Discussion
The presence of HA is well evidenced in the cytoplasm and nuclei of the cells in a number of tissues in vivo to modulate the cell behavior by interacting with specific cell surface receptors- hyaluronic acid binding proteins (HABPs). This phenomenon also plays an important role in maintaining and stabilizing the structural integrity of extracellular matrices (Evanko and Wight, 1999; Toole, 2000; Yoneda, 2001). Recent studies have shown that there are a number of HA binding proteins that may be important in the regulation of cell cycle or gene transcription (Deb et al., 1996; Grammatikakis et al., 1995; Zhang et al., 1998; Huang et al., 2000). It is an established fact that most malignant tumors such as colon carcinoma (Ropponen et al., 1998), breast carcinoma (Auvinen et al., 1997) gliomas (Delpech et al., 1993) lung carcinomas (Horai et al., 1981; Pirinen et al., 1998) and Wilms' tumours (Hopwood and Dorfman, 1978) contain elevated levels of HA than their normal counterparts. Many cell types exhibit receptors for hyaluronan (Stamenkovic and Aruffo, 1994) and an extensive literature documents the ability of HA to modulate cellular functions through these receptors (Knudson and Knudson, 1993; Sherman et al., 1994; Hall et al., 1995; Wang et al., 1998) and HA-binding proteins (HABPs) (Toole, 1990).

The expression of serum H11 antigen in normal, in different cancers and also in different grades was studied by overlay experiment using mAb H11B2C2 (Fig 3.1 to 3.4), bHA pulldown experiment and reacted with mAb H11B2C2 (Fig 3.5) and immunoprecipitation analysis (Fig 3.6). It was found that 57kDa HABP was increasing in the serum of different cancers when compared to normal serum. It was also observed that HABP was increasing with the progression of the cancers from grade I to III.

Previous studies in this laboratory has shown that the nature of mAb H11B2C2 and its affinity towards its antigen in benign, in different cancer tissues and also in different grades of cancer tissues (Sunil et al., 2008).

Scanty information is available on the expression of serum H11 antigen in different cancers. In the present study an attempt has been made to elucidate the serum
H₁₁ antigen expression in the normal, in different cancers and also in different grades of cancers by biochemical methods.

In the present study it was observed that linear over expression and distribution of serum H₁₁ antigen in different human cancer using mAb H₁₁B₂C₂, originally developed through extensive clonal selection of a hybridoma from IVd4 that recognizes HABP (hyaluronic acid binding protein) in cancers of human origin. The linear over expression of the tissue H₁₁ antigen during human tumor progression led to investigate more on the expression levels of serum H₁₁ antigen by biochemical analysis in normal, in different cancers and also in different grades.

The importance of hyaluronan expression during tumor progression was investigated. It explains the positive association of stromal hyaluronan expression with invasive nature of tumors irrespective of their origin. The differential expression of hyaluronan in well differentiated tumour tissues in contrast to the poorly differentiated (Boregowda et al., 2006) is well known. The growing evidence of the presence of increased amounts of intracellular hyaluronan and its interaction with intracellular hyaladherins such as CDC37, IHABP4 (Grammatikakis et al., 1995; Huang, et al., 2000) and further the subsequent loss of hyaluronan interaction with its receptor during late malignancy was well established. This led to study the serum H₁₁ antigen (HABP) expression in different cancers and in different grades of cancers.

Western blot analysis indicated that mAb H₁₁B₂C₂ recognizes mainly serum protein of molecular weight 57kDa irrespective of the origin of the cancer. The serum H₁₁ antigen (HABP) expression of 57 kDa was found in almost all types of cancers studied. A significant increase in the serum H₁₁ antigen (HABP) expression was observed in different cancers and also during progression from grade I to grade III when compared with normal serum.

The 57kDa serum protein which was observed by western blot analysis is a HABP. It was shown by using a specific probe bHA and also using mAb H₁₁B₂C₂ for the detection of HABP. These results were also confirmed by bHA pull down experiment.
The data presented here indicate that serum proteins may act to sequester 57kDa peptide fragments which is a novel hyaluronan binding protein and is overexpressed in the cancer serum. Such sequestered peptides may provide a potentially rich source of cancer-associated biomarkers for clinical evaluation. Presently, there is limited information to provide the mechanism involved but the cumulative data suggests that over-expression of H₁₁ antigen in cancer serum is an important parameter and a clinical marker for all progressive human cancer. Further studies are underway to check its regulatory functions in human tumor progression. Serum-based panels of biomarkers hold a great promise for better and more efficient early diagnosis of cancers. Such tests are not only more convenient and less expensive but also may demonstrate superior sensitivity and specificity in comparison to conventional screening methods. Blood based assays may detect cancer at very early, potentially pre-clinical stages when the probability of efficient therapy and complete cure is the highest. Development of such assays for early detection may shift cancer therapy towards the development of new strategies aimed at treatment of very early or pre-cancerous lesions.
Introduction

Cancer is a multi faceted disease that presents many challenges to clinicians. Cancer researchers searching for more-effective ways to combat its often devastating effects. Among the central challenges of this disease, the identification markers for improved diagnosis and classification of tumors and the definition of targets for more-effective therapeutic measures. New prognostic markers are urgently needed to identify patients, who are at the highest risk of developing metastasis that might enable oncologists to begin tailoring treatment strategies to individual patients.

Cancer starts as a focal disease but can metastasize to distant organs. It is not the primary tumour but its metastasis to distant sites that are the main cause of death. Recently, the rates of metastasis and mortality in cancer patients have decreased as a result of early diagnosis and the implementation of systemic therapy. Detection of cancer is critical for the management of this disease. Early diagnosis of pre malignant lesions is known to directly correlate with increased survival.

Previously, immunohistopathological detection of the continuous over-expression of HA-binding protein in correlation with tumor progression in human tumors of different origin with different grades identified the role of HA-binding protein as a prognostic indicator (Boregowda et al., 2006). A large number of putative prognostic markers (ERBB2, ELAM-1, ICAM-1, VLH-4, CD44 etc) have been reported till date of which only a few of these have so far fulfilled the requirements. The demonstration of a prognostically significant common antigen expression can eventually result in improved diagnosis and treatment. The high-level expression of H₁₁ antigen as detected by mAb H₁₁B₂C₂ to assess tumor progression may be useful. The results confirmed the earlier work carried out in this lab that mAb H₁₁B₂C₂ recognizes the over-expressed 57kDa protein (H₁₁ antigen) in all human tumors and could be a helpful tool in clinical oncology. Present study aims at the purification and characterization of this antigen from normal and cancer serum. This could be of utmost importance and of clinical value. The goal is to be able to screen for and diagnose cancer early, when it is the most treatable and before it has had a chance to grow and spread.
The early diagnosis is a major importance of successful patient management. The search for biomarkers in serum begins with a separation step to remove the abundant high molecular mass contaminating proteins. The problem of detection of serum biomarker is impeded by the presence of highly abundant proteins such as albumin, immunoglobulins, transferring, haptoglobin, lipoprotein and antitrypsin which constitute more than 85% of the serum proteome. However, the circulating carrier proteins such as albumin acquire the carriers longevity in the serum, can become the reservoir for the accumulation and amplification of bound biomarkers. These biomarkers are secreted from cells of metastatic origin into the circulatory system. Intensive investigations on the cancer serum biomarker have been reported, such as CA-125 for ovarian cancer (Jacobs and Bast, 1989), serological mucin assay for pancreatic cancer, (Rhodes, 1999) serum cytokine IL-7, EGF, VEGF, monocyte chemotactic protein (MCP 1) IL6 and 8 in ovarian cancer, serum GFAP (glial fibrillary acidic protein) in glioblastoma multiformis, serum CEA in colorectal cancer (Duffy, 2001; Lambeck et al., 2007; Jung et al., 2007).

Albumin binding of biologically important proteins and peptides is well documented. For example, the amino-terminal peptide of HIV-1, gp41, and the 14-kDa fragment of streptococcal protein G are known to specifically bind with human serum albumin (Sjobrin, 1992; Gordon et al., 1993; Dennis et al., 2002).

Carrier protein bound information contains potential disease related information (Lowenthal et al., 2005; Sahab et al., 2007), in the present study, an investigation comparing the albumin associated peptides from various cancer serum derived from patients with pathologically documented cancer and normal individual was undertaken.

Therefore appearance of cancer biomarker is a molecular event that indicates the pathological changes in a particular tissue or cell type during cancer development. When such molecular events can be detected in the cancer serum as biomarkers, can have a significant effect on clinical outcomes. Recent observation on the role of HA and human cartilage glycoprotein-39 on chronic hepatitis and osteoarthritis have been reported (Mehta et al., 2008; Sandra et al., 2009) the assumption that HABP (hyaluronan binding protein) acts as a protein biomarker and may give an important
diagnostic information in cancer detection led us to the present study. The earlier workers from this laboratory confirmed that mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2} recognizes the over-expressed 57kDa (H\textsubscript{11}antigen) protein from the cancer tissue sections.

Present study aims at the purification and characterization of this antigen(HABP) from various cancers and normal patients using strong anion exchange chromatography, single dimension electrophoresis and elimination or reduction or even association of albumin bound protein biomarker from serum samples using Cibacron blue and affinity antibody related methods.

**Materials and Methods**

**Sample collection:**

(As mentioned in chapter II, Section A)

4.1. Extraction of proteins from normal and cancer serum sample

(As mentioned in chapter II, section B)

4.2. Production of monoclonal antibody [mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2}]

(As mentioned in chapter III)

4.3. Preparation of biotinylated hyaluronic acid (bHA)

(As mentioned in chapter II, Section A)

4.4. Immunoprecipitation

(As mentioned in chapter III)

4.5. SDS-PAGE

(As mentioned in chapter II, Section A)

4.6. Western blotting

(As mentioned in chapter II, Section A)

4.7. Purification of H\textsubscript{11}B\textsubscript{2}C\textsubscript{2} antigen

4.7.a. G-50 (DNA Grade)
4.7. b. Tris buffer
50mM Tris was dissolved in double distilled water and pH was adjusted to 8.0 with 1N HCl.

4.7. c. QAE-Sepharose

4.7. d. Elution buffer
50mM Tris buffer containing 50, 150, 220 and 300mM sodium chloride was used for elution.

4.7. e. Tris buffer (10mM Tris)
The buffer was prepared in double distilled water and pH was adjusted to 8.0 with 1N HCl.

4.7. f. Elution buffer (glycine buffer)
It contains 50mM glycine and 150mM sodium chloride. These chemicals were dissolved in double distilled water and pH was adjusted to 2.5 with 1N HCl.

4.7. g. Elution buffer (Tri ethanolamine buffer)
It contains 50mM triethanolamine and 150mM sodium chloride. These chemicals were dissolved in double distilled water and pH was adjusted to 11.5 with 1N NaOH.

4.7. h. Dialysis buffer
It contains 10mM Tris, 5mM sodium chloride and 0.02% sodium azide. These chemicals were dissolved in double distilled water and pH was adjusted to 7.4 with 1N HCl.

4.8. Cibacron blue affinity column

4.8. a. Elution buffer
Acetonitrile–H₂O–trifluoroacetic acid (70:30:0.2 by volume)

4.8. b. Tris buffer (20mM Tris)
The buffer was prepared in double distilled water and pH was adjusted to 8.0 with 1N HCl.

4.9. G-50 column (Gel permeation chromatography)
G-50 (DNA grade) was swollen in double distilled water for 12hr at 4°C. The swollen gel was packed into a column of bed volume 120mL. The column is then equilibrated with 50mM Tris pH 8.0. The flow rate was adjusted to 15mL/hr. Approximately 100mg of serum sample (normal, stomach and colon /cancer) was loaded onto the column. The protein was eluted from the column with 50mM Tris pH 8.0. Column fractions were read at 280nm absorbance keeping bovine serum albumin as standard. The elution profile was plotted. The peak fractions of G-50 column were tested for HABPs using bHA and mAb H₁₁B₂C₂.
4.10. QAE Sepharose (Ion exchange chromatography)
Preswollen Q-sepharose (fast flow) was packed onto a 10mL column. The column is then equilibrated with 50mM Tris pH 8.0. When the peak fractions of G-50 column were tested for HABP reaction, it was found to be associated with peak fraction I. This peak fraction I with maximum HABP reaction was taken and the sample was loaded on to QAE sepharose column. The column is washed with 50mM Tris pH 8.0 to remove the unbound protein. The column was then eluted with 50mM Tris pH 8.0 containing different salt concentrations (50, 150, 220 and 300mM sodium chloride) sequentially. Column fractions were read at 280nm absorbance keeping bovine serum albumin as standard. The elution profile was plotted. The peak fractions of Q-Sepharose column were tested for HABP using bHA and mAb H11B2C2.

4.11. Cibacron blue affinity column (albumin-specific affinity column)
(Lowenthal et al., 2005)
10 mg of normal and cancer serum protein from Q-sepharose column eluted with 220mM sodium chloride was diluted and run through albumin-specific affinity column twice. The unbound protein was washed thoroughly with 20mM Tris. The bound proteins were eluted from the column by equilibrating with acetonitrile–H2O–trifluoroacetic acid (70:30:0.2 by volume) for 30 min followed by a slow spin-through of the elution mixture, repeated once. The eluate was lyophilized and reconstituted in an H2O–acetonitrile–formic acid (95:5:0.1 by volume) buffer.

5mg of H11B2C2 mAb is taken and dialyzed against coupling buffer for 24hr with 3 changes. The dialyzed antibody is allowed to conjugate with CNBr-activated Sepharose 4B and allowed to mix overnight [16hr] at 4°C. The mixture is packed on to a 1.0ml column. The unbound antibody was washed using coupling buffer. In order to block the free sites the column is equilibrated with glycine. The column is then equilibrated with 50mM Tris pH 8.0. To the column, Q-sepharose column fraction eluted with 220mM NaCl was loaded and re circulated couple of times through the column. The column is washed with 50mM Tris pH 8.0 to remove the unbound protein. The protein from the column is then eluted with 50mM glycine pH 2.5. The
column is washed with 10mM Tris pH 8.0. The protein from the column is then eluted with 50mM Triethanolamine pH 11.5. Both the glycine as well as Triethanolamine fractions were pooled separately and dialyzed against 10mM Tris pH 7.4. After dialysis the samples were lyophilized.

**Scion Image analysis information:**
(As mentioned in chapter II, Section B)

**Detection of HABP’s in normal and stomach and colon cancer serum from column fractions using bHA probe by Western blot analysis:**
The 100μg protein from normal and cancer serum (stomach and colon) from column fractions were run on 10% SDS PAGE under reducing condition at 25mA constant current and transblotted onto PVDF membrane by electroblotting [wet transfer] at 200mA current for 1hr. After transblotting, nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed with TTBS buffer three times extensively followed by incubation with (1:100 dilution) bHA probe over night at 4°C. The blot was washed and incubated with streptavidin peroxidase (HP09) at 1: 20,000 dilutions for 1 hr at room temperature. The proteins were detected using an ECL detection kit.

**Detection of H11 antigen in normal and cancer serum from column fractions using mAb H11B2C2 by Western blot analysis:**
The 100μg protein from normal and cancer serum (stomach and colon) from column fractions were taken and electrophoresed on a 10% SDS-PAGE at 25mA constant current and electrotransferred to a PVDF membrane at 200mA for 1hr at room temperature. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed extensively with TTBS buffer. The membrane was incubated with H11B2C2 mAb (1:500 dilutions) for 1hr at room temperature and overnight at 4°C. Next day the membrane was washed with TTBS. The membrane was then incubated with secondary antibody (goat anti mouse IgG biotin conjugated) 1:10000 dilution for 1hr at room temperature. The membrane was washed with TTBS. The membrane was treated with HPO-9 (streptavidin peroxidase,) 1:20000 dilution for 1hr at room temperature. After extensive washing with TTBS, the immuno-reactive proteins were visualized with ECL.
bHA pull down protein from 220mM Q sepharose column fraction in normal and cancer serum samples reacted with mAb H_{11}B_{2}C_{2} and HA competition with mAb H_{11}B_{2}C_{2}:

100 μg of 220 mM QAE sepharose fraction protein from normal and stomach and colon cancer serum samples was mixed with 10 μg of bHA probe and incubated for 1hr at 4°C. Then 50% of saturated (NH_{4})_{2}SO_{4} was added and sample was mixed thoroughly and incubated over night at 4°C. Then centrifuged the sample and the pellet was separated. The pellet was dissolved in 1% SDS and loaded in SDS sample buffer and ran on 10% SDS-PAGE on two identical lanes. Then the proteins were transblotted to PVDF membrane. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed extensively with TTBS buffer and separated in two strips. One of the membranes was incubated with cold HA (500 μg/ml) for 3hr at room temperature and then mAb H_{11}B_{2}C_{2} was mixed with cold or unlabelled HA (500 μg/ml) and spreaded on the membrane and incubated over night at 4°C. To other strip mAb H_{11}B_{2}C_{2} (1:500 dilution) was added and kept over night at 4°C. Next day the blot was washed and incubated with goat anti mouse IgG-biotin conjugated (1:10,000 dilution) for 1 hr at room temperature. Later, the membrane was washed and incubated with streptavidin peroxidase (1:20, 000 dilution) for 1 hr at room temperature, after which they were detected using an ECL plus Western Blotting Detection System.

Immunoprecipitation analysis of H_{11} antigen from 220mM Q sepharose column fraction of normal and cancer serum samples and reacted with mAb H_{11}B_{2}C_{2}:

(Green et al., 1988)

200μg of normal and stomach and colon cancer serum protein obtained from Q-Sepharose column eluted with 220mM NaCl. Dilute the sample if required with lysis buffer. Add 10μl of protein A-CL agarose and 5μl of protein G-CL agarose and incubated at 4°C with intermittent mixing. Spin the tube for 10min. in a cooling centrifuge and transfer the supernatant to a fresh tube. To the supernatant add 25μl [5μg] of mAb H_{11}B_{2}C_{2} and incubate the tube at 4°C for overnight with intermittent mixing. After overnight incubation the antigen-antibody complex is pulled down using 20μl of protein A-CL agarose and 10μl of protein G-CL agarose for 1hr at 4°C on a mixer. Spin the lysate for 20min. in a cooling centrifuge at 10000rpm and discard the supernatant. Wash the pellet by resuspending in 1ml of TTBS and placing on a
rotary mixer at 4°C for 5min. Spin in a cooling centrifuge for 5min. and repeat the wash steps twice or more. After final wash resuspend the pellet in 30µl of 1X sample buffer and elute the antigen by heating the tube to 100°C for 5min in a water bath. Run samples under reducing conditions on a 10% SDS-PAGE at 25mA constant current. And electrotransferred to a PVDF membrane at 200mA for 1hr at room temperature. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed extensively with TTBS buffer, and then it was incubated with mAb H₁₁B₂C₂ over night at 4°C. The blot was washed and incubated with goat anti mouse IgG-biotin conjugated (1:10,000 dilution) for 1 hr at room temperature. Later, the membrane was washed and incubated with streptavidin peroxidase (1:20, 000 dilution) for 1 hr at room temperature, after which they were detected using an ECL plus Western Blotting Detection System.

**Immunoprecipitation analysis of H₁₁ antigen from 220mM Q sepharose column fraction of normal and cancer serum then reacted with bHA probe:**

This was done as described by Green, et al., 1988, 200µg of 220mM Q sepharose column fraction of normal and cancer serum. Dilute the extract if required with lysis buffer. To the extract add 10µl of protein A-CL agarose, 5µl of protein G-CL agarose and incubated at 4°C with intermittent mixing. Spin the tube for 10min. in a cooling centrifuge and transfer the supernatant to a fresh tube. To the supernatant added 25µl [5µg] of mAb H₁₁B₂C₂ and incubate the tube at 4°C for overnight with intermittent mixing. After overnight incubation, the antigen-antibody complex is pulled down using 20µl of protein A-CL agarose and 10µl of protein G-CL agarose for 1hr at 4°C in a mixer. Spin the lysate for 20min. in a cooling centrifuge at 10000rpm and discard the supernatant. Wash the pellet by resuspending in 1ml of TTBS and placed on a rotary mixer at 4°C for 5min. Spin in a cooling centrifuge for 5min. Repeat the washing steps twice or more. After final wash, resuspended the pellet in 30µl of 1x sample buffer and eluted the antigen by heating the tube to 100°C for 5min. in a water bath. Run samples under reducing conditions on a 10% SDS-PAGE at 25mA constant current. The proteins separated in the gel were transferred onto a PVDF membrane in a western blotting unit at 200mA constant current for 1hr. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed extensively with TTBS buffer, and then the membrane was incubated with bHA (1:100 dilution) for overnight at 4°C. Next day both membranes
were washed with TTBS. The membrane was washed and incubated with streptavidin peroxidase (1:20, 000 dilution) for 1 hr at room temperature, after which they were detected using an ECL plus Western Blotting Detection System.

**Detection of H11 antigen in normal and cancer serum from cibacron blue eluted protein using mAb H11B2C2 and HA competition with mAb H11B2C2:**

Acetonitrile–H2O–trifluoroacetic acid eluted protein from normal and stomach and colon cancer serum samples were run on 10% SDSPAGE under reducing conditions at 25mA constant current in two identical lanes for each sample and transblotted onto PVDF membrane by electroblotting [wet transfer] at 200mA for 1hr. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed with TTBS buffer three times extensively and separated in two strips. One of the membranes was incubated with cold HA (500 μg/ml) for 3hr at room temperature and then mAb H11B2C2 was added later and mixed with cold or unlabelled HA (500 μg/ml). Then spreaded on the membrane and incubated over night at 4°C. To other strip mAb H11B2C2(1:500dilution) was added and kept over night at 4°C. Next day both membranes were washed and incubated with goat anti mouse IgG-biotin conjugated (1:10,000 dilution) for 1 hr at room temperature. Later, the membrane was washed and incubated with streptavidin peroxidase (1:20, 000 dilution) for 1 hr at room temperature after which they were detected using an ECL Plus Western Blotting Detection System.

**Detection of H11Antigen from affinity purified sample using mAb H11B2C2 by Western blot analysis:**

Glycine and Trietanolamin eluted protein from stomach and colon cancer serum samples were run on 10% SDSPAGE under reducing conditions at 25mA constant current in two identical lanes from each sample and transblotted onto PVDF membrane by electroblotting [wet transfer] at 200mA for 1hr. Nonspecific binding was blocked with 5% non-fat dry milk and 1% BSA for 1hr at room temperature. The membrane was washed with TTBS buffer three times extensively and separated in two strips. One of the membrane was incubated with cold HA (500 μg/ml) for 3hr at room temperature and then mAb H11B2C2 was added later and mixed with cold or unlabelled HA (500 μg/ml) and spreaded on the membrane and incubated over night at 4°C, to other strip (1:500) mAb H11B2C2 was added and over night at 4°C, next day the blot was washed
and incubated with goat anti mouse IgG-biotin conjugated (1:10,000 dilution) for 1 hr at room temperature. Later, the membrane was washed and incubated with streptavidin peroxidase (1:20, 000 dilution) for 1 hr at room temperature, after which they were detected using an ECL Plus Western Blotting Detection System.

**Results**

Serum samples from normal and stomach and colon cancers were taken and protein was extracted. The extracted crude protein was loaded onto the column G-50 (1.5x100cm) and eluted with 50mM Tris buffer, pH 8.0 (15ml/hr). The eluted samples were read at 280nm for protein. Elution profile plotted in the graph showed three peaks. These results for normal and stomach and colon cancer serum samples were presented in Fig.4.1, 4.2 and 4.3 respectively Proteins from the peak fractions separated on 10% SDS-PAGE under reducing conditions and transblotted on PVDF membrane and reacted with bHA and mAbH11B2C2. Only the first peak showed the protein of interest.

First peak fraction of G-50 column of normal, stomach and colon cancer samples were loaded on Q-Sepharose (Ion - exchange column chromatography) column and these results are presented in Fig.4.4, 4.5 and 4.6 respectively Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (1.5x15cm). The unbound protein was removed by washing the column with 50mM Tris buffer pH 8.0. The column was eluted with elution buffer containing different NaCl concentrations (50,150,220 and 300mM). Flow rate was adjusted to 30 ml/hr and 1.5ml fractions were collected. Fractions were screened for protein at 280nm and also for HABPs by western blotting using bHA probe and mAbH11B2C2. 

**Fig.4.7, 4.8 and 4.9** show the western blotting analysis of HABPs from I peak fraction from G-50 column and different Q-Sepharose fractions of normal, stomach and colon cancer serum samples using bHA probe. (Lane 1: G-50 I peak, Lane 2,3 and 4 Q-sepharose fractions eluted with 50mM, 150 and 220mM NaCl respectively).It was found that HABPs were eluted only in the I peak fraction from G-50 column and fractions eluted with 50,150 and 220 mM NaCl but not in 300mM. HABPs expression was also more in cancer serum when compared to normal serum sample. However the fraction eluted with 220mM.
100mg of crude protein extracted from normal serum sample was loaded on to a G-50 column (1.5x100cm) and was eluted with elution buffer containing 50mM Tris buffer pH8.0. Flow rate was adjusted to 15ml / hr. 2ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
100mg of crude protein extracted from stomach cancer serum sample was loaded on to a G-50 column (1.5x100cm) and was eluted with elution buffer containing 50mM Tris buffer pH8.0. Flow rate was adjusted to 15ml/hr. 2ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
100mg of crude protein extracted from colon cancer serum sample was loaded on to a G-50 column (1.5x100cm) and was eluted with elution buffer containing 50mM Tris buffer pH8.0. Flow rate was adjusted to 15ml / hr. 2ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
Fig. 4.4
Elution profile of Q-Sepharose (Ion-exchange column Chromatography) of first peak fraction of G-50 column of normal serum sample

Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (1.5x15cm) and was eluted with elution buffer containing different NaCl concentrations (50, 150, 220 & 300 mM). Flow rate was adjusted to 30ml/hr. 1.5ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
Fig. 4.5
Elution profile of Q-Sepharose (Ion-exchange column Chromatography) of first peak fraction of G-50 column of stomach cancer serum sample

Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (1.5x15cm) and was eluted with elution buffer containing different NaCl concentrations (50, 150, 220 & 300 mM). Flow rate was adjusted to 30ml/hr. 1.5ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
Fig. 4.6
Elution profile of Q-Sepharose (Ion-exchange column Chromatography) of first peak fraction of G-50 column of colon cancer serum sample

Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (1.5x15cm) and was eluted with elution buffer containing different NaCl concentrations (50, 150, 220 & 300 mM). Flow rate was adjusted to 30ml/hr. 1.5ml fractions were collected. Fractions were screened for protein at 280nm and also for HABP by western blotting using bHA and mAb H11B2C2.
Western blotting analysis of HABPs from first peak of G-50 and Q-Sepharose fractions eluted with 50, 150, 220 mM NaCl from normal serum samples using probe bHA.

Lane 1: G 50 first peak
Lane 2: 50mM
Lane 3: 150mM
Lane 4: 220mM

Graphs showing the intensity of 57kDa protein (HABP)

Western blotting analysis of HABPs from first peak of G-50 and different Q-Sepharose fractions of normal serum samples using bHA probe. (Lane 1: G-50 1 peak, Lane 2: 50mM, Lane 3: 150mM and Lane 4: 220mM). It was found that HABPs were eluted only in the 50, 150, 220 mM fractions.
Western blotting analysis of HABPs from first peak of G-50 and Q-Sepharose fractions eluted with 50, 150, 220 mM NaCl from stomach cancer serum samples using probe bHA.

Lane 1: G 50 first peak
Lane 2: 50mM
Lane 3: 150mM
Lane 4: 220mM

Graphs showing the intensity of 57kDa protein (HABP)

Western blotting analysis of HABPs from first peak of G-50 and different Q-Sepharose fractions of stomach cancer serum samples using bHA probe. (Lane 1: G-50 I peak, Lane 2: 50mM, Lane 3: 150mM and Lane 4: 220mM). It was found that HABPs were eluted only in the 50, 150, 220 mM fractions.
Western blotting analysis of HABPs from first peak of G-50 and Q-Sepharose fractions eluted with 50, 150, 220mM NaCl from colon cancer serum samples using probe bHA.

Lane 1: G-50 first peak
Lane 2: 50mM
Lane 3: 150mM
Lane 4: 220mM

Graphs showing the intensity of 57kDa protein (HABP)
Western blotting analysis of HABPs from first peak of G-50 and different Q-Sepharose fractions of colon cancer serum samples using bHA probe. (Lane 1: G-50 I peak, Lane 2: 50mM, Lane 3: 150mM and Lane 4: 220mM). It was found that HABPs were eluted only in the 50, 150, 220 mM fraction.
showed major reaction when compared with other two fraction indicating the HABP expression was more in this third peak. Image analysis were also carried out.

Q-Sepharose column fractions were screened for H11 antigen by western blotting using mAbH11B2C2. **Fig.4.10, 4.11 and 4.12** show the western blotting analysis of HABPs from different Q-Sepharose fractions of normal and stomach and colon serum samples respectively using mAbH11B2C2. (Lane 1, 2 and 3 Q-sepharose fractions eluted with 50, 150 and: 220mM NaCl respectively) It was found that H11 antigen was present mostly in the fraction eluted with 220 mM NaCl. H11 antigen expression was also more in cancer serum samples when compared to normal serum sample. Image analysis were also carried out.

In order to ascertain that the H11 antigen has HA binding region, 220 mM Q-Sepharose fraction protein from normal and, stomach and colon cancer serum samples was mixed with 10 μg of bHA probe and incubated 1hr at room temperature. Then 50% of saturated (NH4)2SO4 was added. The sample was mixed thoroughly and incubated for over night at 4°C in order to pull down the proteins. Then centrifuged the sample and the pellet was separated. The pellet was dissolved in 1% SDS and loaded in SDS sample buffer and ran on 10% SDS-PAGE. Then proteins were transblotted to PVDF membrane and separated in two strips. One of the membranes was incubated with cold HA and later added mAbH11B2C2. To other strip only mAbH11B2C2 was added. The membrane that was incubated with cold HA binds to HABPs and not allowed to bind to mAbH11B2C2. Therefore, no bands were observed because cold HA competed with mAbH11B2C2 for HABPs (Lane 4, 5 and 6). The other strip which was incubated only with mAbH11B2C2 showed clear bands (Lane 1, 2 and 3). This blot confirmed 57kDa protein is a HABP which was pulled down by bHA probe and detected with mAbH11B2C2. This figure also shows the over expression of the H11 antigen in cancer serum when compared with normal serum. The Image analysis was also carried out these results were presented in **Fig.4.13**.

Proteins from 220 mM Q-Sepharose fraction protein from normal and stomach and colon cancer serum samples were immunoprecipitated with mAbH11B2C2. Lane 2 and 3 showed over expression of H11 antigen in stomach and colon cancer serum samples and in lane1 normal serum, less expression of H11 antigen was observed. Graphs showing the intensity.
Western blotting analysis of $H_{11}$ antigen from Q-Sepharose fractions eluted with 50, 150, 220mM NaCl from normal serum samples using mAb $H_{11}B_2C_2$

Lane 1: 50mM, Lane 2: 150mM, Lane 3: 220mM

Graphs showing the intensity of 57kDa protein ($H_{11}$antigen)

Western blotting analysis of $H_{11}$antigen from different Q-Sepharose fractions of normal serum samples using mAb$H_{11}B_2C_2$. (Lane 1: 50mM, Lane 2: 150mM and Lane 3: 220mM). It was found that $H_{11}$antigen were eluted mostly in 220 mM fraction.
Fig. 4.11
Western blotting analysis of H_{11} antigen from Q-Sepharose fractions eluted with 50, 150, 220 mM NaCl from stomach cancer serum samples using mAb H_{11}B_{2}C_{2}

Lane 1: 50mM, Lane 2: 150mM, Lane 3: 220mM

Graphs showing the intensity of 57kDa protein (H_{11} antigen)
Western blotting analysis of H_{11} antigen from different Q-Sepharose fractions of stomach cancer serum samples using mAb H_{11}B_{2}C_{2}. (Lane 1: 50mM, Lane 2: 150mM and Lane 3: 220mM). It was found that H_{11} antigen were eluted mostly in 220 mM fraction. H_{11} antigen expression was also more in cancer serum when compare to normal serum samples.
Western blotting analysis of H₁₁ antigen from Q-Sepharose fractions eluted with 50, 150, 220 mM NaCl from colon cancer serum samples using mAb H₁₁B₂C₂.

Lane 1: 50mM, Lane 2: 150mM, Lane 3: 220mM

Graphs showing the intensity of 57kDa protein (H₁₁ antigen)

Western blotting analysis of H₁₁ antigen from different Q-Sepharose fractions of colon cancer serum samples using mAb H₁₁B₂C₂. (Lane 1: 50mM, Lane 2: 150mM and Lane 3: 220mM). It was found that H₁₁ antigen were eluted mostly in 220 mM fraction. H₁₁ antigen expression was also more in cancer serum when compare to normal serum samples.
Graphs showing the intensity of 57kDa protein (H11 antigen)

Western blotting analysis of 220 mM Q-Sepharose fraction protein from normal and stomach and colon cancer serum samples was pulled down with bHA and reacted with cold HA and later mAb H11B2C2 to one strip. The other strip only mAb H11B2C2 was added. Over expression of the H11 antigen in cancer serum was observed when compared.
Fig. 4.14

Immunoprecipitation analysis of H11 antigen from 220mM Q sepharose column fraction of normal and stomach and colon cancer serum samples and reacted with mAb H11B2C2

Lane1: normal, Lane2: ca. stomach, Lane3: ca. colon

Graphs showing the intensity of 57kDa protein (H11antigen)

Western blotting analysis of 220 mM Q- Sepharose fraction protein from normal and stomach and colon cancer serum samples were immunoprecipitated with mAb H11B2C2. Lane 2 and 3 showed over expression of H11antigen in stomach and colon cancer serum samples and in lane1 we observed less expression of H11antigen.
of 57kDa protein in each lane of the blots were done using Image Analysis Software.

Fig.4.14.

Fig.4.15, shows the Western blotting analysis of proteins obtained from 220mM Q-Sepharose column fractions of normal and stomach and colon cancer serum samples. They were immunoprecipitated with mAb H$_{11}$B$_2$C$_2$, and separated on 10% SDS-PAGE under reducing conditions and transblotted on PVDF membrane. They were treated with bHA and developed by ECL. The mAbH$_{11}$B$_2$C$_2$ immunoprecipitated protein was reacted with the probe bHA therefore the H$_{11}$ antigen has HA binding region. Lane 2 and 3 showed over expression of 57kDa H$_{11}$ antigen in stomach and colon cancer serum samples respectively and in lane1 less expression of H$_{11}$ antigen was observed in normal serum sample. Image analysis was also carried out.

Proteins eluted from cibaron blue gel column by TFA/Acetonitrile from normal and stomach and colon cancer serum samples were resolved in a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAb H$_{11}$B$_2$C$_2$. Protein bounded to the cibacron blue gel along with serum albumin was H$_{11}$ antigen or 57kDa HABP.T the result conformed with HA competition experiment. Image analysis showed over expression of 57kDa H$_{11}$ antigen in stomach and colon cancer serum samples when compared with normal serum. Fig.4.16.

Western blotting analysis of proteins obtained from stomach and colon cancer serum Immuno-affinty column chromatography were separated on 10% SDS-PAGE under reducing conditions, transblotted on PVDF membrane, reacted with mAb H$_{11}$B$_2$C$_2$ and developed by ECL They were eluted with TEA followed by glycine . The TEA eluted fraction from the affinity column reacted with mAb H$_{11}$B$_2$C$_2$ showed less expression of 57 kDa protein whereas, the glycine eluted fraction when reacted with mAb H$_{11}$B$_2$C$_2$ showed more expression of 57 kDa protein. These results were given in Fig.4.17 and Fig.4.18.
Immunoprecipitation analysis of $H_{11}$ antigen from 220mM Q sepharose column fraction of normal and stomach and colon cancer serum samples reacted with bHA probe

Lane1: normal  Lane2: ca. stomach  Lane3: ca. colon

Graphs showing the intensity of 57kDa protein ($H_{11}$antigen)

Western blotting analysis of proteins obtained from 220mM Q-Sepharose column fractions of normal and stomach and colon cancer serum samples. They were immunoprecipitated with mAb$H_{11}B_2C_2$. Then mAb $H_{11}B_2C_2$ immunoprecipitated protein was reacted with the probe bHA indicating the $H_{11}$antigen has HA binding region. Lane 2 and 3 showed over expression of 57kDa $H_{11}$antigen in stomach and colon cancer serum samples respectively and in lane 1 it was observed less expression of $H_{11}$antigen.
Western blotting analysis of $H_{11}$ antigen from cibacron blue eluted protein of normal and cancer serum samples using mAb $H_{11}B_2C_2$ and HA competition experiment

Lane 1, 2 & 3 showed the blot reacted with mAb $H_{11}B_2C_2$ only
Lane 4, 5& 6 showed the blot competed with cold HA and mAb $H_{11}B_2C_2$ was added later
Lane1 & 4 normal  
Lane2 &5, ca stomach, 
Lane3&6, ca- colon

Graphs showing the intensity of 57kDa protein ($H_{11}$antigen)

Western blotting analysis of proteins obtained from cibacron blue gel column eluted by TFA/Acetonitrile from normal, stomach and colon cancer serum samples were reacted with mAb $H_{11}B_2C_2$. It was found that protein bounded to the cibacron blue gel along with serum albumin was $H_{11}$antigen or 57kDa HABP, the result conformed with HA competition experiment. Image analysis showed over expression of 57kDa $H_{11}$antigen in stomach and colon cancer serum samples when compared with normal serum.
Western blotting analysis of stomach cancer serum proteins obtained from Immuno-affinity column eluted with TEA and glycine and reacted with mAb H_{11}B_{2}C_{2}

Lane 1: TEA eluted fraction  
Lane 2: Glycine eluted fraction

Graphs showing the intensity of 57 kDa protein (H_{11}antigen)

Western blotting analysis of proteins obtained from stomach cancer serum. Immuno-affinity column chromatography fraction eluted first with TEA followed by glycine. They were reacted with mAb H_{11}B_{2}C_{2} and developed by ECL. The TEA eluted fraction from the affinity column reacted with mAb H_{11}B_{2}C_{2} showed less expression of 57 kDa protein, whereas, the glycine eluted fraction when reacted with mAb H_{11}B_{2}C_{2} showed more expression of 57 kDa protein.
Western blotting analysis of colon cancer serum proteins obtained from immune-affinity column eluted with TEA and glycine and reacted with mAb H_{11}B_{2}C_{2}

Lane 1, TEA eluted fraction
Lane 2, Glycine eluted fraction

Graphs showing the intensity of 57kDa protein (H_{11}antigen)

Western blotting analysis of proteins obtained from colon cancer serum. Immuno-affinity column chromatography fraction eluted first with TEA followed by glycine. They were reacted with mAb H_{11}B_{2}C_{2} and developed by ECL. The TEA eluted fraction from the affinity column reacted with mAb H_{11}B_{2}C_{2} showed less expression of 57 kDa protein, whereas, the glycine eluted fraction when reacted with mAb H_{11}B_{2}C_{2} showed more expression of 57 kDa protein.