Introduction

Hyaluronan (hyaluronic acid, HA) is a major macromolecular polysaccharide component of the extracellular matrix that confers structural frameworks for all cells. Despite its relatively simple chemical composition, hyaluronan mediates many other important functional aspects including signalling activity during embryonic morphogenesis, cellular regeneration and wound healing. Abnormalities in hyaluronan metabolism have been implicated in many diseases, such as inflammatory disorders, cardiovascular diseases and cancer. To date, it has become increasingly clear that hyaluronan production in vertebrates is tightly regulated by three hyaluronan synthases and that hyaluronan catabolism is regulated by an enzymatic degradation reaction involving several hyaluronidases. Together, these discoveries have provided key insights into the physiological roles of hyaluronan and a deeper understanding of the mechanisms underlying altered hyaluronan turnover in diseases (Itano, 2008).

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 (Laurent and Fraser, 1986; Laurent et al., 1986). 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 in liver cirrhosis (Laurent et al., 1985; Frebourg et al., 1986) and when the tissues produce increased amounts of hyaluronan in inflammatory disease (Laurent et al., 1985), and in cancer (Inger et al., 1988).

As mentioned earlier, HA is ubiquitously present in the extracellular matrices (ECMs) of animals, plays an important role in ECM organization and cell behaviour through binding to HABPs. The family of HA binding proteins are termed as hyaladherins (Toole, 1990), which include matrix HA binding proteins and cell surface HA receptors that exhibit high HA binding affinity. Extracellular hyaladherins include ECM proteins such as versican, aggrecan, neurocan, brevican, fibronectin, hyaluronan link protein and TSG-6 (tumor necrosis factor stimulated gene-6) and soluble protein
such as α-trypsin inhibitor. Cellular hyaladherins include intracellular proteins such as CDC37, P32, RHAMM, HBP (hepatocyte binding protein) and IHABP4. Cellular receptors for the extracellular matrix component hyalurona (HA) are involved in a broad spectrum of biological processes i.e., organogenesis (Fenderson et al., 1993), development of embryonic structures (Gakunga et al., 1997), migration of normal cell (Turley et al., 1992) and activation of the immune response (DeGrendele et al., 1996). Transmembrane proteins such as CD44 family, RHAMM, members of the hyaladherins (Turley, 2002), are the extracellular hyaladherins, although present in smaller proportions, may participate in cartilage matrix assembly and maintenance of matrix integrity (Knudson and Knudson, 1993). Cellular hyaladherins have been detected in several cell types from a wide verity of tissues (Toole, 1990; Underhill; 1992).

Detection of cancer is critical for the management of the disease. Early diagnosis of pre-malignant lesion is directly proportional to chances of survival. The presence of HA and its interaction with receptor HABP are involved in establishing and modifying the properties and function of ECM. Several intracellular hyaladherins e.g. CDC37, IHABP4, RHAMM have been characterized. Each of these proteins interacts with kinases, important in regulation of the cell cycle and may be involved in HA synthase activity. Over expression of soluble HABPs act as an interactive link for displacement of endogenous HA from its receptor and inhibit putative downstream events. Support for the hypothesis comes from the various experimental evidences implicating hyaluronan and HABP in progression of a variety of cancers (Toole et al., 2002; Culry et al., 1994; Hardwick et al., 1992; Knudson et al., 1989). New prognostic markers of cancer metastasis are urgently needed to avoid over treatment or under treatment of newly diagnosed patients.

HA plays a structural role and mediates signalling events via interactions with cell surface receptors, such as RHAMM and CD44 (Turley et al., 2002). CD44 and RHAMM are established signal-transduction receptors that influence cell proliferation, survival and motility, and are known to be relevant to cancer. Other cell-
surface hyaladherins, such as lymphatic-vessel endothelial hyaluronan receptor 1 (LYVE1) and TOLL4, might also have roles in cancer pathogenesis. Interactions of hyaluronan with CD44 and RHAMM lead to numerous cellular responses including those that involve tyrosine kinases, protein kinase C, focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase, nuclear factor-κB and RAS as well as cytoskeletal components (Turley et al., 2002; Ponta et al., 2003; Bourguignon, 2001; Thorne et al., 2004). Both RHAMM and CD44 mediate hyaluronan signalling and participate in growth factor regulated signalling. However, they likely regulate signalling by different mechanisms because they are not homologous proteins and are compartmentalized differently in the cell and differ in the mechanisms by which they bind to hyaluronan. Additional cellular hyaladherins have been identified, but their role in cell signalling has not yet been reported.

The potential marker should be tested retrospectively in large number of patients with a long follow-up period. Multivariate analysis needs to be done in conjunction with established markers to assess its independent value. Subsequently, an independent group of researchers, and, ideally, a prospective study should validate the findings to confirm the prognostic significance of the tested marker. A large number of putative prognostic markers (ERBB2, ELAM-1, ICAM-1, VLH-4, CD44 etc) have been reported till date, but 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.

Despite advances in diagnostic imaging technology, surgical management and therapeutic modalities, cancer remains a major cause of mortality worldwide. Currently, it is estimated that cancer kills over 6 million people per year worldwide with over 10 million new cases being diagnosed every year. Mortality is mainly attributed to dissemination of primary cancer to distant organs on which no effective treatment is available. The dilemma in oncology practice is the large number of patients presenting macro- or micro-metastases at primary diagnosis, and the fact that in some cases (e.g. breast cancer) metastases are seen in patients free of metastasis-positive axillary lymph nodes (Braun et al., 2000).
A small number of clinically approved biomarkers are available for early diagnosis and or for successful monitoring of treatment and relapses (Table 3.1) (Alaoui et al., 2006), so that they contributed significantly to reduced mortality rates and increased overall survival for cancers such as prostate cancer (Ludwig and Weinstein, 2005; Sidransky, 2002). However, most solid tumours, the absence of selective biomarkers hamper efforts to improve early detection and therapeutic management.

The assumption that HABP (hyaluronan binding protein) acts as a protein biomarker and may give important diagnostic information in cancer detection led to the present study. Here the investigations were described for the detection of serum HABP (H₁₁ antigen) from various cancers, in different grades of cancers and also in normal patients using mAbH₁₁B₂C₂.

**Materials and Methods**

**Chemical:**

Hyaluronan (Na salt human umbilical cord) was procured from Sigma, USA. Streptavidin-horseradish peroxidase conjugated (HPO9) was purchased from Invitrogen, Media and glasswares for mammalian cell culture were purchased from Gibco, BRL, Nunclon and Millipore, Germany. The secondary antibody (goat anti mouse IgG biotin conjugated) was purchased from Genei, Bangalore, India. 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 purchased were from Sigma, USA.

**Sample collection:**

(As mentioned in chapter II, Section A)

**3.1 Production of monoclonal antibody [mAb H₁₁B₂C₂] (Boregowda etal., 2006)**

**3.1a. DMEM [Dulbecco’s modified Eagle’s media]**

It contains 3.7g sodium bicarbonate, 10mM glutamine and 110mg of sodium pyruvate. They were dissolved in 800ml of sterilized triple distilled water and pH was adjusted to 7.4 with 1N HCl. The final volume was made up to 1000ml with triple distilled water. This was filtered through 0.2µm filter.
Table 3.1
Common serum cancer markers used in primary care

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cancer type</th>
<th>Specificity</th>
<th>Example of non-cancer pathology</th>
<th>Primary clinical use</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-fetoprotein</td>
<td>Hepatocellular, non-seminomatous testicular</td>
<td>Moderate</td>
<td>Prostatitis</td>
<td>Staging</td>
</tr>
<tr>
<td>Human chronic gonadotropin-β</td>
<td>Testicular, ovarian</td>
<td>Low</td>
<td>Pregnancy</td>
<td>Staging</td>
</tr>
<tr>
<td>CA15-3</td>
<td>Breast</td>
<td>Poor</td>
<td>Cirrhosis, benign diseases of ovaries and breast</td>
<td>Disease monitoring</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Gastro, pancreatic, stomach</td>
<td>Poor</td>
<td>Gastritis</td>
<td>Disease monitoring</td>
</tr>
<tr>
<td>CA125</td>
<td>Ovarian, cervical, uterine, fallopian tube</td>
<td>Moderate</td>
<td>Pancreatitisis, kidney or liver disease</td>
<td>Disease monitoring</td>
</tr>
<tr>
<td>CA27-29</td>
<td>Breast</td>
<td>Low</td>
<td>Non-malignant disorders</td>
<td>Disease monitoring</td>
</tr>
<tr>
<td>CEA</td>
<td>Colorectal, pancreas, lung, breast, medullary thyroid</td>
<td>Low</td>
<td>Non-malignant disorders</td>
<td>Disease monitoring</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>Colon, non-small cell lung cancer</td>
<td>Low</td>
<td>Non malignant disorders, such as benign prostatic hyperplasia</td>
<td>Selection of therapy</td>
</tr>
<tr>
<td>Her2/Neu</td>
<td>Breast, ovarian</td>
<td>Moderate</td>
<td>Benign breast disease</td>
<td>Disease monitoring; selection of therapy</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate</td>
<td>High</td>
<td>Benign prostatic hyperplasia</td>
<td>Screening; disease monitoring</td>
</tr>
<tr>
<td>Thyrogblobulin</td>
<td>Thyroid</td>
<td>Poor</td>
<td>Grave’s disease thyroiditis</td>
<td>Disease monitoring</td>
</tr>
</tbody>
</table>

CA: Cancer antigen; CEA: Carcinoembryonic antigen; PSA: Prostate-specific antigen
Adapted from (Alaoui et al., 2006)
3.1b. Phosphate buffered saline [PBS]
(As mentioned in chapter II, section A)

3.1c. bovine serum

3.1d. Ammonium sulphate

The antibody was originally produced by the fusion of a myeloma variant NS1 with spleenic lymphocytes from SJL/J mice, immunized with semi-purified hyaluronic acid binding protein (Banerjee and Toole, 1991). Hybridomas producing IVd4 antibody were selected, whose interaction with antigen was competed out by hyaluronic acid and hyaluronan oligomers (Banerjee and Toole, 1991). Subsequent hybridomal clonal selections were performed by heat shock treatment, growing them in bovine serum and finally subcloned in filtered human serum of different blood groups received from the hospitals. Furthermore the hybridoma was selected in HAT and HT media in DMEM. One of the clones H11B2C2 was selected. The antibody production in human serum of any blood groups did not affect H11B2C2 antibody in recognizing the human antigen expressed in tissues derived from malignant tumours. The clone H11B2C2 were grown in DMEM containing 10% (v/v) human serum. After 14 days the media was collected. The media collected was taken and an equal volume of cold saturated ammonium sulphate solution was added with constant stirring at 4°C overnight and centrifuged at 12000rpm for 30min. The pellet was dissolved in PBS and dialyzed. After dialysis the antibody solution was lyophilized and antibody was dissolved in PBS whenever required.

3.2. Extraction of proteins from normal and cancer serum samples
(As mentioned in chapter II, section B)

3.3. Preparation of biotinylated hyaluronic acid (bHA)
(As mentioned in chapter II, section A)

3.4. Immunoprecipitation

3.4. a Protein A-CL agarose
3.4. b Protein G-CL agarose

3.5. SDS-PAGE
(As mentioned in chapter II, section A)

3.6. Western blotting
(As mentioned in chapter II, section A)

Scion Image analysis information
(As mentioned in chapter II, section B)
**H₁₁ antigen expression in normal and cancer human serum:**

After extraction of protein, equal amounts of protein (100µg) from each serum sample was 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 mAbH₁₁B₂C₂ (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 1:20000 dilution for 1hr at room temperature. After extensive washing with TTBS, the immuno-reactive proteins were visualized with ECL.

**Immunoprecipitation analysis of normal and cancer serum H₁₁antigen and reacted with mAb H₁₁B₂C₂ :** (Firestone and Wingoth, 1990)

Proteins from normal and cancer serum samples were immunoprecipitated with mAb H₁₁B₂C₂. Antigen-antibody complex was pulled down by protein-A. Beads were washed extensively and loaded in SDS sample buffer and ran on 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 mAbH₁₁B₂C₂ over night at 4°C. The blot was washed and incubated with goat anti mouse IgG-biotin conjugated (1:20,000 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.

**bHA pull down protein reacted with mAb H₁₁B₂C₂ :**

250 µg of protein from normal and cancer serum samples was mixed with 10 µg of bHA probe and incubated for 1hr at 4°C. Then 50% of saturated (NH₄)₂SO₄ was added and sample mixed thoroughly and incubated over night at 4°C. Then centrifuge
the sample and the pellet was separated. Dissolve the pellet in 1% SDS and loaded in SDS sample buffer and ran on 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 incubated with mAbH11B2C2 antibody 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.

**Results**

The expression of H11 antigen (HABP) as detected by mAbH11B2C2 was analyzed by western blotting method in different cancer serums. They are also compared with normal serum samples. 100 μg protein was resolved in 10% SDS-PAGE. The samples screened for H11 antigen (HABP) are from normal and different cancer serums. The cancer samples are from, colon GI, stomach GI, II, tongue GI and cervix GII, GIILI.The expression of 57 kDa protein was observed mainly in all samples and also over expression of H11 antigen in cancer was found when compared with normal individual and during tumor progression. Graphs showing the intensity of 57kDa protein in each lane of the blots and they were done using Scion Image Analysis Software. These results are presented in Fig.3.1.

Fig.3.2 presents the differential expression of the 57kDa H11 antigen in the serum of normal and in different cancers at different grades as quantified by western blot analysis. This was depicted in biogram, mean and pixel values were used with a standard deviation of 1044.4.

The H11 antigen expression was analyzed by western blot in some of the normal and cancer serum samples. These results are depicted in Fig.3.3 Proteins were resolved in a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAbH11B2C2. This blot shows the expression level of the H11 antigen in different cancer serums. The H11 antigen expression was analyzed in normal and different cancer samples, including buccal mucosa, ovary, thyroid, and colon. A significant
increase in the expression level of the H11 antigen can be seen in the cancer samples. In normal serum, very low expression was observed. Graphs showing the intensity of 57kDa protein in each lane of the blots and they were done using Scion Image Analysis Software.

The H11 antigen expression was analyzed by western blot in some of the normal and cancer serum samples. These results are given in Fig.3.4. Proteins were resolved in a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAb H11B2C2. This blot shows the expression level of the H11 antigen in different cancer sera. The H11 antigen expression was analyzed in normal and different grades of cancer samples, including breast GII and stomach G I, II and III. A significant increase in the expression level of the H11 antigen can be seen in the cancer samples during tumour progression When compared within grades, grade III showed more expression but in normal serum, very low expression was observed. Graphs showing the intensity of 57kDa protein in each lane of the blots. They were done using Scion Image Analysis Software.

Western blot analysis of bHA pull down protein reacted with mAb H11B2C2. Protein from normal, colon cancer and different grades of stomach cancer serum samples were mixed with 10 μg of bHA probe then pulled down with 50% of saturated (NH4)2SO4. Then proteins were resolved in a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAb H11B2C2. This blot shows the over expression of the H11 antigen in cancer serum samples when compared with normal serum samples. This also confirmed the protein which was pull down with bHA probe is a 57kDa HABP. Graphs showing the intensity of 57kDa protein in each lane of the blots and they were done using Scion Image Analysis Software. These results are presented in Fig.3.5.

Proteins from normal and different grade of breast and stomach cancer serum samples were immunoprecipitated with mAb H11B2C2. Lane 2 to 5 showed over expression of H11 antigen in stomach GI,GII and breast GI,GII cancer serum samples, whereas, in lane1 it was observed less expression of H11 antigen. It was also detected linear over expression of H11 antigen (HABP) during cancer progression. Graphs showing the intensity of 57kDa protein in each lane of the blots and they were done using Scion Image Analysis Software. These results are presented in Fig.3.6.
Western blotting analysis of serum H₁₁ antigen expression in normal, in different cancers and also in different grades of cancer using mAb H₁₁B₂C₂

Lane 1, 2, 3: normal  Lane 4: ca. stomach GI
Lane 5: ca. tongue GI  Lane 6: ca. colon GI
Lane 7: ca. stomach GII  Lane 8: ca. cervix GII
Lane 9: ca. cervix GIII  Lane 10: Mol. Wt. marker

Scion Image analysis of the 57kDa HABP (H₁₁antigen)

Expression of H₁₁antigen was detected by western blot analysis in normal and cancer serum samples. Proteins were resolved on 10% SDS PAGE and transblotted on to PVDF membrane and reacted with mAb H₁₁B₂C₂. The levels of H₁₁antigen were elevated in serum of cancer patients. All cancer serum samples showed the over expression of 57kDa protein (H₁₁antigen) during tumor progression from grade I to III. And in normal serum, very low expression was observed.
Fig 3.2
Graph shows the expression of 57kDa serum H11 antigen (HABP) in normal, in different cancers and also in different grades of cancers.

Lane 1, 2, 3: normal                      Lane 4: ca. stomach GI
Lane 5: ca. tongue GI                     Lane 6: ca. colon GI
Lane 7: ca. stomach GII                   Lane 8: ca. cervix GII
                                                Lane 9: ca. cervix GIII
Western blotting analysis of H\textsubscript{11} antigen expression in normal and in different cancer serum samples using mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2}

Lane 1: normal  
Lane 2: ca. ovary  
Lane 3: ca. buccal mucosa  
Lane 4: ca. thyroid  
Lane 5: ca. colon

Expression of H\textsubscript{11} antigen was detected by western blot analysis in normal and cancer serum samples. Proteins were resolved on 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2} the levels of H\textsubscript{11} antigen were elevated in serum of cancer patients. All of the cancer serum samples showed the over expression of 57kDa protein (H\textsubscript{11} antigen) in compare with normal serum sample.
Western blotting analysis of serum H₁₁ antigen expression in normal, in different cancers and also in different grades using mAb H₁₁B₂C₂

Lane 1: normal        Lane 2: ca. stomach GI
Lane 3: ca. breast GII   Lane 4: ca. stomach GII
Lane 5: ca. stomach GIII

Scion Image analysis of the 57kDa HABP (H₁₁ antigen)

Expression of H₁₁ antigen was detected by western blot analysis in normal and cancer serum samples. Proteins were resolved on 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with mAb H₁₁B₂C₂ the levels of H₁₁ antigen were elevated in serum of cancer patients. All cancer serum samples showed over expression of 57kDa protein (H₁₁ antigen).
Western blot analysis of bHA pull down protein and reacted with mAb H_{11}B_{2}C_{2}.

Lane 1: normal
Lane 2: ca. colon
Lane 3: ca. stomach GII
Lane 4: ca. stomach GIII

Scion Image analysis of the 57kDa HABP (H_{11}antigen)

bHA probe was added to protein from normal and different grades of cancer serum samples and the reaction mixture was incubated for 1 hr at 4°C. Saturated (NH_{4})_{2}SO_{4} was added to the sample and they were mixed and incubated overnight at 4°C followed by centrifugation. The pellet so obtained was dissolved and loaded in SDS sample buffer and ran on 10% SDS-PAGE. Then the proteins were transblotted onto PVDF membrane and reacted with mAb H_{11}B_{2}C_{2} the levels of H_{11}antigen were elevated in serum of cancer patients. All cancer serum samples showed the over expression of 57kDa protein (H_{11}antigen) in compare with normal serum sample.
Fig. 3.6

Immunoprecipitation analysis of serum H\textsubscript{11} antigen in normal, in different cancers and also in different grades of cancer using mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2}.

Lane 1: normal  
Lane 2: ca. stomach GI  
Lane 3: ca. breast GI  
Lane 4: ca. breast GII  
Lane 5: ca. stomach GII

Scion Image analysis of the 57kDa HABP (H\textsubscript{11} antigen)

Proteins from normal and different cancer serum samples was immunoprecipitated with mAb H\textsubscript{11}B\textsubscript{2}C\textsubscript{2}. Lane 2 to 5 showed over expression of H\textsubscript{11} antigen in ca. stomach GI, GII, ca. breast GI, GII cancer serum samples and in lane 1 less expression of H\textsubscript{11} antigen in normal serum. Linear over expression of H\textsubscript{11} antigen (HABP) during cancer progression was also observed.