

## CIRCULATORY AUTOANTIBODIES AGAINST HYALURONIC ACID BINDING PROTEINS: A NOVEL SERUM BIOMARKER

VENKATRAO NUNNA<sup>1</sup>, BANERJEE SD<sup>1</sup>, KARUNA KUMAR M<sup>1\*</sup>,

Department of Studies in Biochemistry, University of Mysore, Manasagangothri, Mysore.

Email: kaarunamkk@rediffmail.com

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### ABSTRACT

**Objective:** Circulating autoantibodies have been extensively investigated as possible markers for early diagnosis of cancer. The present study was carried out to investigate whether anti-HABPs autoantibodies could be classified as a serum biomarker for malignant tumors.

**Methods:** An enzyme-linked immunosorbent assay, western blot was used to detect anti-HABPs autoantibodies in sera from 99 patients with various types of cancers and 50 healthy subjects matched by age and gender.

**Result:** Our results clearly indicated that patients with cancer have significant higher circulating levels of anti-HABPs antibodies as compared to control subjects ( $P < 0.001$ ). Receiver operating characteristic plot test has exhibited 91.9% sensitivity and 76.3% specificity.

**Conclusion:** anti-HABPs autoantibodies are promising biomarker for malignant tumors and could play a role in the development of multimarker assay for the early detection of cancer.

**Abbreviations:** HABPs, hyaluronic acid binding proteins; ELISA, enzyme linked immune sorbent assay; AAbs, autoantibodies; BSA, bovine serum albumin; HA, hyaluronic acid; bHA, biotinlyted hyaluronic acid; ROC, receiver operating characteristics; AUC, area under curve; TAA, tumor associated antigen; ABTS, 2,2'-azino bis (3ethylbenzothiazoline 6 sulphonic acid); MES buffer, 2 (N morpholino) ethanesulfonic acid; EDC, 1 Ethyl 3 (3 dimethylamino propyl) carbodiimide; DMSO, dimethyl sulfoxide; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence

**Keywords:** autoantibodies, cancer, HABPs, HC, serum.

### INTRODUCTION

Early detection and diagnosis are of utmost importance in cancer management. Despite significant improvements in treatment, early detection remains the most important prognostic factor prediction [1,2] Current cancer screening methods, including colonoscopy for colon cancer [3], computed tomography for lung cancer, papanicolaou stains for cervical cancer, mammography of better outcome for breast cancer, PSA for prostate cancer and have demonstrated some limitations in terms of sensitivity, specificity and complexity [4]. Serum tumor associated antigens (TAA) have been extensively studied for early cancer detection because of simplicity and reliability of test used for their determination, such as western blot and enzyme-linked immunosorbent assay (ELISA). Unfortunately, they are transiently secreted and rapidly eliminated from blood circulation [5] and usually reach a detectable concentration only in the advanced stage of cancer [6].

In conjunction with TAAs, autoantibodies are frequently detected in sera from patients affected by different types of tumors [7]. This finding has been interpreted as an attempt of the immune system to block invasion and spreading of cancer cells in the organism. Circulating autoantibodies have biochemical and biological characteristics that relinquish them particularly suitable to screen subjects at early risk of cancer. In a point of fact, they may develop early in the process of tumorigenesis, when premalignant or malignant cells inaugurate to express altered proteins as a result of cell transformation [8]. In addition, they can easily be detected in the serum because of the usual high concentration and long-time stability [7]. For these reasons, great efforts have been made in recent years to identify circulating autoantibodies directed against cancer-related proteins in order to build up tests for the early detection of tumors [9].

In present study, we investigated the production of autoantibodies against HABPs in patients affected by different types of cancer. These HABPs, also has been largely regarded as a TAA, since their present at elevated concentrations in the blood of cancer patients and is over expressed in the vast majority of cancer serum/tissues [10]. In fact, HA can bind important proteins associated with the membrane of tumor cells, such as H11, CD44, cdc37. [11]. HA-HABPs has also been implicated in cancer progression by modulating tumor cell adhesion, cell proliferation and invasion processes. Here we show that patients with different types of cancer, but not healthy controls, develop autoantibodies against HABPs. This finding discloses the capability of HABPs to trigger a humoral immune response in cancer patients and provides the basis for further investigation on a possible use of anti-HABPs antibodies as biomarkers for early diagnosis of cancer.

### PATIENTS WITH METHODS

#### Subjects

We tested 149 serum samples from patients with cancer (n= 99) and normal HC (n=50), ninety nine consecutive patients with early (N = 40), (N = 37) and (N = 22). Related cancer includes (male 32 and female 67), HC (male 28 and female 22). All human samples were collected prospectively at Preethi center for oncology, Bharath cancer hospital and KR hospital. The cancers were various cell types breast (n = 20, mean age  $\pm$  SEM = 50.6  $\pm$  14.4 years), cervical (n = 23, mean age  $\pm$  SEM = 47.2  $\pm$  15.5 years), colon (n = 18, mean age  $\pm$  SEM = 52.2  $\pm$  12.2 years), ovary (n = 13, mean age  $\pm$  SEM = 59.5  $\pm$  15.1 years), stomach (n = 12, mean age  $\pm$  SEM = 56.5  $\pm$  15.6 years), Rectum (n= 4, mean age  $\pm$  SEM = 59.7  $\pm$  9.3 years), lung (n =9, mean age  $\pm$  SEM = 45.2  $\pm$  15.5), healthy control (n = 50, mean  $\pm$  SEM = 38.7

± 15.5 years), (shown in table 1). All the samples were handled and stored essentially with the same protocol. Written informed consent was obtained from each subject. The study protocol conformed to the Institutional Ethical Review Board-approved by University of Mysore

#### Preparation of biotinylated hyaluronic acid probe

500µg of the hyaluronic acid (across USA) was dissolved in 500µL of 0.2 M MES buffer (pH 5.5). To this solution, 1mM biotin-LC-hydrazide (dissolved in DMSO) and 10mM EDC were added. The reaction mixture was incubated at 4°C for 16h. This was dialyzed against PBS pH 7.4 for 36h at 4°C. Finally, the dialyzed bHA was stored in glycerol at -20°C [12].

#### Detection of hyaluronic acid binding proteins by bHA polymer

HABPs were detected by solid phase ELISA using Maxi Sorp flat-bottom high protein-binding capacity polystyrene 96 well plates. The plates were coated with 100µl of diluted circulating cancer, other diseases (other than cancer) and healthy controls serum (5µg/mL concentration in 50mM carbonate/bicarbonate buffer pH 9.6.) Each sample was coated in triplicate and half of the plate served as control, coated samples were incubated overnight at 4 °C. Then plates were washed with washing buffer (50 mM Phosphate buffer, 0.1% bovine serum albumin (BSA), 0.05% tween-20 pH 7.4. After washing, non specific binding sites were blocked with 100 µL of 5% inactivated serum, 5% non fat dry milk, 1% BSA, 0.05% Tween-20 for 1 hr at room temperature and washed thrice with washing buffer. Then incubated with 100 µL of 1:1000 dilution bHA for 1 hr at 37°C. After extensive washes again incubated with 100 µL of 1:15,000 diluted goat anti-human biotin conjugate, for 1hr at room temperature, Finally the reaction was developed using 100 µL of ABTS substrate (1% ABTS 0.1 M citric acid 0.2 M Na<sub>2</sub>HPO<sub>4</sub> and 30% H<sub>2</sub>O<sub>2</sub>).The reaction was stopped by adding 100 µL of 200 mM sodium citrate buffer and the absorbance was read at 450nm on an ELISA reader (micro plate reader Texan Magellan, Sweden). Absorbance was mentioned as mean ± SEM.

#### Partial purification of hyaluronic acid binding proteins (HABPs)

Pre-swollen QAE Sepharose (fast flow sigma) was packed onto a (1.5x15cm) column. The column was then equilibrated with 50mM Tris pH 8.0. 100mg (diluted to 1 mL) of cancer sera was loaded and was eluted with elution buffer containing different NaCl concentrations (50, 150, 220 & 300 mM). Fractions were read at 280nm. Each peak was dialyzed and lyophilized.

#### Affinity purification of HA-Binding proteins (Anders Tengblad, 1979)

Preparation of HA-Sepharose column: 100mg of hyaluronic acid was dissolved in 30ml of 0.05M sodium acetate containing 0.15M NaCl (pH 5.0). For complete dissolving of HA, keep it overnight. To the dissolved HA add 2mg of hyaluronidase and incubate the mixture at room temperature for 3hours and then boil it for 10min. Centrifuge at 10,000 rpm for 15min and take the supernatant. Take 10mL of EAH-Sepharose 4B and add the digested HA solution and 250mg of EDC to it. Keep the mixture in a stirrer and mix it for 6hr keeping pH constant at 5.0. Adjust the pH with 0.1M HCl. After 6 h add 2mL of glacial acetic acid and incubate for 6hr. Later wash the gel with 1M NaCl and then with 0.05M formate buffer pH 3.0. Wash with distilled water and store the mixture at 4°C. Equilibrate the HA-Sepharose column with 4.0M guanidium HCl containing 0.5M sodium acetate pH 5.0. Load the 50,150mM Q-Sepharose fraction onto the column. Re-circulate at least 3 times. Wash the column to remove the unbound fraction. Elute the protein with 4M guanidium HCl. [13-15]

#### Detection of anti-HABPs autoantibody by ELISA

Autoantibodies against HABPs were measured by solid phase ELISA using Maxi Sorp flat-bottom high protein-binding capacity polystyrene 96 well plates. The plates were coated with 100µl of diluted circulating purified HABPs with 5µg/mL concentration in

50mM carbonate / bicarbonate buffer pH 9.6. Each sample was coated in triplicate and half of the plate served as control of the purified human HABPs and were incubated overnight at room temperature. Then plates were washed with washing buffer (50 mM Phosphate buffer, 0.9% NaCl, 0.1% bovine serum albumin (BSA) of pH 7.4. After washing, the plates were blocked with 100 µL of blocking buffer (5% inactivated serum, 1% BSA, 0.05% Tween-20) for 1 hr at room temperature and washed thrice with washing buffer. Then incubated with 100 µL of 1000 fold diluted patient and control serum samples (dilution buffer: PBS pH 7.4, 0.25% BSA and 0.05% Tween 20) for 1 hr at 37°C. After three washes with washing buffer and again incubated with 100 µL of 15,000 fold diluted goat anti-human IgM horseradish peroxidase conjugate in PBS, 0.9% NaCl, and 1% BSA for 1hr at room temperature, followed by five times washes with washing buffer. Finally the reaction was developed using 100 µL of ABTS substrate (1% ABTS 0.1 M citric acid 0.2 M Na<sub>2</sub>HPO<sub>4</sub> and 30% H<sub>2</sub>O<sub>2</sub> ).The reaction was stopped by adding 100 µL of 200 mM sodium citrate buffer and the absorbance was read at 450 nm on an ELISA reader (micro plate reader Texan Magellan, Sweden). Absorbance was mentioned as mean ± SD.

#### Immunoblot analysis

The purified human circulatory HABPs (50 µg) were subjected to 10% SDS-PAGE, under reducing conditions at constant voltage (100) and electro blotted onto PVDF membrane (Millipore USA) for 45 min at 50 volts, then membrane was blocked, (5% CaCl<sub>2</sub> inactivated human serum, 1% BSA, 5% non fat dry milk) for 1 hr at room temperature and the membrane was washed with washing buffer (TTBS-Tris, NaCl and Tween-20 pH 8.0) and the blotted strips were incubated with 1:100 dilution cancer patients and HC sera in T-TBS for overnight at 4°C. Next strips were incubated with bHA, mAb CD44, and cdc 37 antibody. The strips were washed and then incubated with secondary antibody (goat anti-human IgM-horseradish peroxidase conjugate 10,000 fold dilutions) for 1 hr at room temperature and immune reaction was detected by ECL (enhanced chemiluminescence (Amersham Pharmacia).

#### Statistical analysis

The comparison of different cancer and HC result are expressed as mean ± SEM and were analyzed by the use of unpaired *t*-test, one way ANOVA test and the difference was consider statistically significant when *p* < 0.05. Multivariate analysis was based on receiver operating characteristics (ROC) curves, which allow the characterization of discrimination between two well defined groups. The receiver operating characteristic criterion [16,17] finds the immense linear and area under the curve ( AUC) is maximized. Sensitivity, which represents its ability to detect the diseased population and specificity, represents its ability to detect the non diseased population. Statistical analysis was done with the use of graphpad prism software 5.0 [18].

#### Results

##### HABPs detection

To detect HA-binding proteins we adapted a simple ELISA technique, biotinylated HA polymer reacted with HABPs in cancer when compared with HC and positive other diseases (*p* < 0.001), has shown (Fig.1a).

##### HABPs purification

HABPs were partially purified by strong anion exchanger Q Sepharose column, fractionated with increasing salt gradient and each pooled peak was tested for HABPs activity (Fig.1b) only in 50,150mM fractions shown maximum reaction (Fig.1c), therefore we have pick out 50,150mM fractions and further affinity purified by EAH Sepharose column. Guanidinium HCl eluted fractions was run under reducing condition and transblotted reacted with bHA polymer, multiple HABPs (120 kD, 80 kD, 57 kD, 47 kD and 37 kD ) was shown with strong reaction (Fig.7 lane 4)

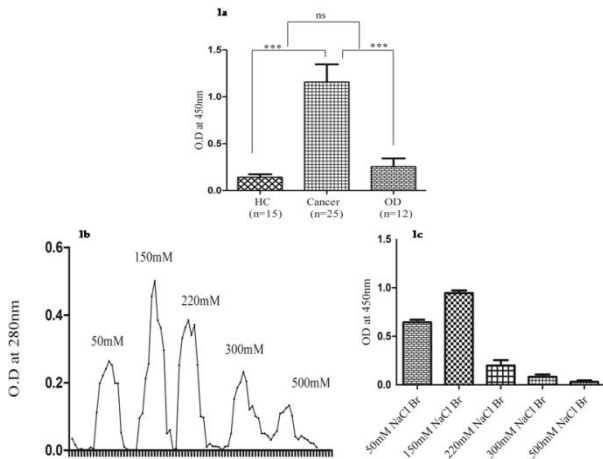


Fig.1

Figure.1a shows detection of HA-binding proteins by biotinylated bHA polymer, bHA binds to HABPs, HC as a negative control and other diseases as a positive control. There is a significant difference between HC vs cancer, cancer vs other diseases ( $p < 0.001$ ). **1b.** Elution profile of different NaCl concentrated (50 mM, 150,200,300 and 500 mM) strong anion exchange Q Sepharose column fractions. **1c.** Partially purified Q Sepharose fractions were screened for HA-binding proteins by biotinylated HA polymer

**Anti-HABPs autoantibodies detection in the sera of patients with cancer:**

Serum anti-HABPs autoantibodies levels were significantly higher in patients with cancer ( $0.98 \pm 0.51$ ) compared to those with HC ( $0.257 \pm 0.22$ ) ( $P$  value 0.001). HA-HABPs excretion or secretion might elicit the induction of serum autoantibodies. Let us to develop a novel ELISA method to measure the anti-HABPs autoantibody, sera obtained from 99 patients with cancer and 50 healthy subjects were investigated for the presence of anti-HABPs autoantibodies. The reactive proteins most commonly observed in patient with cancer sera but not significantly with HC.

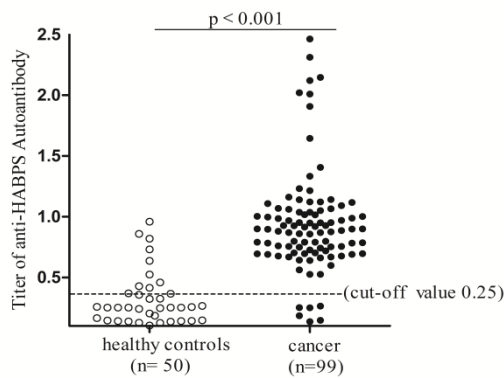


Fig.2

Figure.2 shows titers of anti-HABPs autoantibody in sera of patient with different types of cancer and HC. Anti-HABPs autoantibody was measured by ELISA. Autoantibody titers were arbitrary expressed as a mean absorbance values = 0.257 (broken line) was considered as a positive. The ELISA experiments were done thrice with each cancer and HC. Total sample size ( $n=149$ ); various cancer ( $n= 99$ ) and as a control normal sera ( $n=50$ ). The different between groups (patient versus control) were calculated to have significance at  $p$  value 0.05

**Distribution of anti-HABPs autoantibodies**

To evaluate the specificity and sensitivity we employed ELISA. The titers for anti-HABPs autoantibody in sera of Patients with cancer

have significant higher circulating levels of anti-HABPs antibodies as compared to control subjects ( $p < 0.001$ ). ROC plot test has exhibited 91.9% sensitivity and 76.3% specificity (Fig.1). We also examined whether the level of anti-HABPs autoantibodies were detectable as a function of tumor types. The titers of anti-HABPs autoantibodies detected into patients with different cancer in comparison to the HC are depicted (Fig.2) In HC the mean  $\pm$ SEM level was ( $0.25 \pm 0.03$ ) among cancer patients were mean  $\pm$ SEM ( $0.99 \pm 0.14$ ,  $p$  value 0.001) for breast, mean  $\pm$ SEM ( $0.97 \pm 0.13$ ,  $p$  value 0.015) for cervix, mean  $\pm$ SEM ( $1.0 \pm 0.11$ ,  $p$  value 0.035) for colon, mean  $\pm$ SEM ( $0.80 \pm 0.4$ ) for ovarian, mean  $\pm$ SEM ( $0.90 \pm 0.05$ ,  $p$  value 0.041) for stomach, mean  $\pm$ SEM ( $1.4 \pm 0.38$ ) for rectum, mean  $\pm$ SEM ( $0.91 \pm 0.08$ ,  $p$  value 0.001) for lung cancer.

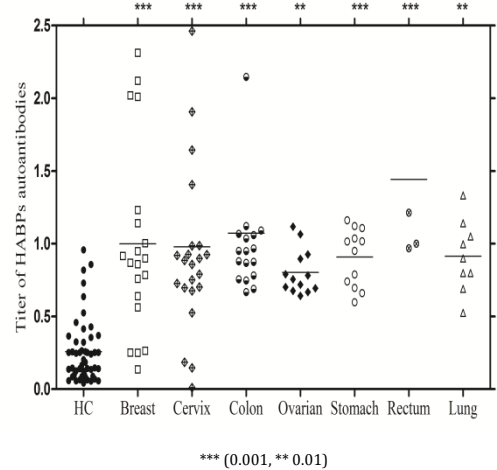


Fig.3

Figure.3 Dot plot showing levels of anti-HABPs autoantibodies as detected by ELISA, in patients with breast, cervix, colon, ovarian, stomach, rectum, lung, and HC. Each dot represents the datum for an individual subject. The solid and dotted horizontal lines indicate the mean level.

The sensitivity and specificity of the anti-HABPs autoantibody detected by ELISA was evaluated using ROC plots, exhibited high sensitivity with 91.9% (95% confidence interval) and also maintaining high specificity of 76.3% (95% confidence interval) in discriminating between patient with cancer and HC and area under curve (AUC) was 0.85. So, this study clearly demonstrates the specificity of AAbs to HABPs in cancer (Fig.5).

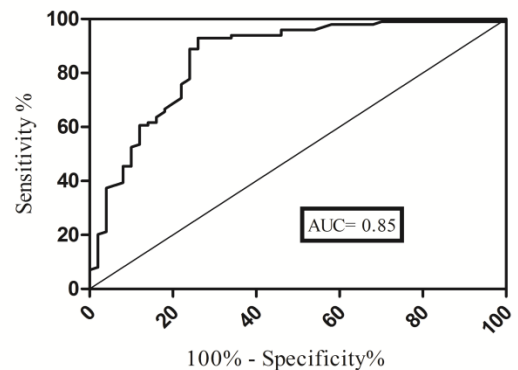


Fig.4

Figure.4 ROC plots of HABPs autoantibody by EIA in patients with cancer and healthy control sera. The sensitivity and specificity of anti- HABPs autoantibody EIA (from the data of Fig. 1) was presented. The autoantibody EIA had a sensitivity of 91.9% and specificity of 76.3% (AUC, 0.85).

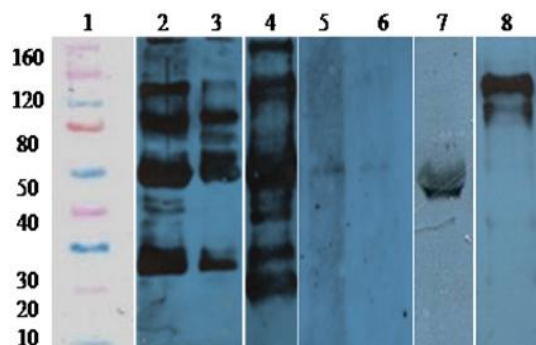


Fig.5

Figure. 5 immune blotting of anti-HABPs autoantibody in sera from patients with cancer and HC. Lane 1, molecular weight marker; lane 2, breast cancer, Lane 3, cervix cancer (1:100 fold dilution); lane 4, blotted with bHA, lane 5 & 6, blotted with HC sera (1:100-fold dilution); lane 7, mAb cdc37 antibody and lane 8, mAb CD44 antibody (HCAM).

#### Immune blot analysis of anti-HABPs autoantibodies

We also examined the immunologic identification of anti-HABPs autoantibodies present in the cancer sera and in HC. selected same patient sera exhibited immune reactivity towards purified HABPs and this result was correspond to bHA as multiple bands (120 kD, 80 kD, 57 kD, 40 kD and 37 kD), whereas there was no such significant immune reactivity in HC. (Fig.6). In order to say that, autoantibodies could also have high affinity to known HA receptors we used mAb CD44 and mAb cdc37 as a control, this result was compare with (lane 2-4).

#### DISCUSSION

Numerous studies have demonstrated that cancer detection and diagnosis is a serious step in cancer management. Therefore, we vigorously validated the method and carefully evaluated the data. We measured elevated level of circulatory autoantibodies which is generated against HABPs. Circulatory serum autoantibodies recognize relevant HABPs in patients with cancer, suggesting that autoantibody-based tests may be used to differentiate patient with cancer from inflammation and HC [19]. Immunoblot analysis identified 120 kD, 80 kD, 57 kD, 47 kD and 37 kD as major HABPs and their relevant autoantibodies in patient with cancer and these proteins are directly associated with bioactive fragments or polymer HA and are involved in cell proliferation, cell-cell adhesion, differentiation and migration during tumorogenesis [20], and also these multiple HABPs (Hyaladherins) are known to be directly associated with metastasis and reported to play a vital role in carcinogenesis [14]. The over expression of HABPs were observed during tumorogenesis but, not in HC. Hence HABPs could act as a protein biomarker and might provide important diagnostic information in cancer detection. The consistent results have been observed in cancer of breast, cervix in which the autoantibodies have been associated with cancer progression. In this study, we have used a simple ELISA technique, which allow the identification of autoantibodies and their corresponding tumor associated antigen (HABPs) and showed the elevated levels of autoantibodies directed against HABPs in the sera of patients with cancer but not significantly in the HC.

Beside these, many of the well-known available tumor-associated serum biomarkers was studied, such as CEA for colon cancer, AFP for liver cancer, PSA for prostate cancer, CA19-9 for gastrointestinal cancer and CA-125 for ovarian cancer, [21]. Cancer testes (CT) antigens [22] Elevated serum protein level of any one of the above markers is less than 36% and combination of these markers range from 20-70% [19]. As such, the clinical application of these biomarkers is limited when assayed independently, although using combinations of markers have somewhat enhanced diagnostic value. Similarly, an antibody response to a single protein is not expected to be universal biomarker [23]. In spite of that, few of these biomarkers are acceptable for routine clinical applications. Owing to conflicting

results from different studies showing low sensitivity and specificity [24,25]. Therefore, it is essential to identify clinical reliable biomarkers for tumor to develop an effective approach for early diagnosis. Finally, these markers are mostly used in conjunction with diagnostic imaging and physical examination to establish the initial diagnosis, to subsequently assess the efficacy of treatment and to monitor evidence of relapse. Therefore, it is of high clinical importance to develop an easy-to-perform screening test that can identify patients with cancer in early curable stages.

In the present investigation, anti-HABPs autoantibodies were elevated with tumor progression. This elevation indicates that the serum AAbs against HABPs might represent cellular status. These results suggest that detection of circulating AAbs against HABPs could potentially serve as a useful serum biomarker for detecting cancer. Detection of anti-HABPs AAbs by immunoblot may be of limited value for routine use in most diagnostic laboratory. We used a simple and rapid ELISA method. The assays were used to measure these biomarkers need to have high sensitivity and specificity, anti-HABPs AAbs were elevated in all malignantly transformed cells irrespective of cancers [26]. To our knowledge, no investigations have been reported on the autoantibodies against HABPs in the serum of patients with cancer. It has been established that anti-HABPs autoantibodies are useful serological biomarkers in monitoring and diagnosis of cancer.

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#### CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to disclose.

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