins in various types of malignant conditions that include malignant melanoma [24, 25], breast cancer [18, 26], colorectal [27], gynecological [16], oral cancers [28]. Therefore, present study results are in consonance with previously reported findings.

Another study conducted in 161 patients with ovarian cancer reported elevated levels of serum lipid-associated sialic acid [29]. López-Morales et al. [30] reported high levels of serum sialic acid in the cervical cancer patients and an increase in total sialic acid concentration has been reported in case of cervical neoplasia and cervical cancer. Proposed mechanism for high levels of sialic acid could be the change in sialylation that occurs before cancer development. Recently, An et al. [31] developed the glycomic approach to identify oligosaccharide markers for ovarian cancer. Globally released oligosaccharides from O-linked glycoproteins can be harvested and could serve as biomarkers for ovarian cancer. When cells become cancerous, alteration in glycosylation patterns occurs and some glycoproteins are shed from tumors and detected in systemic circulation.

Table 2 Summary of overall ROC curve analyses, estimates, and associated 95 % confidence intervals (95 % CI's)

Ovarian cancer patient versus healthy subjects					
Serum glycoprotein markers	AUC	Std. error[a]	Asymptotic sig[b]	Asymptotic 95 % confidence interval	
				Lower bound	Upper bound
PBH	0.595	0.075	0.271	0.447	0.742
PBHA	0.608	0.076	0.207	0.459	0.757
PBF	0.569	0.079	0.424	0.414	0.724
PBSA	0.613	0.076	0.189	0.464	0.761
PBC	0.610	0.076	0.201	0.460	0.759

Serum Glycoprotein Marker (s): PBF has at least one tie between the positive actual state group and the negative actual state group. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1

All the other cutoff values are the averages of two consecutive ordered observed test values

[a] Under the nonparametric assumption

[b] Null hypothesis: true area = 0.5

Significantly elevated levels of plasma total sialic acid, protein-bound hexoses, hexosamine and fucose were observed in 30 ovarian cancer patients as compared to the apparently healthy controls. Increase in these carbohydrate moieties of glycoproteins reflect the stage of cancer and may act as an additional tool in the diagnosis and prognosis of ovarian carcinoma [32].

Abnormal protein glycosylation is very important in cancer transformation and metastasis. In the present study, reported high levels of serum glycoproteins i.e. PBH, PBHex, PBF, PBS, and PBC in ovarian cancer patients could be due to abnormal protein glycosylation which ultimately indicates malignant transformation of the cells in ovarian cancer.

Thus, present study results support the previous study findings. Results clearly show the diagnostic importance of serum glycoproteins in the diagnosis of ovarian cancer. On the basis of present study findings it can be concluded that serum glycoprotein levels may serve as an indicator of ovarian cancer. Moreover fall in the level of serum glycoproteins in response to anticancer chemotherapy can also be used as an indicator of efficacy of treatment as well as clinical improvement. In the current study, the serum glycoprotein fractions were analyzed by conventional ultraviolet spectrophotometry methods. In future work, further analysis of serum glycoproteins will be done with more advanced LC–MS/MS techniques.

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Conflict of interest The authors report no declarations of interest.

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