Validation and evaluation of common biomarker in human cancers sera protein detected by a monoclonal antibody UNIVMab

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Research note

Keywords: ELISA, Western blot, Hyaluronic acid binding protein, H11 (sera antigen), UNIVMab. Common Biomarker, Cancers Sera

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Abstract

Objective: Management and diagnosis of multiple human cancers remain a challenge and search for a common biomarker are still debatable. We described a method and evaluated the use of monoclonal antibody UNIVMab, to detect the protein (H11) as a common biomarker for all cancer irrespective of grade and origin . H11 protein identified as a unique Hyaluronan binding protein not detected earlier. We applied this test both with ELISA, Western blot, fractionated in anion exchange, cibacron gel exclusion, b-Hyaluronan interaction and HA-Oligo competition from various grades of Human cancers sera and processed for the detection of hyaluronan binding protein H11, reacted with Monoclonal antibody UNIVMab and with b-HA.

Results: Studies from ELISA, Western blot and b-Hyaluronan interactions showed a definitive positive over-expression of UNIVMab reacted H11 antigen in all fortyfive cancer sera compared to normal sera and can be used as a common biomarker. We believe, UNIVMab detected H11 antigen, a unique hyaluronan binding protein, is a common biomarker for all cancer sera. Keywords: ELISA, Western blot, Hyaluronic acid binding protein, H11 (sera antigen), UNIVMab. Common Biomarker, Cancers Sera

Introduction

The appearance of cancer serum biomarker is a molecular event that indicates thepathological changes in a particular tissue orcell type during cancer development. The most important part of the screening test is the ability to detect cancer as early as possible. Diagnosis of disease based on the quantification or localization of particular antigen in cells, tissues or body fluids (1, 2, 3, 4, 5). The widely used blood test for early detection of cancer in the prostate for PSA (prostate-specific antigen) and the proper use of the test are highly debatable(6) However, diagnosis using circulatory serum antigens makes the assay more feasible, and relatively non-invasive method to acquire experimental sample (4, 5, 7, 8, 9, 10, 11).

The development of monoclonal antibodies (mAb's) that are reacting with specific antigens expressed by cancer cells offer to establish a diagnostic technique for early cancer detection (12). MAb's are widely used reagents in the clinical diagnostic laboratory to develop the sensitive immunoassays for the detection of their target antigen in the circulation, their levels relate with the disease progression (11, 13, 14, 15,16).(Drugs/GuidanceComplianceRegulatoryInformation/Guidances CM070107.pdf

Analysis of human embryonic, adult tissues and in human serum revealed the presence of hyaluronan (HA) and its receptors. Hyaluronan is a nonsulfated, high molecular weight glycosaminoglycan consisting of the D-glucuronic acid and N-acetyl D-glucosamine (17). HA is present in the extracellular matrix of most tissues and is enriched in many tumors (17, 18). It is well documented that HA and its receptors, known as hyaladherins (hyaluronan binding proteins) are involved in matrix regulation, cell proliferation, migration and malignant tumor progression (18). These hyaladherins not only interact with hyaluronan at the matrix proper but also with hyaluronan at the plasma membrane as cell surface receptors and thus influence cell physiology, including secretion of this protein into the circulatory system

(19,20,21). Previously we identified H11B2C2 clones reactive hyaluronan binding protein and revealed its association with tumor progression using immunohistochemistry (13). The antigen expression increases during tumor progression irrespective of cancer origin. Because of the antibody produced by the clones detected the antigen in all human cancer tissues and serums we renamed the antibody as UNIVMab The assumption that UNIVMab reactive H11 acts as a protein biomarker in various tumor tissues may give crucial diagnostic information in cancer detection led us to the present study. We described the procedures for fractionation of serum proteins, mainly HABP, from various cancer patients and healthy subjects using single dimension electrophoresis for detection of H11 antigen using UNIVMab and even association of the protein of interest to develop into a common biomarker for multiple cancers.

Materials And Methods

Hyaluronan (Na salt) (Acros Organics, New Jersey, USA). Guanidium hydrochloride, bovine testicular hyaluronidase type I S, EDC [1, ethyl 3-(3-dimethyl aminopropyl) carbodiimide hydrochloride, MES buffer (2-N-morpholino ethane sulfonic acid) Protease inhibitors cocktail and biotinylated goat anti-mouse IgG's HRP conjugate were procured from Sigma chemicals, USA. EZ-Link Biotin LC hydrazide purchased from Pierce, Rockford, USA. Anti-human CD44 (H-CAM, Clone 1M7.8.1) antibody was purchased from Fisher Scientific, USA. Streptavidin-peroxidase conjugate (HRP) obtained from Invitrogen, USA.

Sample collection and preparation

The study consisted of 70 normal subjects and 45 cancer patients (Lymphoma,(2) Salivary(1), Tongue(4), Thyroid(1), Buccal Mucosa(1), Lung(1), Breast(6), Stomach (3), Gallbladder(1), Oesophagus (3), Colon(3), Pancreas (1), Rectal(2), Urinary bladder(2), Prostate(4), ovary(5), endometrial(3) and cervix(2). Serum samples from normal subjects and cancer patients accessed from cancer hospitals in Mysore, India (Preethi Centre of oncology, and KR Hospital, Logic and Clue Diagnostic center) and the protocol approved by the ethical review committee (Mys–00340-AA-NH,an d IHEC-UOM NO 35) and the patient's consent was taken. Blood samples were collected from each patient before any treatment. Samples centrifuged at 2000xg for 30 min at room temperature for 1 hr, and the separated sera were stored at –80^0C. The tumor sections from patients after H&E stain was graded using the TNM grading system. Serum samples treated with 4X lysis buffer, containing 0.2 M Tris-HCl (pH 8.0), 80 mM EDTA, 4 mM PMSF, 4 mM Benzamidine-HCl and 2% Triton X 100 plus protease inhibitor cocktails and centrifuged at 10,000xg for 30 min at 4^0C. The supernatant was stored either at –80^0C until further analysis. The amount of protein estimated at UV 280 nm and Bradford reagent using BSA as a standard.

Preparation of biotinylated hyaluronic acid according to Boregowda et al.2013 and Srinivas et al. 2016, (13,24) In brief, HA dissolved in PBS-A dialyzed in MES buffer and reacted with biotin_LC-hydrazide and EDC in DMSO. Incubated for 16hrs, dialyzed against PBS-A and stores in glycerol at –20C

Production of monoclonal antibody UNIVMab

Hybridoma and the antibody were prepared according to Boregowda et al. *(*18, 22, 23). In brief, the hybridoma grown in DMEM under pathogen and complement free human serum received from the hospitals. The antibody production in human serum of any blood groups did not affect UNIVMab in recognizing the human antigen. The clones were grown in DMEM containing 10% (v/v) inactivated human serum. After 21 days, the media was collected and precipitated with cold saturated ammonium sulphate solution (final 50%) at 4^0C overnight and centrifuged at 12000xg for 30 min. The pellet dissolved in PBS and dialyzed against PBS.

The statistical evaluations from Western blots were analyzed using the two-tailed Student-test. Statistical P values defined as follows: $P<0.05$ = significant; $P<0.01$ = highly significant; and $P<0.001$ = extremely significant. Data are presented as means±SEM

Results

Detection of H11 antigen by ELISA using UNIVMab

MaxiSorp flat-bottom high protein binding capacity polystyrene–96 well plates were used. Serum samples dissolved in 0.05M carbonate-bicarbonate buffer pH at 9.6 at a concentration of 1ug/ml. 100ul Samples in triplicate plated on to the 96well and incubated overnight. Blocked with skimmed milk in PBS for 1 hour and reacted with UNIVMab at 1:10000, incubated overnight at 4^oc, washed with0.2%Tween-PBS followed by incubation with b-goat anti-mouse antibody at 1:20000 for 1 hour and reacted with streptavidin-peroxidase at 1:50000 for one hour, reactive colour developed using 100ul of 1.0mg/ml ABTS in 0.1M citrate buffer at pH4.0 and 5%. Hydrogen peroxide. The reactions stopped after one hour with 0.2M citric acid, and the absorbance was measured at 405nm. Figs 1.Experiments were repeated at least three times. Protein levels were measured by quantitative ELISA.

Western blot analysis of serum according to Boregowda et al, Fekry et al (15, 16)

50 µg proteins from serum lysate were resolved on 10% SDS-PAGE transferred to PVDF membrane and reacted with mAb (1:1000 dilution) or anti HCAM mAb (1:1000 dilution) or with bHA probe (1:100 dilution) overnight at 4 ^{0}C. Following day, the blot was developed and the proteins detected using Enhanced ChemiLuminescence (ECL). Since the isolation of antigen is vital to understand its property, we used the circulating antigen purification by antibody conjugated CNBR activated sepharose and Cibacron blue affinity purification method and finally UNIVmab reactive protein cross reacted with bHA and HA-oligo (500ug Oligo) competition. These work may identified the presence of Hyaladherins.

Discussion

We validated the clones H11B2C2 reactive novel HABP in human cancer tissues. HABP overexpression was related to poor tumor outcomes (13, 25). The antibody produced by the clones detected the antigen

in all human cancer tissues and cancers sera; we renamed the antibody as UNIVMab. We have also identified soluble hyaluronan bonding proteins in colon cancer serums with a molecular mass of 57 and 30 kDa (14). We believed that the soluble 57-kDa H11 protein from colon cancer might have relation with multiple cancer samples. UNIVMab reactive H11 proteins maybe a new species related to Hyaluronan binding protein and thus investigated the present study to understand its expression and the nature of H11 protein in various cancers sera and further to characterize using biochemical analysis, using its application as a universal cancer biomarker. Table 1 showed the detailed results of H11 expression from various cancers sera.

From ELISA experiment (Fig 1), normal showed an average of triplicate OD at 405nm is 0.175, However from sera Grade 1–0.25, Grade 2–0.46, and Grade 3- 0.52 showed increased expression of the antibody reactive antigen. Even though eight samples from 18 different cancers shown for the determination for H11 antigen activity with UNIVMab, Similar results observed with the remaining cancer samples.

UNIVMab reactive H11 proteins were overexpressed in human cancer serum shown in Fig 2

To detect whether UNIVMab reactive antigens are present in the circulation, we conducted western blot analysis for normal and various cancer serum samples using mAb. The mAb showed reactivity reduced in normal sera at 57kD with UNIVMab (Figure 2. Panel A), however the intensity of reaction enhanced during tumor progression in high-grade cancers (Figure2. Panel D vs panel B & C). We experimented with 70 normal and 45 cancer patients of different grade. However, we have shown 12 different healthy individuals sera showed in figure 2 (pane A lanes 1 to 12). The mAb reaction with serum proteins of six different cancer sera of different cancer patients showed in panel B, C & D. The presence of mAb reactive protein in normal and eighteen cancer subjects suggested that it might have several biological functions. Similar results observed in the tumor/normal tissues (13). Progressive cancer sera showed overexpression of the activity.

Further, we find western blot analysis with bHA probe detected serum HABP expression as H11 antigen (Fig not shown) and HA-Oligo competition showed 80%reduction of antigen expression in all cancers sera. This result indicates the 57kD, named H11 may be a hyaluronan binding protein (data not shown). We performed immunoprecipitation and affinity purification of normal and cancer patient’s serum proteins with mAb to prove that mAb is reacting with 57-kDa protein with high specificity. As expected the normal sera showed low levels of circulatory 57 kDa on western blot, whereas, advanced cancer sera showed overexpression of 57-kDa antigen. To show that the cancer antigen 57kD is associated with carrier protein such as Albumin we used Cibacron blue gel exclusion method. Eluted fractions were tested for reactivity with b-HA and mAb, showing a strong reaction at 57 kDa (data not shown). To investigate UNIVmab reacted cancer antigen may be related to a known Hyaladherin, CD44 (std); Whether, UNIVMab immunoprecipitated protein can pull down CD44 reactive protein, we used stomach grade lll cancer

sample, which overexpressed for Mab reacted H11antigen and a normal sera were tested. We find no reaction to HCAM antibody to the H11 antigen (data not shown).

Conclusions

Blood-borne metastasis is the greatest obstacle to cure in patients with cancer.. The abundant blood proteins, such as albumin, immunoglobulin, etc; may mask the less abundant proteins, which are usually potential markers. There are many known specific serum markers but not as a common biomarker for various cancers sera.

Thus, this UNIVMab, which detected the overexpression serum specific H11 antigen in various cancers sera, offers significant advantages. In the present study after screening serum samples from 70 normal and 45 cancer patients samples (18 different cancer types of various grade) of different grades we predicted that the UNIVMab might be used as a common cancer biomarker. This Mab detected a protein H11 and is unique HABP not identified earlier in serum and serum albumin may be the carrier protein of H11 antigen. Still unanswered question is, what is the nature of 57kD H11 antigen. We investigated the H11 antigen by proteomic analysis from grade ll adenocarcinoma of the colon. We observed the presence of IgGH1, member of immunoglobulin super family (data not shown). The present data showed that UNIVMab detected the H11 might be a unique hyaladherins can be used as a common biomarker for progressive human cancers sera.

Limitations

At present, there are no common biomarkers for cancer detection. The present study could reflect the UNIVMab reactive serum antigen a common biomarker for multiple cancers. In addition, future experiment on the proteomic analysis will help the nature of the antigen as a potential common biomarker.

Declarations

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Author’s contributions

HNA, SBK and SDB made contributions to conception, design and interpretation. DM performed the experiments. SBK, SHV, AT and SDB reviewed and commented on the manuscript. All the authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

Ethics approval and consent to participate

The ethical review committee of Preethi center of oncology approved the work (MYS–00340-AA-NH) and the patients written consent was taken

Consent of publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Tables

Evaluation of UNIVMab reacted H11 expression in multiple Tunors

Table-1

Tumor types	Grade	Number of the experiment from each patient studied	H11 expression
Salivary	I	3	x
tongue	II	3	xx
	III	3	xxx
Thyroid (papillary)	II	3	xx
Buccal mucosa	II	3	xx
lung	II	2	xx
	III	3	xxx
Breast	I	4	x
	II	3	xx
	III	4	xxx
stomach	II	3	xx
	III	3	xxx
Gall bladder	I	3	x
oesophagus	II	3	xx
	III	3	xxx
colon	I	2	x
	II	3	xx
	III	4	xxx
pancreas	II	3	xx
Rectum	II	3	xx
ovary	II	3	xx
	III	3	xxx
Endometrium	I	2	x
	II	3	xx
	III	4	xxx
cervix	II	3	xx

Urinary bladder	I	3	x
prostate	I	2	x
	II	3	xx
lymphoma	II	3	xx

xxx-very strong, xx-moderate, x-weak

Note: we also detected H11 expression from astrocytoma glioblastoma and medulloblastoma not included into the table. XXX= Overexpression of H11

Figures

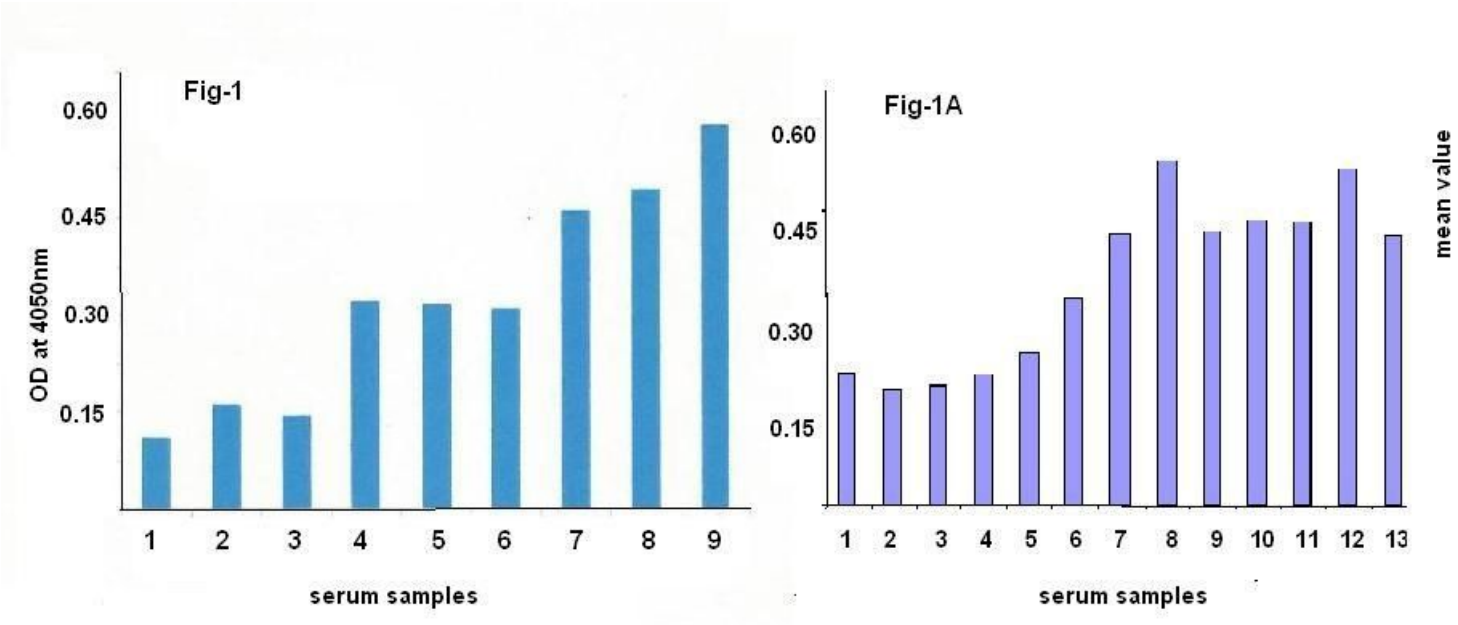

Figure 1

Lane 1, 2, 3 Normal Serum (each average of three determinations Lane 4.Ca Stomach Grade 1. Lane 5. Ca Tongue Gr.1. Lane 6. Ca Colon Gr.1, Lane 7.Ca Stomach Gr.2, Lane 8.Ca Cervix Gr.2, Lane 9. Ca Cervix Gr 3. 1A 1-4, Normal serum, 5-&6 Grade 1,Tounge, 7-9 Grade 2,Breast, 10-13 Grade3 10-Colon, 11-Lung, 12-Oesophagus, 13-Ovary. (Average of 4 samples from each serum). There is gradual over-expression of H11 in sera as the tumor progress.

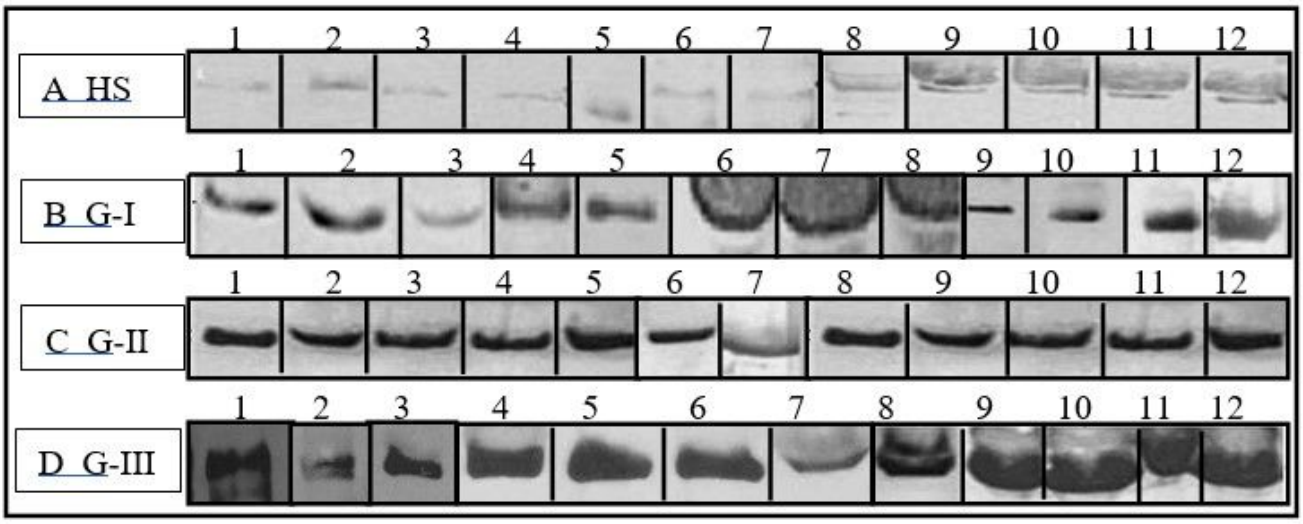

Figure 2

Fig-2 UNIVMab reactive human sera antigen expression by western blot Pane A: Healthy controls serum from 12 different individuals. Panel B, C & D: Grade 1,Grade 2, and Grade 3.Lanes 1 & 2 - Tongue, Lanes 3 & 4- Salivary, Lanes 5 & 6 -Breast, Lanes 7 & 8 - Colon, Lanes 9 & 10 -Cervix, Lanes 11 & 12 - Ovary,