Original Article

Purification of Hyaluronan Binding Proteins from Human Normal and Cancer Serum

Fekry B[1], Siddaiah M[1], Srinivas KP[1], Nunna RV[1], Banerjee SD[2], Mortha KK[1]

Abstract

Background: Analysis of human cancer serum has revealed the presence of high amounts of hyaluronan (hyaluronic acid, HA) when compared to human normal serum, It is well documented that HA and its receptors, known as hyaladherins (HABPs) are involved in matrix regulation, cell proliferation, migration and malignant tumour progression. These hyaladherins not only interact with hyaluronan at the matrix proper but also with hyaluronan at the plasma membrane as a cell surface receptors and thus influence cell physiology including secretion of this protein into the circulatory system.

Methods: Normal serum and colon cancer serum samples were included in this study, using biochemical techniques such as gel permeation, strong anion exchange chromatography, single dimension electrophoresis and western blot analysis.

Results: This study is based on the clinical work of normal serum and colon cancer serum. The description of the procedure was given for the fractionation of serum proteins mainly HABPs from 20 normal and 15 colon cancer patients by using special biotinylated hyaluronan probe.

Conclusion: To evaluate whether serum HABPs levels could be used as diagnostic marker for human cancer. The semi purified serum from normal and colon cancer patients showed mainly a major protein (57kDa) and a minor one (30kDa) by overlay experiments with b-HA probe and these results were confirmed by competition experiments with cold HA.

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Key Words: Hyaluronan (Hyaluronic acid, HA) Hyaluronic acid binding protein (HABP), Biotinylated hyaluronan probe (bHA), Cold HA.

1. Department of Biochemistry, University of Mysore, Mysore-570 006, India
2. Tufts University School of Medicine, Boston, USA

Corresponding Author:
Mortha Karuna Kumar, PhD
Professor, Department of Studies in Biochemistry,
University of Mysore
Tel: (+91) 99-00 45 16 42
Email: karunamkk@rediffmail.com

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Introduction

Hyaluronan (hyaluronic acid, HA) is a glycosaminoglycon of the extracellular matrix (ECM).HA is a prominent factor in serum whenever rapid tissue growth occurs particularly during embreogenesis and tumor growth and its spread. [1-3]. HA promotes the detachment process that permits cells to migrate [4]. Similarly, HA takes up a large volume of water during hydration, opening up tissue space that is permissive for cell migration. It is considered a factor that will enhance wound healing and facilitate tumor growth and metastasis [1,5].

The turnover of hyaluronan in serum has been elucidated. The, polysaccharide is produced in the tissues and carried by lymph flow to the local lymph nodes, where, part of it is taken up and degraded [6, 7]. The remaining fraction is carried to the general circulation from which it is rapidly extracted by the liver endothelial cells. Conditions are known in which serum hyaluronan is elevated, when liver function is impaired as in the case of liver cirrhosis [8, 9] and also when the tissues produce increased amounts of hyaluronan as in the case of cancer [10].

It is well established that the large array of functions that a tumour cell has to fulfil to settle as a metastasis in a distant organ requires cooperative activities between the tumour and the surrounding tissue. Several classes of molecules are involved such as cell–cell and cell–matrix adhesion molecules and matrix degrading enzymes. Furthermore, metastasis formation requires concerted activities between tumour cells and surrounding cells as well as matrix elements and possibly concerted activities between individual molecules of the tumour cell itself [11].

The families of HABPs are termed as hyaladherins [2]. Consequently, evidence was sought and obtained for the presence of HA receptors on the surface of cells. Subsequent investigations led to the molecular characterization of two classes of cell surface HA receptors, namely, CD44 and RHAMM [12, 13].Most of the malignant solid tumors contain elevated levels of hyaluronan products [1], which are correlated, with poor differentiation of cells [14].

Hyaluronan receptors have been widely implicated in tumorigenesis but their involvement varies. Recent evidences suggest that CD44-mediated events can enhance [15] or inhibit [16] tumor progression in different types of tumors.

The circulating proteins acquire the longevity in the serum and 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 biomarkers have been reported such as CA-125[17], serological mucin assay for pancreatic cancer [18].

Here a procedure was described for the partial purification of serum HABP from normal and colon cancer patients by gel permeation and strong anion exchange chromatography, single dimension electrophoresis and western blotting analysis to determine as a possible biomarker from cancer serum samples.

Materials and Methods

Sephadex G-50, QAE-Sepharose, hyaluronan (Na salt, human umbilical cord) and protease inhibitor cocktail were procured from Sigma, USA. Streptavidin-horseradish peroxidase conjugated was purchased from Invitrogen, Molecular weight markers were purchased from Fermentas USA, PVDF membrane from Millipore, biotin LC hydrazide, EDC purchased from sigma, DMSO purchased from SRL, Mumbai, India. ECL plus western blotting detection system was purchased from Amersham Biosciences, USA. All other chemicals were purchased from Sigma, USA.

Serum collection and preparation:

The study consisted of 20 normal and 15 second grade of colon cancer patients. Serum samples were collected from normal volunteers for normal serum and colon cancer patients from hospitals (Bharath Cancer Hospital and Institute of Oncology, Mysore, India) The protocol was approved by the Ethical Review Committee (IHEC-UOM NO.35/PhD/ 2009-10 dated 2nd December 2009) and the patients consent was also taken. Serum sample was taken from each patient prior to any treatment e.g. radiation or chemotherapy. Both normal and cancer patient's blood samples were centrifuged at 2000rpm for 30 min. after standing at room temperature for one hr and the supernatant serum was stored at -80°C in liquid nitrogen. The tumour sections from patients after H and E stain was graded using TNM grading system .Serum samples were homogenized in 4X lytic buffer containing 0.2M Tris-HCl (pH:8.0), 80mM EDTA,4mM PMSF, 4mM benzamidine-HCl and 2% Triton X100 plus protease inhibitor cocktails. The homogenate was centrifuged at 10,000rpm for 30 min at 4°C. The supernatant was stored at either at (-80) until further analysis was carried out. The amount of protein was estimated.

Sephadex G-50 gel permeation column chromatography:

Sephadex G-50 was swollen in double distilled water for 12hr at 4°C. The swollen gel was packed into a column of bed volume 85ml. The column is then equilibrated with 50mM Tris pH 8.0. The flow rate was adjusted to 17ml/hr. 100mg of crude serum extract from normal and colon cancer patients was loaded onto the column. The protein was eluted from the column with 50 mM Tris pH 8.0. The protein elutions were monitored in a spectrophotometer at 280nm absorbance. The elution was followed by assay for protein of interest.

QAE-Sepharose ion exchange column chromatography:

Preswollen QAE-Sepharose (fast flow) was packed on to a 10ml column. The column was then equilibrated with 50mM Tris pH 8.0. The major peak fraction (approximately 20mg) recovered from G-50 column was loaded onto QAE-Sepharose column. The column was washed with 50mM Tris pH 8.0 to remove the unbound protein. The column was then eluted step wise with different molarities of sodium chloride (50,150, 220 and 300mM) in 50mM Tris buffer pH 8.0. Column fractions were monitored in a spectrophotometer at 280nm absorbance. The elution profile was plotted.

Preparation of biotinylated hyaluronic acid:

Fifty (50) mg of hyaluronic acid was dissolved in 10ml of filtered PBS-A buffer (Ca and Mg free). The dissolved hyaluronan solution was dialysed against 0.1M MES buffer pH 5.5 for 16hrs at 4°C. Later hyaluronan solution was mixed with 50mM biotin-LC-hydrazide dissolved in DMSO to give a final concentration of 1mM. 50mM EDC was added to

give a final concentration of 10mM and incubated for 16hrs at 4°C and then dialysed against PBS-A for 36hrs at 4°C.Finally the dialysed bHA was stored in glycerol at-20°C.

Detection of serum HABP's in normal and cancer serum using bHA probe by Western blot analysis:

The 100µg protein from crude serum and column fractions samples were run on 10% SDS PAGE under reducing conditions. Then proteins were transblotted to PVDF membrane and incubated 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 and the membrane was extensively washed and the protein complexes were detected using an ECL detection kit.

bHA competition experiment:

To confirm proteins which are reacting with HA are really HABPs, HA competition experiment was carried out. In this experiment 100 µg protein from 220Mm column fraction of normal and colon cancer serum samples were run on 10% SDS-PAGE under reducing conditions in two identical lanes as duplicates. This was followed by transblotting onto PVDF membrane, which was then separated in two strips. To one group, the membrane was incubated with cold HA (500 µg/ml) for 3hr at room temperature and then with the probe bHA, (1:100, mixed with 500µg of cold or unlabelled HA) overnight at 4°C. To another group, 1:100 dilution of bHA was added followed by overnight incubation at 4°C. Routine western blot analysis and detection by ECL (Amersham Biosciences) were carried out as mentioned earlier.

Results

Crude serum protein was separated on 10%SDS-PAG and transblotted on to the PVDF membrane and reacted with bHA probe. The Lane 1: is from normal serum and shows two minor bands expressing very little HABPs, whereas, the Lane 2 is from ca-serum from colon cancer patients that shows over expression of HABPs. The Lane 3 shows the molecular weight markers. The graph shows image analysis of 57kD band (HABPs) from normal and colon cancer patient serum. These results are presented in Fig1.

The crude serum protein from normal and colon cancer patients run on Sephadex G-50 column chromatography. The elution profile is given in Fig2. Three peaks were observed in both normal and cancer serum, however, more protein was observed in the first peak of colon cancer serum. Only the first peak of column showed proteins of interest (HABPs), these proteins in different peaks were separated on 10%SDS-PAG and transblotted on to the PVDF membrane. After reacting with bHA probe, it was found that HABPs were eluted only in the first peak but not in other two peaks. HABPs expression was also more in cancer serum when compare to normal serum samples.

Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (Ion - exchange column chromatography).Figure 3 presents the elution profile of Q-Sepharose column. When eluted with different concentrations of sodium chloride, four fractions were obtained in both normal and cancer serum. These proteins in different peaks were separated on 10%SDS-PAG and transblotted on to the PVDF membrane. After reacting with bHA probe, it was found that HABPs were eluted only in the first, second and third peaks only but not in fourth peak. HABPs expression was also more in cancer serum when compare to normal serum sample However the fraction eluted with 220mM 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.

When western blot analysis was carried out with 1st peak protein from G-50 column and 1st, 2nd and 3rd peak proteins from Q-sepharose column, two bands were appeared, one major protein (57kDa) and a minor band at 30kDa in case of colon cancer serum sample . These results were presented in Fig.4. Similar results were obtained with normal serum but expression was low (Fig5). Image analyses were also carried out with both normal and cancer samples.

To confirm these proteins which are reacting with bHA probe are really HABPs or not, HA competition experiment was carried out in both normal and cancer samples with cold HA. Since high amounts of HABP were eluted with 220mM NaCl, this fraction was selected for this experiment with cold HA. Here the proteins separated on 10% SDS-PAGE under reducing conditions and transblotted on PVDF membrane. Lane 1 and 2 samples were allowed to react with bHA, whereas, lane 3, 4 samples were allowed to react first with cold HA and later were exposed to bHA. As lane 3 and 4 were already exposed to cold HA, these proteins did not react with bHA therefore no bands were appeared. These results were given in Fig.6. Image analysis graph also presented. It was found that over expression of this protein in cancer serum sample when compared with normal serum samples.

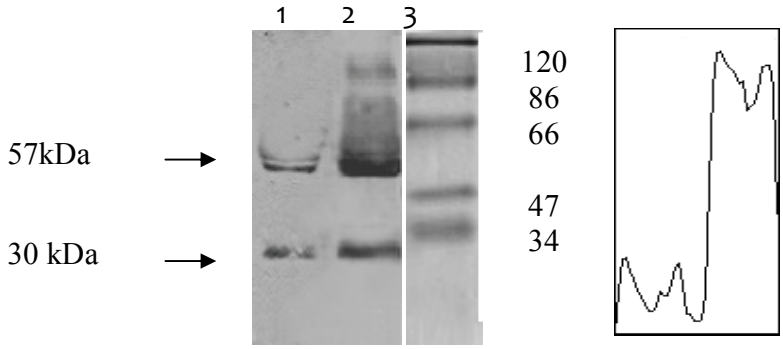

Figure1. One hundred (100) µg of crude serum protein from normal and colon cancer patients was separated on 10%SDS-PAGE,transblotted on to the PVDF membrane and reacted with bHA probe, Lane 1: normal serum, Lane 2: ca-serum (colon), Lane 3: the molecular weight markers. The graph shows image analysis of 57kD band (HABP) from normal and cancer patients' serum

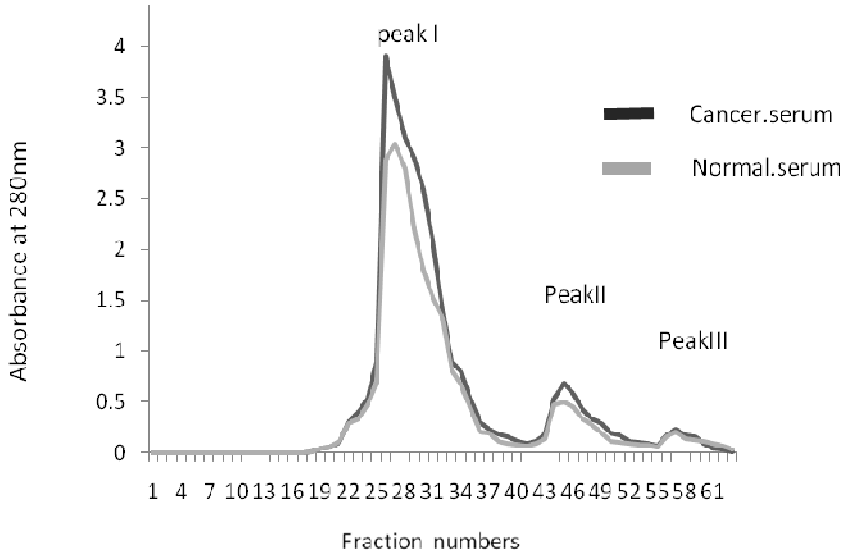

Figure2. Elution profile of Sephadex G-50 column chromatography of crude serum protein from normal and colon cancer patients

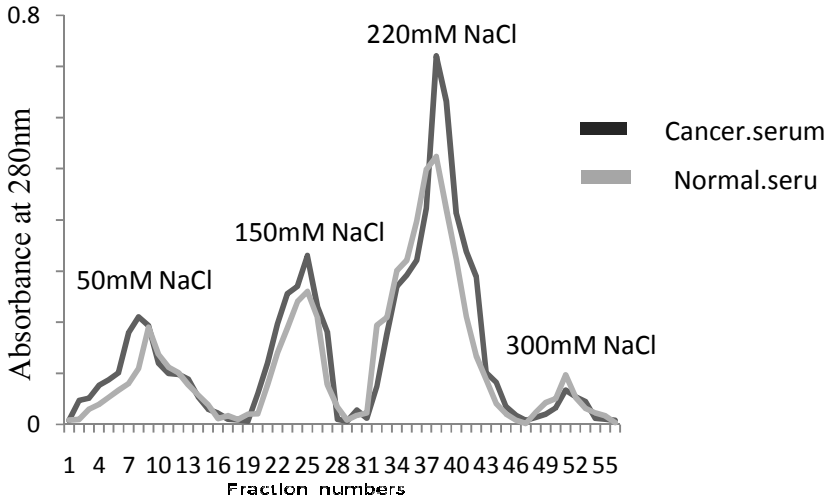

Figure3. Elution profile of Q-Sepharose (Ion - exchange column Chromatography) from normal and colon cancer samples

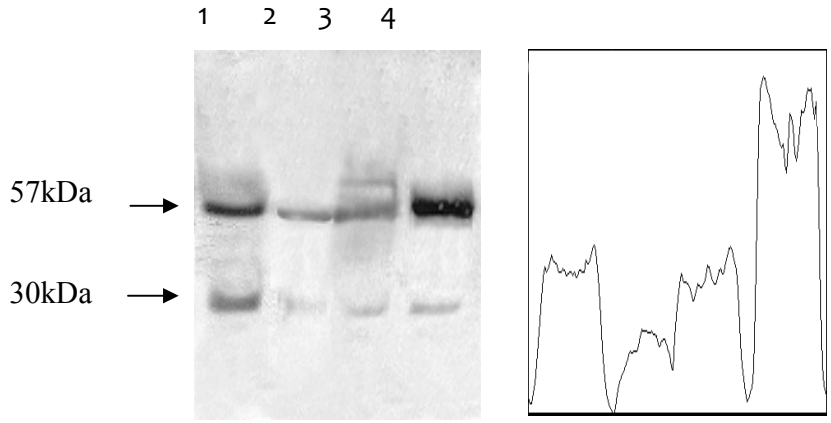

Figure4. Western blotting analysis of HABPs from 1st peak of G-50 and 1st,2nd and 3rd fractions from Q-Sepharose column of colon cancer serum samples using probe bHA,Lane 1: G 50 Ist peak, Lane 2: 50mM , Lane 3: 150mM, Lane 4: 220mM.The graph shows image analysis of 57kD band (HABP)

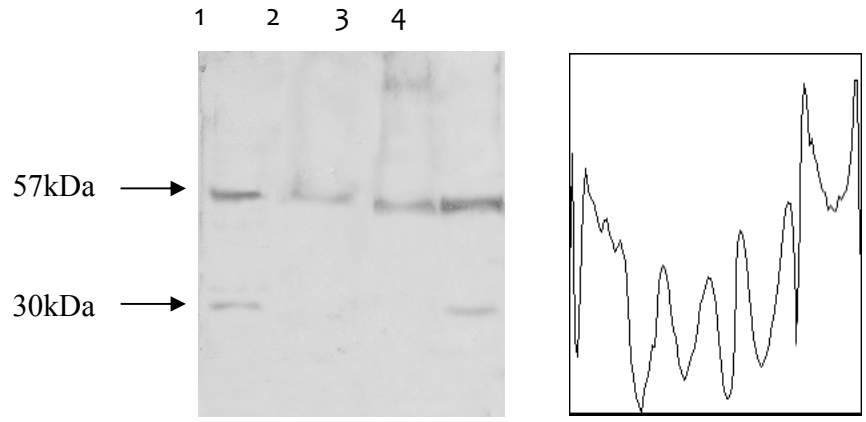

Figure5. Western blotting analysis of HABPs from 1st peak of G-50 and 1st,2nd and 3rd fractions from Q-Sepharose column of normal serum samples using probe bHA ,Lane 1: G 50 Ist peak, Lane 2: 50mM, Lane 3: 150mM ,Lane 4: 220mM . The graph shows image analysis of 57kD band (HABP)

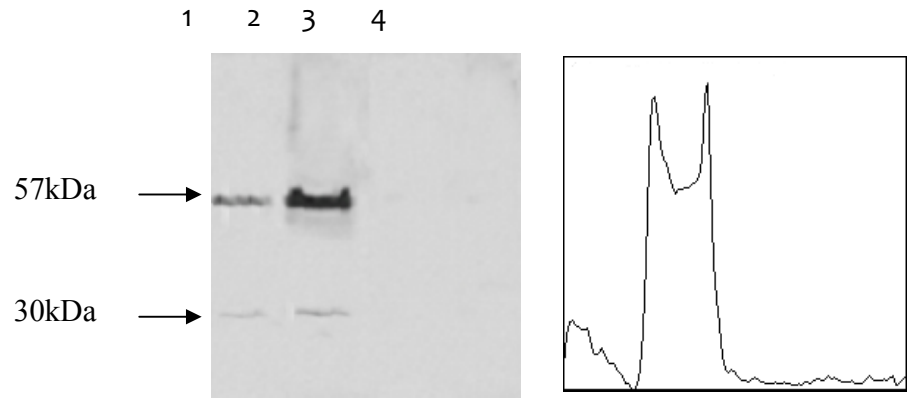

Figure6. Western blotting analysis of HABPs expression in 220 mM Q-Sepharose fractions of normal and Colon cancer serum samples using probe bHA and competition assay using cold HA. Lane 1, 2 showed the blot with bHA , Lane 3,4 showed the blot competed with cold HA and bHA , Lane1,3:normal serum , Lane1,3: ca-serum (colon) .The graph shows image analysis of 57kD band(HABP)

Discussion

High levels of HA might promote tumorigenesis The increased detachment and motility of malignant cells during early and midstages of the tumor are due to the increased accumulation of HA, which in turn caused the deformation of restrictive architecture of the ECM components and provides a hydrated environment to facilitate migration of tumor cells to distant sites [19-21].

Studies have indicated that increased serum HA levels and deposition in tumour tissues are often associated with malignant progression of cancers and multiple transcriptional regulation of HAS genes may allow cancer cells to optimize the extracellular environment for tumour growth and invasion [22].

Western blot analysis of crude normal and colon cancer serum showed the over expression of HABPs mostly 57kDa MW and minor protein with 30kDa MW as recognized by probe bHA.. The image analysis of 57kDa protein from normal and colon cancer serum sample also showed similar trend.To know the nature of this HABP, the serum proteins were purified in a sequential process. First through gel chromatography (G-50) and then through Ion-exchange chromatography (Q-Sepharose). These HABPs came out in the first peak of G-50 column and then re-chromatographed through Ion-exchange column (Q-Sepharose)., When eluted with different concentrations of NaCl, the peak eluted with 220mM fraction showed high amount of 57kDa MW HABP, whereas, peaks eluted with 50mM and 150mM fractions showed a very light reaction when reacted with probe bHA and no reaction was found in 300mM fraction.

When competition experiments were conducted with fractions eluted with 220mM NaCl, the bHA without the presence of cold HA will be binding to HABP. When cold HA was added prior to bHA, the HABP were bound to cold HA first and not allowing bHA to bind indicating HABPs.

Conclusions

The data presented here indicate that serum proteins may act to sequester peptide fragments which are hyaluronan binding proteins and they are over expressed in the colon cancer serum. Such sequestered peptides may provide a potentially rich source of cancer-associated biomarkers for clinical evaluation. Further work is under investigation to characterize these proteins by mass spectrometric analysis and its possible homology with other basement membrane matrix molecules.

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Conflict of Interest

The authors declare that they have no conflict of interest in this article.

Authors' Contribution

The biochemical experiments and writing the manuscript were done by all authors, while the biochemical studies were done by FB.

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