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CLINICAL USEFULNESS OF ALTERATIONS IN SIALIC ACID, SIALYLTRANSFERASE AND SIALOPROTEINS IN BREAST CANCER

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ABSTRACT

Sialic acid, the end moieties of the carbohydrate chains are biologically important and essential for functions of glycoconjugates and are reported to be altered in cancer patients. Two hundred and twenty five breast cancer (BC) patients, 100 patients with benign breast disease (BBD) and 100 healthy females (controls) were enrolled for the study. Eight hundred and twenty four follow-up samples of 225 breast carcinoma patients were also evaluated. The association of sialic acid forms, sialyltransferase and α-2-6 sialoproteins levels with presence and extent as well as prognosis of breast carcinoma was studied. Serum sialic acid forms and sialyltransferase revealed significantly elevated levels among untreated breast cancer patients as compared to the controls, patients with BBD as well as cancer patients in remission. Non-responders showed comparable levels of the markers with those found in breast cancer patients at the time of diagnosis. Higher levels of sialic acid forms at diagnosis were associated with poor prognosis. A positive correlation between serum levels of different forms of sialic acids and extent of malignant disease was observed. The changes in serum proteins with terminal α-2-6 sialic acid correlated well with alterations in the levels of sialic acid forms and sialyltransferase. Malignant tissues showed elevated levels of sialic acid and sialyltransferase as compared to surrounding normal tissues.

The results suggested potential utility of these markers in evaluation of clinical outcome.

KEY WORDS

Breast Cancer, Glycoproteins, Sialic acid, Sialyltransferase, Tumours.

INTRODUCTION

The incidence and mortality rate of cancer is still unacceptably high. This stark fact itself is the strong argument for further research in the field of cancer biology. Immense increase in knowledge of the altered characteristics of malignant cells have shown that altered cell surface is the hall mark of malignant cells (1). Study of biochemical changes during malignant transformation is also referred as a form of chemical biopsy because they facilitate the diagnosis of organ abnormalities through chemical eye. The biology of glycoproteins, the vital components of cell surface, has been the field of intensive investigation since it became evident that they play a significant role during malignant transformation. Majority of the presently known tumour markers are glycoprotein in nature. Various investigators have reported striking differences in cell surface carbohydrate structure in malignant cells (2, 3). Further, molecular changes in carbohydrate antigens have been reported to be associated with cancer (4). Altered glycosylation of glycoconjugates is one of the important molecular changes that accompany malignant transformation (5, 3). Sialic acid, the end moieties of the carbohydrate chain of glycoconjugates are reported to be elevated during malignancy (6, 7). Increased levels of sialic acid in cancer patients can be explained by spontaneous release (shedding) of aberrant sialic acid rich glycoproteins and glycolipids (8, 9, 4).

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Sialic acid moiety of carbohydrate epitope is important for biological interactions including cell adhesion to selectin and lectins (10). Thus, sialic acid is an important constituent for the characteristic changes of transformed cells. The alterations in glycoproteins start at an early stage of tumorigenesis. Gatchev (11) presented first data on sialic acid levels before diagnosis of tumour, and reported elevated levels of sialic acid among men in whom a malignant tumour was diagnosed during the period of eight years after health examination. Sialylation is one of the most common and versatile type of terminal glycosylation (12). The presence of extra sialic acid in glycoproteins of malignant cells has also been demonstrated (13, 3). Structural analysis of tumour associated carbohydrate antigens has shown that sialylated derivatives together with related structural changes are essential carbohydrate epitopes associated with malignant transformation (14). Sialylated oligosaccharides are synthesized by a family of enzyme sialyltransferase. Majority of serum proteins are glycosylated. When disease is present, subtle changes occur in glycosylation of these proteins. The knowledge of the way in which serum proteins are glycosylated in cancer patients can contribute to the understanding of association of glycoprotein with malignant conditions. Thus, the changes in glycoprotein levels could provide clinically useful information. Only a few studies aiming at investigating simultaneous evaluation of serum levels of different forms of sialic acid as well as sialyltransferase in diagnosis and prognosis of cancer patients have been reported. Therefore, the current study aimed to investigate association of alterations in serum levels of sialic acid forms, sialyltransferase and sialoproteins with diagnosis and treatment monitoring of breast carcinoma.

MATERIALS AND METHODS

The study included 225 pathologically proven breast carcinoma patients. Cancer patients were ineligible for the study, if they had received prior anticancer therapy or were suffering from any other disorders. Clinical details were obtained from clinical records (Table 1). Stage of the disease was classified according to the UICC criteria (15). Patients with BBD are highly vulnerable for developing breast cancer. Therefore, measurement of serum levels of the markers was also carried out from 100 age matched patients with BBD. Among the patients with BBD, 60 females were having fibrocystic disease, 5 females had benign ductal disease and 35 females had benign mass. To evaluate baseline circulatory levels of the markers, 100 healthy females without any major illness in recent past were included in the study as controls. Due consent was obtained from all the subjects. Blood samples were collected between 9.0 A.M. and 11.0 A.M. to avoid any possible diurnal variations. Sera were separated and stored at- 80°C till analysis.

Tissue samples of breast cancer patients were collected on ice from operation theatre during surgical removal of the tumours. The normal tissues were selected by a pathologist from free margins of excised surgical specimens keeping 2-3 centimeters away from tumour margins and histopathological examinations were carried out from all the samples. The normal and tumour tissues were kept frozen at -80°C after washing with normal saline.

First follow-up blood sample was collected at least one month after initiation of anticancer therapy. Subsequent, follow-up blood samples were collected during the patient's visit to the hospital. However, minimum one month interval was kept between two follow-ups. Clinical status of the patients during/ after anticancer treatment was evaluated as suggested by Miller et al. (16). Patients were grouped as mentioned below :

Complete Responders (CR) : Patients with disease free survival at-least for one month.

Partial Responders : Patients with decrease in tumour size by > 50%.

Stable Disease : Patients with no objective treatment response i.e. no changes in tumour size for 3 months.

Progressive Disease : Patients having increase in tumour size and/or appearance of new malignant lesions.

The patients with partial response, stable disease and progressive disease were grouped together as non-responders (NR) to simplify the comparisons of the marker levels with disease status. Out of 824 follow-up blood samples, 559 were CR and 265 were NR at the time of follow-up (Table 2).

Estimation of Free Sialic acid (FSA)

Sialic acid levels were analysed using method of Skoza and Mohos (17) as modified by Warren (18). Oxidation of N-acytylneuraminic acid (NANA-Sialic acid) was carried out by incubation of 0.1 ml serum samples with 0.25 ml 0.025 N periodic acid at 37°C for 30 minutes. Formyl pyruvate was formed due to oxidation of N-acetyl neuraminic acid. The reaction was stopped by addition of 0.2 ml of 2% sodium arsenite to react with remaining periodic acid molecules. 1.5 ml of thiobarbituric acid reagent was added and mixture was kept in boiling water bath for 7.5 minutes. Salmon pink thio-barbituric

Table I. Clinical details of breast cancer patients

TNM Stage			I	II	III	IV	Total
No. of patients			2	48	115	60	225
Site :		Left	0	26	67	37	130
		Right	2	22	48	23	95
Menopausal status :		Pre	0	24	53	24	101
		Post	2	23	58	33	116
		Peri	0	1	4	3	8
Histology:	Invasive/Infil. Ductal		2	43	100	51	196
	Invasive/Infil. lobular		0	0	9	5	14
	Others		0	5	6	4	15
Lymphnode Involvement:		Absent	0	25	82	35	142
		Present	2	23	33	2	60
		Unknown	0	0	0	23	23

Table 2. Clinical status and duration after treatment initiation in breast cancer patients at the time of follow-up

Duration in months	CR	NR	Total
1-6	240	95	335
6-12	138	67	205
12-24	119	75	194
24-36	34	11	45
36-48	15	09	24
48-60	10	05	15
60-72	03	03	06
Total	559	265	824

CR - Responders, NR - Non Responders

acid chromophore was obtained due to its reaction with formyl pyruvate. The stability of chromophore was increased by addition of 1.5 ml dimethyl sulphoxide. Intensity of the chromophore was read at 549 nm. To correct potential interference of 2-Deoxy-deribose, optical density of reaction mixture was also measured at 532 nm.

Estimation of Total Sialic acid (TSA)

To release bound sialic acid, serum samples were hydrolysed with 1N H_2SO_4 at 80°C for 1 hour. After hydrolysis, proteins were removed by addition of 2 ml 10% trichloroacetic acid and supernatant was used for estimation of total Sialic acid contents as described by the method for FSA.

Estimation of Protein-bound Sialic acid (PBSA)

Perchloric acid soluble and phosphotungstic acid precipitable fractions were obtained as described by Winzler (19). The fractions were estimated after hydrolysis at 80°C in the presence of 1 N H_2SO_4. The hydrolysed fractions were estimated as per the method of FSA described above.

Sialyltransferase Activity

Activity of sialyltransferase was measured using the method of Kessal and Allen (20) with minor modifications. The assay system for sialyltransferase consisted of 50 mM HEPES-NaOH buffer pH 6.8, 10 mM $MnCl_2$, 2 nmole C^{14}-labelled 5'-cytidine monophosphate N-acetyl neuraminic acid (CMP C^{14}NANA), 4 mg of asialo fetuin and the enzyme source. In control tube, addition of acceptor was omitted. Reaction was terminated by addition of ice cold acid mixture. Precipitates were solubilized in 1.0 ml of NCS tissue solubiliser. After addition of scintillation cocktail, quantitation of incorporated N-acetyl neuraminic acid was calculated by counting the radioactivity of reaction mixture in β-counter. The non-specific binding was nullified by deduction of control readings. Enzyme activities were expressed as CPM/mg protein/hour.

Isolation of α-2-6 sialylated proteins

The lectin affinity chromatography for isolation of glycoproteins was performed as described by

Thompson and Turner (21). Briefly, α-2-6 sialic acid specific lectin (Sambucus Nigra, Sigma) was coupled with CNBr activated sepharose beads (Amersham Pharmacia) in a concentration of 2 mg/ml beads. Immediately prior to coupling with proteins, sepharose beads were washed with acid followed by 0.1 M bicarbonate buffer (pH 8.3). Sialoproteins were separated from 75 µl serum by mixing with Lectin-Sepharose beads. Unbound proteins were removed by washing with 0.05 mol/L Tris-HCl Buffer (pH 7.4) containing 25 mmol/L KCl, 5 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$ and 0.5 % V/V Nonidet P-40. Bound sialoproteins were released by solubilising in Tris Buffer (pH 6.8) containing SDS. Resultant elute was loaded on to 7.5% SDS-polyacrylamide gel and electrophoresis was performed. To visualise protein bands, the gels were stained by silver staining method.

Statistical data analysis

Data were statistically analysed using SPSS statistical software (22). Level of significance was assessed by computing Fisher's two tailed exact test and paired 't' test. 'p' values < 0.05 were considered statistically significant. To study association of the marker levels with clinical stage of malignant diseases, Spearman's correlation coefficients were derived. Log survival were calculated using life table analysis (23). Log-Rank statistic was used to assess significance of survival. Mean values of the markers in patients were considered as cut-off for constructing survival curves. The multivariate analysis was carried out to correlate response to anticancer treatment, clinico-pathological parameters and levels of the biomarkers. For multivariate analysis, Cox's forward Logistic Regression (24) stepwise model was used.

RESULTS

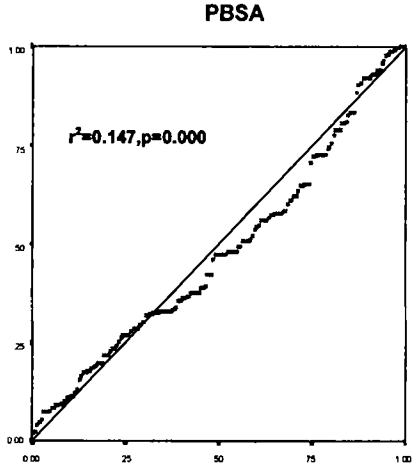
Serum levels of sallic acid forms

Serum levels of different forms of sialic acid were significantly higher in patients with BBD and untreated breast cancer patients as compared to the controls. In comparison between patients with BBD and untreated breast cancer patients, the levels of different forms of sialic acid and its ratio to total proteins were significantly raised in breast cancer patients. Diagnostic values of sialic acid forms were also evaluated by constructing Receiver's Operating Characteristic (ROC) curves. TSA and TSA/TP showed better diagnostic values as compared to PBSA, PBSA/TP and free Sialic acid (FSA). The PBSA and PBSA/TP levels were unable to discriminate between controls and untreated breast cancer patients at 30% or less sensitivity levels (as similar results are documented in previous reports (25, 26, 27) from our laboratory data are not shown).

Correlation of the markers with stage of disease

Spearman's correlation curves were constructed to evaluate association of serum levels of sialic acid and stage of disease in untreated breast cancer patients. The correlation of the variations in PBSA levels with stage of disease is depicted in Fig. 1 as representative pictorial presentation. Other sialic acid forms also showed positive correlation with the stage of malignant disease of breast. The "r^2" and "p" values indicated that the correlation was statistically significant for FSA, PBSA, TSA, PBSA/TP and TSA/TP.

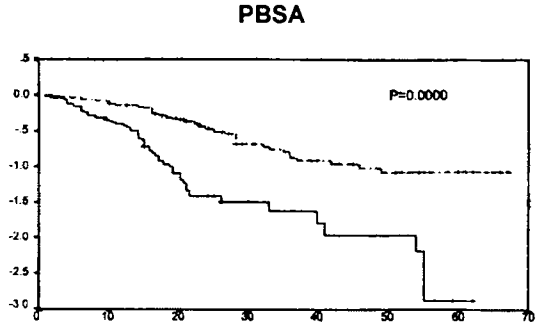
Fig. 1. Sperman's correlation curve showing variations in PBSA levels with stage of disease in breast cancer patients



Parameter	r^2	'p' value
FSA	0.033	0.015
TSA	0.085	0.000
PBSA/TP	0.126	0.000
TSA/TP	0.061	0.001

X-axis - Observed cumilative probability
Y-axis - Expected cumilative probability
PBSA - Protein bound sialic acid
FSA - Free sialic acid
TSA - Total sialic acid
PBSA/TP - Protein bound sialic acid/ Total protein
TSA/TP - Total sialic acid/Total protein

Fig. 2. Survival curve (log survival) of PBSA in breast cancer patients according to mean values as cut off



Parameter	'p' value
FSA	0.350
TSA	0.000
PBSA/TP	0.000
TSA/TP	0.000

_____ Above mean values

---------- Below mean values

X-axis - Duration in months
Y-axis - Log survival
PBSA - Protein bound sialic acid
FSA - Free sialic acid
TSA - Total sialic acid
PBSA/TP - Protein bound sialic acid/ Total protein
TSA/TP - Total sialic acid/ Total protein

Sialic acid forms and survival of breast cancer patients

To evaluate prognostic values of the markers, survival curves (log survival) were plotted for breast cancer patients. Mean pretreatment values of the markers were used as cut off levels. Survival of the patients with levels of biomarkers above cut off values were compared with patients whose pretreatment marker levels were below cut off.

Fig. 2 indicates over all survival curves for PBSA levels in breast cancer patients as representative presentation. TSA, PBSA/TP and TSA/TP also demonstrated significant difference (p = 0.000, 0.000 and 0.000) indicating poor prognosis of the group of the patients whose marker levels were above cut off. The patients having the marker levels below mean values had better overall survival. The results documented that high pretreatment levels of serum sialic acid were indicators of poor prognosis.

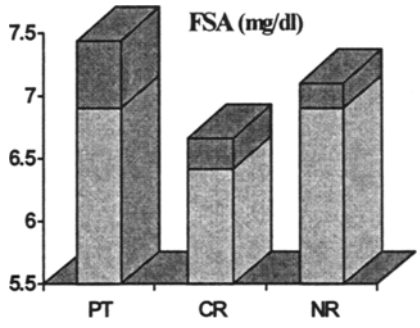
Comparison between pre-treatment and post-treatment serum levels of sialic acid forms

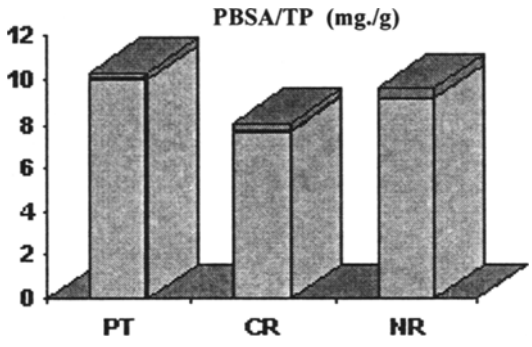
To evaluate efficacy of the biomarkers in treatment monitoring of breast cancer patients, levels of the biomarkers were analysed serially after initiation of anticancer therapy and grouped in to CR and NR. As can be seen from Fig. 3, the CR showed significantly lower mean values of the markers as compared to pretreatment values. However, in NR the marker levels were comparable or elevated as compared to their levels at the time of diagnosis. In comparison between CR and NR, the CR showed lower values. Each follow-up values were paired with the individual's pretreatment marker values and paired "t" analysis was carried out. CR showed significantly lower levels of PBSA, TSA and TSA/TP (p=0.004, p=0.003 and p=0.000 respectively) as compared to the values found at the time of diagnosis. Circulating levels of the markers during follow-up period in NR were comparable or increased than their pre-treatment values (Data not shown). To assess alterations in the biomarkers of individual patients with different treatment out come, levels of the biomarkers during follow-up were calculated as percentage of pretreatment levels. The pretreatment values were considered as 100%. Serum levels of all the markers remained below pretreatment level throughout the follow-up period in CR and remained above pretreatment level through out follow up duration in NR. Among the patients with initial response and then recurrence of the disease, marker levels remained below pretreatment values initially but then started rising. Importantly, the rise in the marker levels was observed 3-4 months prior to the clinical detection of the metastasis.

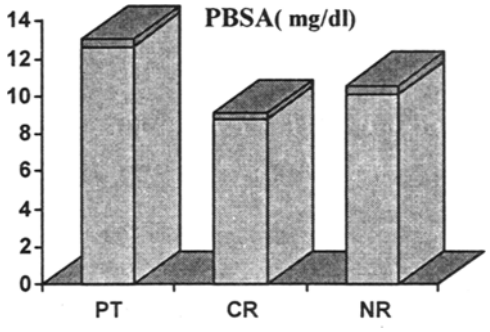
Lectin affinity chromatography for sialoproteins

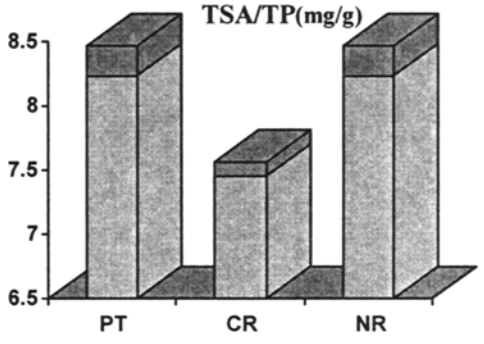
Proteins with terminal α-2-6 sialic acid residues were separated using sialic acid specific lectins (Sambucus Nigra). The lectin was coupled with CNBr activated sepharose beads prior to incubation with serum. Various control elutes were electrophoresed to confirm isolation of specifically desired proteins. Fig. 4 shows separation of proteins by SDS-PAGE of various elutes which were

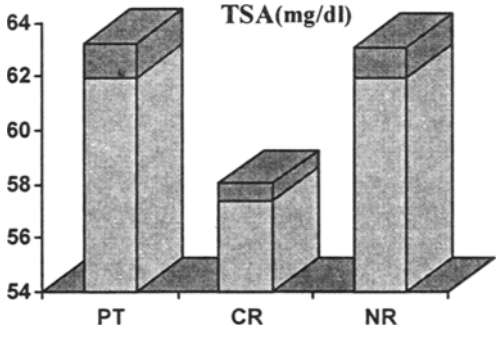
Fig. 3. Comparison of serum sialic acid levels (mean ± S.E.) in untreated breast cancer patients, complete responders and non-responders







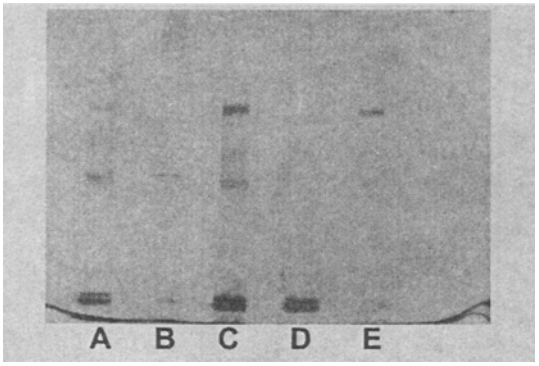




CR	- Complete responders
NR	- Non-responders
FSA	- Free sialic acid
PBSA	- Protein bound sialic acid
TSA	- Total sialic acid
PBSA/TP	- Protein bound sialic acid/ Total protein
TSA/TP	- Total sialic acid/Total protein
BC pts.	- Breast cancer patients

			FSA	PBSA	TSA	PBSA/TP	TSA/TP
Untreated	BC pts. VS CR	"t"	4.053	8.394	8.803	0.101	16.93
		"p"	0.045	0.004	0.003	0.751	0.000
Untreated	BC pts. VS NR	"t"	1.007	0.011	0.385	2.966	0.236
		"p"	0.086	0.921	0.122	0.056	0.310
	CR VS NR	"t"	1.582	5.970	5.367	3.780	14.84
		"p"	0.098	0.015	0.021	0.053	0.000

Fig. 4. SDS-PAGE of serum protein showing specific affinity to the lectins



Lane A : Elute of Lectin incubated with sepharose

Lane B : Elute of CNBr activated sepharose without lectin and serum

Lane C : Elute of lectin coupled with CNBr activated sepharose with serum

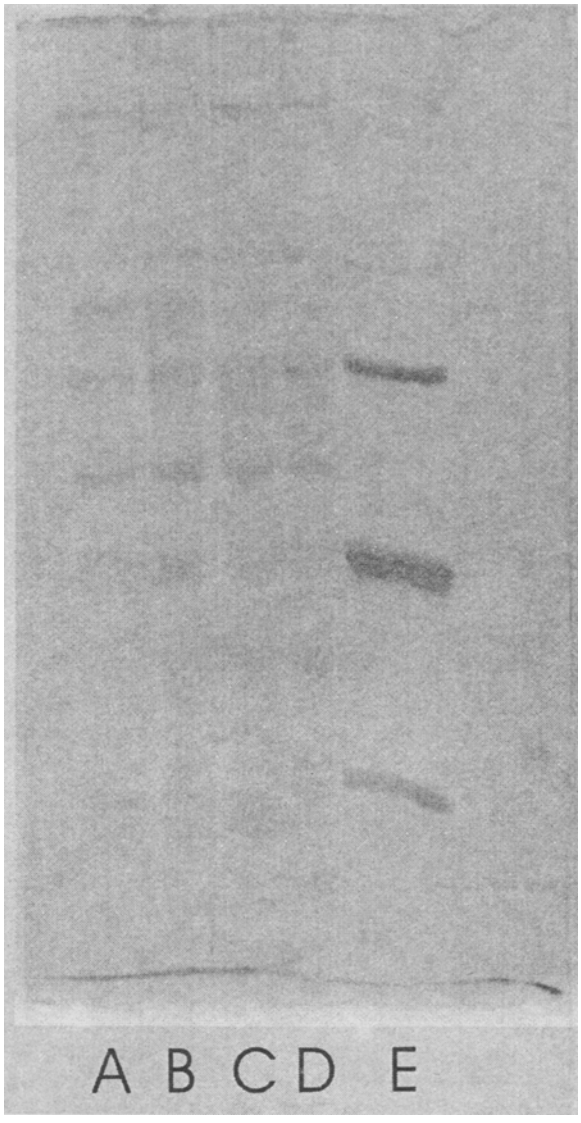
Lane D : Elute of serum incubated with only lectin

Lane E : Elute of serum incubated with CNBr activated sepharose

stained by silver staining. Lane A represents elute of lectin incubated with sepharose, lane B represents elute of CNBr activated sepharose without lectin and serum, lane C represents elute of lectin coupled with CNBr activated sepharose with serum, lane D represents elute of serum incubated with only lectin and lane E represents elute of serum incubated with CNBr activated sepharose. It is clear from the figure that lectin coupled with sepharose were able to bind with more number of proteins as compared to lectin alone. Further, the elutes of only sepharose, only lectin and serum with sepharose showed only one band which indicate that resultant separated proteins were only lectin binding proteins without impurities of other proteins.

Fig. 5 shows 1D electrophoretic pattern of lectin (Sambucus Nigra) extracts of serum from a breast cancer patient at diagnosis and during follow-up. Lane A shows sialoproteins of serum from control. Lane B shows sialoproteins of serum from untreated breast cancer patient. Lane C and D exhibit serum sialoproteins of cancer patient during/after anti cancer treatment. Lane E represents molecular

Fig. 5. Serum sialoprotein electrophoretic pattern in a breast cancer patient at diagnosis and during follow-up (using lectin-sambucus nigra)



Lane A : Serum Sialoprotein pattern in control

Lane B : Serum Sialoprotein pattern in untreated breast cancer patient

Lane C-D: Serum Sialoproteins pattern in the breast cancer patient during/after treatment.

Lane E : Molecular weight marker

Table 3. Mean values of serum sialyltransferase (CPM/mg protein/hour) in controls, patients with BBD and breast cancer patients

Group	Mean	S.E.	Groups compared	't' Value	'p' Value
Controls	2419.9	369.12	Controls vs BBD	1.913	0.1
Patients with BBD	3380.6	355.41	Controls vs BC	3.207	0.001
Untreated BC pts.	4680.4	419.52	BBD vs BC	2.918	0.01
CR	3219.7	380.37	BC vs CR	2.519	0.05
NR	4289.5	423.89	BC vs NR	0.199	NS
			CR vs NR	2.930	0.05

BBD - Benign Breast Diseases, Pts. - Patients, BC - Untreated Breast cancer patients
TP - Total Protein, CR - Complete Responders, NR - Non responders, vs. - versus

Table 4. Association of treatment response with biomarkers and clinicopathological parameters in breast cancer patients

Variable	Significance
Histology	0.4555
Stage	0.0000
Lymph node involvement	0.0118
Site	0.8838
Free sialic acid	0.5784
Protein bound sialic acid	0.1788
Total sialic acid	0.0010
Protein bound sialic acid/ Total protein	0.8040
Total sialic acid/ Total protein	0.0020

weight marker. Clinically the patient was having disease free survival. As clear from the figure, more number of bands were present in the elutes of sera from untreated breast cancer patients at the time of diagnosis as compared to that of follow-up, in patients with favorable treatment outcome.

Variations in serum sialyltransferase activity

Mean values of sialyltransferase in the controls, patients with BBD and breast cancer patients at the time of diagnosis and during follow-up are shown in Table 3. Untreated breast cancer patients revealed elevated levels of serum sialyltransferase as compared to the controls and patient with BBD (p=0.001 and 0.01, respectively). The patients with BBD showed higher mean values of the enzyme as compared to the controls. When sialyl-transferase levels were compared between breast cancer patients at diagnosis and during follow-up, CR exhibited significantly lower levels of the

Table 5. Comparison of glycoprotein constituents and enzyme levels between nonmalignant and malignant tissues

Parameters	Group	Mean	S.E.	't' value	'p' value
FSA	NM	1.429	0.185	2.921	0.097
(mg/g protein)	MT	1.606	0.364		
Sialyltransferase	NM	268.99	65.34	4.26	0.047
(CPM/mg protein/hr)	MT	1058.7	508.8		

FSA - Free sialic acid, MT - Malignant tumour tissue, NM - Non malignant Tissue

enzyme as compared to the untreated breast cancer patient (p=0.05) and NR (p=0.05). The enzyme levels were comparable between untreated breast cancer patients and NR.

Multivariate analysis : Clinico-pathological parameters and biological markers with treatment outcome

Cox's regression model was used to evaluate correlation between established clinico-pathological parameters and variations in serum tumour markers in predicting the prognosis (Table 4). TNM classification, lymph node involvement, TSA and TSA/TP showed significant association with treatment outcome. However, histopathological examination, site of the disease, PBSA, PBSA/TP and FSA levels did not show any relation with treatment response. In this model, prognostic significance of TNM stage was followed by TSA/TP and TSA.

Comparison of the markers between nonmalignant and malignant tissues

The levels of FSA and sialyltransferase were analysed from malignant and surrounding normal tissues to evaluate its association with the circulatory levels. As clear from Table 5, the glycoprotein constituents and enzyme levels were higher in malignant tumour tissues as compared to the surrounding normal tissues. The elevations in sialic acid and sialyl-transferase were statistically significant (p=0.097 and 0.047, respectively). The increased values of FSA and sialyltransferase in tumour tissues confirmed that the elevations in the markers in circulation were due to the presence of malignant tumours.

DISCUSSION

Glycosylation has been demonstrated to play a critical role during malignant transformation (28, 29). We found that breast cancer patients had significantly higher levels of different forms of sialic acid as compared to the controls. The results on serum sialic acid levels in present study were in accordance with the reports from our laboratory as well as of other workers (6, 30, 31). Previous reports on sialic acid in cancer have mostly compared the levels with healthy individuals. The goal of present study was to understand association of glycoprotein changes with malignancy. Therefore, the patients with BBD served as pathological controls in the current study. Current study also revealed that serum sialic acid have significant positive correlation with stage of the malignant disease which is in accordance with previous reports (6, 30, 32).

The present investigation also studied for association of serum concentrations of glycoprotein constituents on overall survival of patients which was assessed by Kaplan Meier survival curves. Except FSA, statistically significant relationship was observed between elevated serum levels of the markers and survival of the breast cancer patients. This is a poorly studied part of the previous work on glycoprotein constituents. Only a few reports showing association of pretreatment levels of these markers with survival of the patients are available in the literature (33).

Various investigators have reported significant correlation between continuously raised levels of sialic acid and a worse prognosis in cancer patients (31, 34). However, the numbers of subjects in these reports were less. Our observations on a large population showed that NR had higher levels of the biomarkers than CR.

Previous studies were centered largely around the demonstration of increased levels of carbohydrates of the carbohydrate-protein complexes in sera of cancer patients. As sialic acid occupies terminal position, any change in glycoprotein will account for the changes in sialic acid and vice versa. The data from our laboratory and others indicate elevations in protein bound sialic acid levels in various malignancies (30, 31). The elevations in sialic acid contents during malignancy can not be explained only by the production of new proteins because very less new glycoproteins are seen on electrophoresis (35). The elevations found in sialic acid forms in cancer patients could be due to an overall higher amount of sialic acid or due to selective increase in existing specific sialylated sequence or a tumour associated de novo synthesis of specific sialylated sequence. Considering the fact that electrophoretic analysis can provide a comprehensive view on multiple proteins, we have analysed electrophoretic patterns of serum sialoproteins.

Variations in the structure of an oligosaccharide glycan have been previously referred to as microheterogenicity. Minor microheterogenicity can be caused by variations in sialic acid, galactose and/or fucose contents (21). The changes in serum sialoglycoproteins that profile malignancy are shared by other disease status, but correlation of malignant cells with increased or abnormal sialoproteins is found to be different (36, 37). Elevations in sialylation has been reported to be associated with poor prognosis and resistance to cancer therapy (38). High sugar specificity of lectins makes them ideal tool for identifying structural features of the oligosaccharide moieties. It was reported by Muryama *et al.* (39) that α-2-6 sialylation recognised

by Sambucus Nigra is different than sialylated antigens. Selective binding of terminal sialic acid residues via α-2-6 linkage during malignancy, has been reported (39). The current investigation found more number of sialoprotein bands among cancer patients as compared to controls as well as patients with BBD. We have observed more number of proteins having terminal α-2-6 sialic acid residues in patients with malignant breast tumours as compared to controls and patients with BBD. The current findings on sialic acid and sialoproteins revealed its significant use in prognostication and treatment monitoring.

Analysis of the enzymes involved in addition of terminal sugars can provide useful information for better understanding of the mechanism of elevations in sialic acid values during malignancy. Gessner *et al.* (40) demonstrated that determination of sialyltransferase in colorectal tumour tissue and sera of cancer patients may be a new means for tumour detection and monitoring (41). Increased excretion of glycosidically bound sialic acid in urine of cancer patients reflects elevations of sialyltransferase activity in tumour tissue. Increased circulatory sialyltransferase activities have been reported in patients with various cancer (42, 43). Serial estimations of sialyltransferase were carried out to evaluate its importance in assessing response to anticancer therapy. Serum sialyltransferase levels significantly correlated with disease status during follow-up in breast cancer patients. A significant decline in the levels of sialyl-transferase was associated with favourable treatment response which is in accordance with previous reports (44, 45). Increased sialyl-transferase activities may be responsible for increased expression of cell surface glycoconjugates. The current results support the notion that the alterations seen in cell surface glycoconjugates during oncogenic transformation can be the result of altered expression of glycosyl transferases. The presence of sialyltransferase in malignant cells could lead to altered or even unique glycoconjugates.

To examine the origin of altered serological concentrations of biomarkers, present study has also included malignant and adjacent normal tissues for analysis of glycoprotein constituents. The study revealed significantly higher concentrations of sialic acid in tissue homogenates of malignant tumours as compared to that of surrounding normal tissues. Significantly higher levels of sialic acid in tumour tissue have also been reported by various workers (6, 46). Similar observations for abnormal levels of glycoprotein constituents have been documented in tissue extracts of liver cancer (34, 47). Higher expression of sialyltransferase was also reported in malignant brain tumours and was undetectable in normal brain tissues (43). An increase in sialyl-transferase activity of malignant tumours have been documented (48). The results suggested that sialylation by the sialyltransferase is dominant in tumour cells. However, previous reports have not correlated presence of circulating markers with their concentrations in tumour tissues. Present study exhibited that the alterations in glycoprotein constituents in serum are reflection of the alterations in the tumour tissues. Current study addressed role of serum levels of sialic acid and sialyltransferase in prognosis and therapeutic monitoring of breast cancer patients. To the best of our knowledge, the present study validated data on the largest population of the patients with long-term post-treatment follow-ups which is an important feature of the work.

In conclusion, elevations in serum levels of sialic acid and sialyltransferase in breast cancer were associated positively with presence of malignant tumour and negatively with response to anticancer treatment. Altered sialic acid and sialyltransferase levels correlated well with alterations in the sialylation changes. The results revealed that measurement of sialic acid forms and sialyltransferase are of clinical value in monitoring clinical course as well as in assisting the diagnosis of breast cancer. Analysis of the markers can be an additional tool for diagnosis, prognostication and treatment monitoring of cancer patients. The present study indicated altered glycosylation during malignant transformation in terms of sialylation at α-2-6 position at termini. It may contribute to malignant growth by altered glycosylation of glycoconjugates during tumorigenesis.

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