East African Medical Journal Vol. 84 No. 5 May 2007
SERUM TOTAL SIALIC ACID AND HANGANUTZIU-DEICHER ANTIBODY IN NORMALS AND IN CANCER PATIENTS
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SERUM TOTAL SIALIC ACID AND HANGANUTZIU-DEICHER ANTIBODY IN NORMALS AND IN CANCER PATIENTS

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ABSTRACT

Objective: **To determine the levels of both TSA and HD antibody in sera of patients with various malignancies and evaluate their potential role as diagnostic and/ or prognostic markers.**
Design: **Laboratory based analysis.**
Settings: **Kenyatta National Hospital, Kenya Medical Research Institute and the Department of Biochemistry, University of Nairobi.**
Subjects: **A total of 909 serum samples, 420 from cancer patients recruited at Kenyatta National Hospital and 509 from normal blood donors recruited at Nairobi Hospital.**
Results: **The mean age for the patients and controls was 36 and 37 years respectively. Carcinoma patients constituted 54%, sarcoma 12.1%, lymphoma 16.4% and 17.4% had other types of tumours. The mean TSA in patients was 0.86 mg/ml ± 0.026 compared to 0.82 mg/ml ± 0.014 in controls. The TSA level was significantly higher in patients compared to controls (Student's t-test p = 0.031 at 0.05 confidence level). The TSA increased with age in both study groups. In patient sera, both gender gave the same mean of 0.83 mg/ml while it was 0.82 mg/ml and 0.83 mg/ml in control females and in males respectively. Sarcomas had the highest amount of 0.93 mg/ml but there was no significant statistical variation between tumour types (p = 0.076). The HD antibody mean readings were 0.004 in pathologic sera compared to 0.011 in controls. The values were significantly elevated in patients (p=0.03) with females giving a higher value for both study groups (p = 0.628). HD antibody readings was significantly higher in carcinomas (p = 0.017) compared to those of sarcomas and lymphomas. There was no association between antibody readings and age of patient (p = 0.601).**
Conclusion: **Both TSA and HD antibody values were significantly elevated in patients compared to clinically healthy controls and while TSA levels increased with age and was independent of gender, HD antibody levels were independent of age, gender and also tumour type. The study demonstrates that although TSA is normally elevated in malignancy, most of the sialic acid shed is of N-acetyl type as some patients do not express HD antibody directed to the N-glycolyl sialic acid. The reason why some tumours would express Neu5Gc at any one time needs further evaluation.**

INTRODUCTION

Blood TSA may be regarded as a useful marker for a variety of cancers and could be reliable in predicting early malignant change, progression and metastasis during both therapy and follow-up (1). Sialic acids are components of glycoproteins and glycolipids (2,3), the so-called sialoglycoconjugates that range throughout the animal kingdom. The simplest sialic acid is N-acetylneuraminic acid (Neu5Ac), and it can be modified into more complex sialic acids (4). Neu5Ac is the only sialic acid normally present in

human and chicken tissues and fluids. However, several human cancers are known to express N-glycolylneuraminic acid (Neu5Gc), another sialic acid made from the CMP-glycoside of Neu5Ac or from free Neu5Ac by enzymes CMP-Neu5Ac hydroxylase and Neu5Ac hydroxylase respectively (5-7). Such Neu5Gc-containing sialoglycoconjugates are called Hanganutziu and Deicher (HD) antigens. Cancer patients occasionally generate antibodies against HD antigens and these are referred to as HD antibodies (8,9). Chickens with Marek's lymphoma also express Neu5Gc (10) and the fact that chickens lack Neu5Gc was confirmed by the production of high-titer antibody in chickens immunised with Neu5Gc-containing gangliosides (11).

It has been previously demonstrated that sialic acid is a useful tumour marker (12-14), but the elevated levels has not been correlated with HD antibody titers. This study compared the pathological levels of both TSA and HD antibody in malignant sera and in apparently normal healthy individuals. In addition, the relationship of the two parameters to age of patient, sex and type of tumour was assessed.

MATERIALS AND METHODS

Normal human sera: Serum samples from 509 blood donors was obtained from Nairobi Hospital Blood Bank and used as controls. These samples had been earlier screened for HIV, Hepatitis B Surface Antigen and TB. All the samples were stored at –20°C till usage.

Malignant sera: Sera from 420 individuals diagnosed with various types of malignancy were obtained with written consent from outpatients attending the Haematology and Oncology Outpatients' Clinic or from in-patients hospitalised at Kenyatta National Hospital for a period of 12 months. The sera were extracted from about 3-5 ml of blood drawn from the anterior cubital vein. Analysis of the samples was carried out in the Department of Biochemistry, University of Nairobi and at Kenya Medical Research Institute (KEMRI). Relevant clinical details were noted and recorded in a proforma questionnaire.

Determination of TSA: TSA was measured colorimetrically in sera from 420 patients and 509 controls by a modification of the method described earlier (15). To each test tube, 0.1 ml of serum was added to 0.9 ml of distilled water, 0.2 ml of Ehrlich's reagent prepared by dissolving 0.7 g of p-dimethylaminobenzaldehyde in 250 ml of concentrated HCl:H_2O mixtures (1.5:1) was added to the test tube while blank tubes contained 0.2 ml of the HCl:H_2O mixture. The tube contents were then vortexed, sealed with parafilm and incubated at 56°C for 24 hours with occasional agitation. After this incubation, 3 ml of a 0.9 ml NaCl solution were added to each tube, vortexed and centrifuged at 5000 x g for 10 mm. The colour of the supematant was read at 525 nm using a supernatant Perkin Elmer spectrophotometer.

Enzyme-Linked Immunosorbent Assay (ELISA): This was done as previously published (8) using sterile microtitre plates with 96 flat-bottomed wells (Nunclon Intermed., Denmark). Fifty µl of chloroform:methanol (2:1) solution containing 25 µg of antigen obtained from horse erythrocyte membrane (8) and 25 µg of sodium taurodeoxycholate was dispensed into each well. For the assay of each serum sample, three wells were coated with antigen while the other three wells were coated with sodium taurodeoxycholate. The plate was left to dry overnight at 37°C. A 200 µl of PBS-containing 1% egg albumin was dispensed into each well and the plate incubated for 1 hour at 37°C. The plate was then washed thrice with 200 µl per well of 0.05% Tween 20 in PBS before 50 µl of serum diluted ten times with 1% egg-albumin in PBS was added. The first immunoreaction was allowed for 1 hour at 37°C. Washing was repeated similarly and 50 µl of 2000-fold diluted horse radish peroxidase-labelled goat anti-human IgG (Fab fragment) was added to each well. The second immunoreaction was performed for 1 hour at 37°C. Subsequent washings were repeated before 100 µl of 0.06 g/l in a glycine buffer of 2,2'-azino-di [3-Ethyl-benzathiozoline sulfonate] (ABTS) containing 0.02% hydrogen peroxide in a citric acid buffer was discharged into each well. The enzyme reaction was allowed for 1 hour at 37°C after which the product was directly determined by measuring the absorbance at 405 nm using an ELISA reader (Labsystems Ltd).

Statistical analysis: The data obtained was grouped and entered in SPSS version 7.5 for statistical analysis. It was subjected to statistical tests of significance

such as Students t-test in order to make comparisons between study subjects while differences in various means was by Analysis of Variance (ANOVA). Differences between non-parametric variables were arrived at by use of chi-squares (x^2). Differences associated with $p<0.05$ were considered significant. Correlation analysis (designated *r)* was achieved by use of Spearman's correlation method.

RESULTS

Data on the study subjects: The mean age of the patients and controls was 36 and 37 years and the range was 13-67 and 17-67 respectively. Male patients were 110 (38%) while 182 (62%) were females. As shown in Table 1, 54% of patients studied had carcinoma, 12.1% sarcoma, 16.4% lymphoma and 17.4% had other types of tumours.

TSA levels of normal and malignant sera: The distribution of TSA in both study groups is shown in Figure 1. In controls, the range of TSA concentration was between 0.52-1.20 mg/ml with a mean and a standard deviation of 0.82 ± 0.03. The range in patients was 0.48-1.72 mg/ml with a mean and a standard deviation of 0.87 ± 0.03 respectively. Statistical analysis by Student's t-test revealed that the malignant levels were significantly elevated compared to those of control cases ($t = 14.82$, $p = 0.03$). The individual patients were grouped into five according to age. The mean TSA level for the five groups ranged from 0.79-0.91 mg/ml while those of controls ranged from 0.73-0.87 mg/ml (Table 2). When both the tests and controls were compared in terms of age group, the malignant sera had higher TSA levels in all age groups. Patients above 56 years had the highest levels of TSA. The test of difference in mean levels between age groups in patients by ANOVA was $F\ 4,209 = 3.864$, $p = 0.005$ while it was $F\ 4,241 = 2.021$, $p = 0.092$ in controls.

The mean TSA level by gender was the same in both sexes in both study groups. Students t-test analysis for the difference was $t = 0.067$, $p = 0.947$ (Table 3).

The TSA levels were also compared for both the tests and controls according to gender (Table 3). Although the malignant sera had higher levels than controls (Table 2), both genders gave the same mean readings giving a no significance verdict by ANOVA ($F\ 1,246 = 0.59$, $p = 0.44$). Further, the TSA levels were compared against tumour types. Sarcoma cases had the highest mean TSA level at 0.93 mg/ml compared to carcinoma (0.85 mg/ml) and lymphoma (0.81 mg/ml) (Table 4). Statistical test of significance for any difference in means by ANOVA within cancer types was $F\ 3,255 = 2.321$, $p = 0.076$ implying insignificance. However, TSA levels in patients with sarcomas were significantly different from levels in patients with lymphomas ($p = 0.037$) but not from carcinoma.

HD antibody levels: In controls, the HD antibody mean level was -0.055 compared to 0.014 in patients. Levels of HD antibody were significantly elevated in patients compared to controls (Student's t-test $t = 2.613$, $p = 0.009$) (Figure 2). The mean level

Table 1

Histological classification of tumour types studied

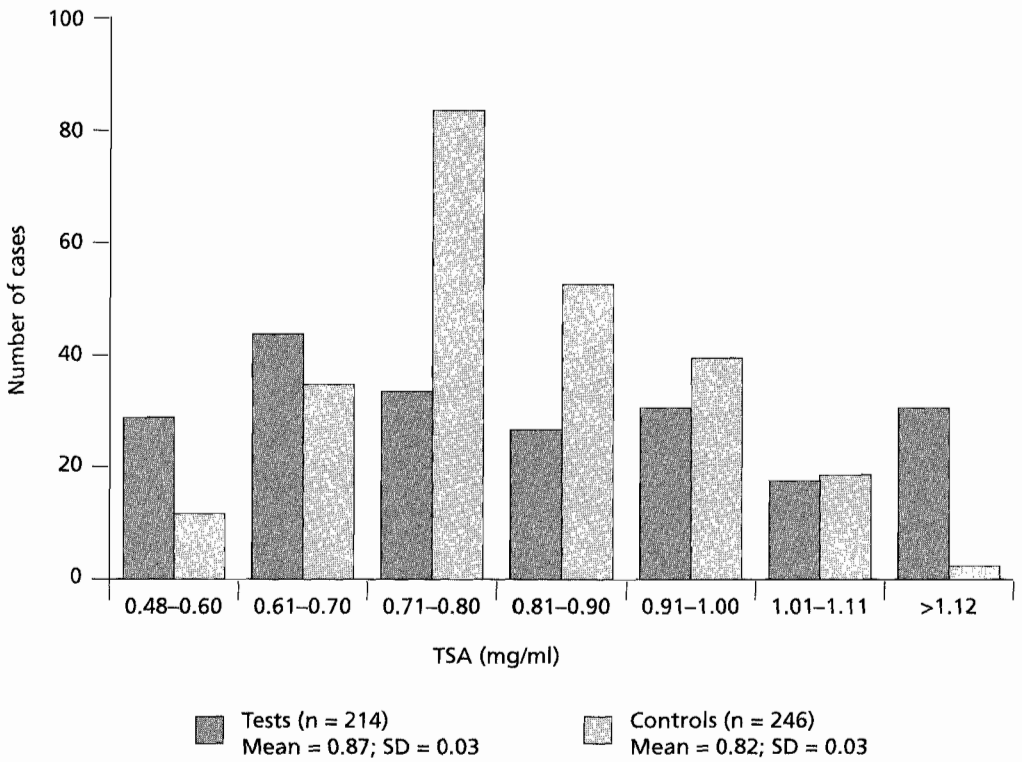
Tumour	No. of cases	(%)	Location of tumour
Carcinoma	227	54	Carcinomas of cervix, ovary, breast, oesophagus, larynx, rectal and post nasal space
Sarcoma	51	12.1	Kaposi's sarcoma, osteogenic sarcoma, rhabdomyosarcoma and fibrosarcoma
Lymphoma	69	16.4	Hodgkins, non-Hodgkins and other lymphomas
Others	73	17.4	Tumours such as meningioma, haemangioma and spinal among others
Total	420	100	

292 cases had age-matched controls while 246 cases matched both for age and sex

was higher in females than in males in test cases compared to controls (Table 5). Patients of the age between 26-35 years showed higher levels of HD antibody of 0.02 compared to other age groups (Table 6). There was no significant mean difference within age groups in controls. The mean HD antibody level in carcinomas and sarcomas was 0.01 while the level in lymphomas was -0.01 (Table 7). The correlation between HD antibody ELISA values and TSA levels was not significant (Spearman's correlation, $r = -0.36$, $p = 0.565$).

Figure 1

TSA concentrations of patients and control sera



The TSA readings ranged from 0.52 – 1.2 mg/ml in controls and 0.48 – 1.72 mg/ml in the tests. Over 75% of the patients had levels above 1.0 mg/ml compared to 0.9 mg/ml in 100% of the controls

Table 2

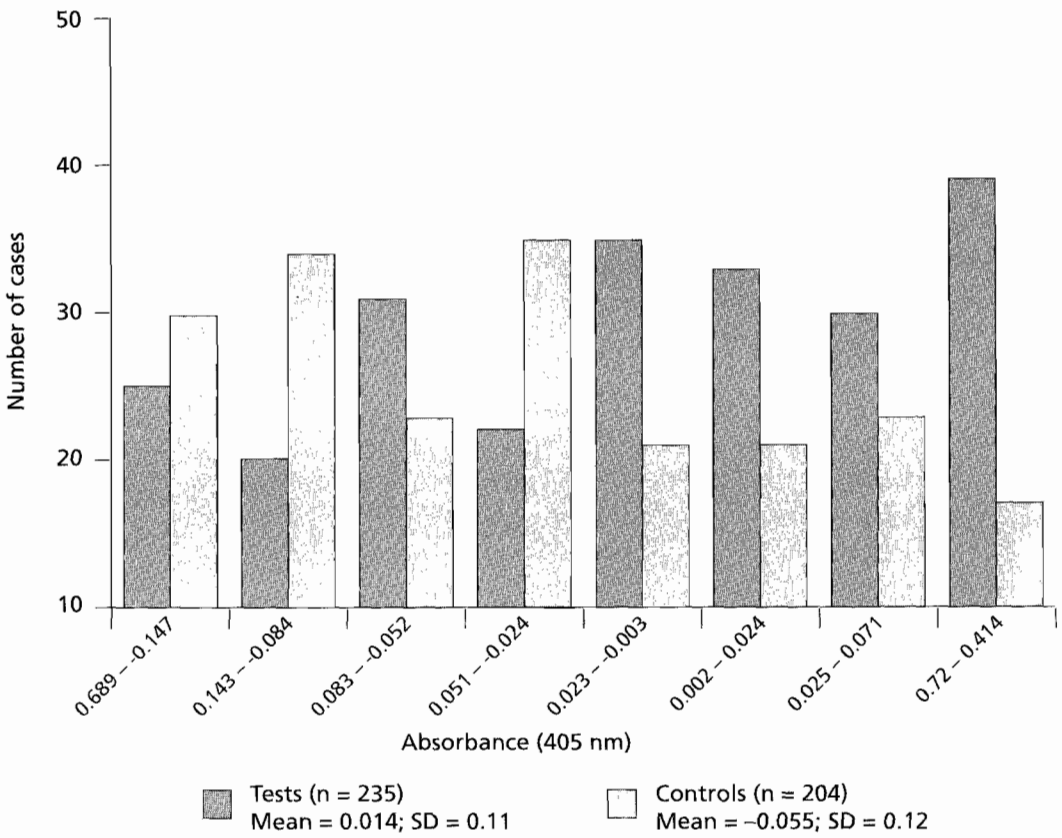
TSA levels of various age groups

Age (years)	Controls			Tests		
	No.	Mean	(SD)	No.	Mean	(SD)
15-25	51	0.73	(0.01)	34	0.79	(0.02)
26-35	75	0.83	(0.01)	72	0.88	(0.03)
36-45	61	0.84	(0.01)	50	0.90	(0.02)
46-55	49	0.84	(0.01)	48	0.88	(0.03)
56+	10	0.87	(0.01)	10	0.91	(0.03)

The mean difference between patients age groups was significant by one way ANOVA ($F_{4,209} = 3.864$, $p = 0.005$)

Figure 2

ELISA reading for HD antibody



HD antibody was significantly elevated in patient sera compared to healthy sera (Student's t-test; t = 2.613, p = 0.009)

Table 3

TSA levels by gender

Gender	Controls		Tests	
	No.	Mean (mg/ml)	No.	Mean (mg/ml)
Females	103	0.82	129	0.87
Males	143	0.83	85	0.87

The mean difference in the two gender types by ANOVA was F 1,246 = 0.59, p = 0.44.

Table 4

TSA levels of various tumour types

Tumour type	No. of cases	Mean (mg/ml)	(SD)
Carcinoma	139	0.85	(0.03)
Sarcoma	34	0.93	(0.03)
Lymphoma	37	0.81	(0.02)
Others	49	0.92	(0.03)
Total	259	0.87	(0.03)

The variation in mean TSA between tumour types was not significant (F 3,255 = 2.321, p = 0.076).

Table 5

HD antibody by gender

Gender	Controls		Tests	
	No.	Mean	No.	Mean
Females	91	-0.02	139	0.01
Males	132	-0.01	102	0.00

The statistical mean difference in HD antibody titres in the two genders was not significant (ANOVA F 1,255 = 0.24, p= 0.63)

Table 6

HD levels by age

Age (years)	Controls		Tests	
	Mean	(SD)	Mean	(SD)
15-25	-0.02	(0.08)	-0.012	(0.05)
26-35	0.00	(0.11)	0.019	(0.06)
36-45	-0.03	(0.1)	-0.014	(0.05)
46-55	0.00	(0.11)	0.004	(0.06)
56+	-0.02	(0.09)	0.005	(0.03)

Patients of the age 26-35 years gave a higher mean of HD antibody (0.019) than other age groups.

Table 7

HD antibody according to tumour type

Tumour type	Carcinomas	Sarcomas	Lymphomas
Mean	0.01	0.01	-0.01
(SD)	(0.05)	(0.051)	(0.05)

HD antibodies were not detected in lymphoma type of tumour. Carcinomas and sarcomas gave significantly higher values (p = 0.013)

DISCUSSION

Tumour markers can be of great clinical utility in enhancing early diagnosis, prognosis and in follow-up of post treatment of many types of cancer. An elevation or reduction of the marker in serum would be a good indicator of disease stage and therapeutic response thereby reflective of tumour burden.

In most cancers, chemotherapy, radiation and surgery are the definitive approaches employed in the treatment of nearly all cases. Basically, anticancer drugs cause inhibition of the growth of cancer cell lines, reduction of tumour mass and complete and partial remissions in patients. This therapeutic response would be reflected in a change in the tumour mass and activity leading to alteration of the marker such that these patients would show a decline in marker levels. Marker levels may be increased from normal tissues in response to certain physiological stimuli while they could vary between tumours since there is heterogeneity within tumours represented by the type of the tissue and rate of growth. The above observations necessitated the investigation of TSA and HD antibody levels in relation to certain patients' factors such as age, gender and type of tumour.

A significant difference in levels of TSA was found in patients compared to healthy controls (p = 0.03). This is consistent with earlier findings that cancer patients have increased TSA concentration reflected by significantly elevated levels arising from shedding or secretion of this marker into circulation thus increasing their concentrations in blood.

Neoplasms often have increased concentration of TSA on the tumour cell surface occurring as sialoglycoconjugates. These are shed or secreted by some of these cells thus increasing their concentrations in blood (12-14).

A significant elevation of TSA at advanced age in patients compared to the controls was observed. TSA level increase with age has been reported in patients with squamous cell carcinoma (16). The TSA levels were however independent of gender as the mean levels in the two gender groups was the same. With regards to histological tumour types, the data showed levels to be increased in all tumour types. Sarcomas had the highest level compared to carcinomas and lymphomas though the difference was not significant. The slight differences observed in different histological types could be explained by the membrane composition of the different tissues. It is possible that normal tissues have a varying degree of sialoglycoconjugates and thus different amounts of total sialic acid component. These findings imply that TSA is not specific to one type of tumour and can perhaps be used for general screening for malignancy.

Since reduction of marker levels in patients responding to therapy has been reported (1,13,14,17), this could reflect the observed decrease in TSA levels since the majority of patients were on treatment. This, however, needs to be verified in follow-up cases, which are underway. Detection of HD antibodies in malignant sera was an indication that antibody-producing cells are stimulated in many patients following expression of HD antigens. Antibodies were detected in carcinomas and sarcomas but not in lymphomas.

This observation calls for further study. HD antibody level was shown to be different in the two gender groups as female patients had higher levels than males. This difference was however not significant and could be more due to the effect of tumour type rather than gender difference since most patients suffering from carcinomas were females.

The level profile of HD antibody in different age groups did not show any significant trend. Since production of antibodies is triggered by the presence of an antigen and has nothing to do with the age of the patients, this observation may largely be attributed to the tumour rather than age of the patient. Further studies are called for.

CONCLUSION

This study has demonstrated significant differences in circulating levels of TSA and HD antibody in cases with malignancies compared to clinically healthy cases. The TSA levels were significantly dependent on age of patient and were independent of the gender and the tumour type. Conversely, HD antibody levels were independent of gender, age and tumour type. There was no relationship between HD antibody and TSA levels in the studied subjects meaning that most of the expressed type of sialic acid is the N-acetyl type which has no immunological correlation with the HD antibody. Overall, this work indicates that TSA and HD antibody levels can be used for following up but not for elucidation of specific type of tumours. It remains to be established why at some stage of malignancy, the tumour cell expresses N-glycolyl sialic acid and subsequent production of HD antibody.

ACKNOWLEDGEMENTS

To the Nairobi Hospital Blood Bank Unit and Kenyatta National Hospital Cancer Outpatients Clinic for the provision of control and malignant sera respectively and to the VRC, KEMRI where ELISAs were done. Our gratitude goes to Mr. F.U. Mmbaya of the Department of Biochemistry, University of Nairobi, for technical assistance.

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