SECTION – A:

Screening of hyaluronic acid binding proteins (HABPs) in different cancer tissue samples

Introduction

The malignant solid tumors contain elevated levels of hyaluronan (Hyaluronic acid, HA) (Knudson et al., 1989). HA levels have been shown to be elevated in lung tumor, Wilms tumor and breast carcinoma. Increased levels of hyaluronan correlate between poor differentiation in ductal carcinomas of human breast (Auvinen et al., 1997) and with poor survival rates in human colorectal adenocarcinomas (Ropponen et al., 1998). 2.5 to 6.5 folds elevated HA levels were observed in bladder cancer patients and serve as a highly sensitive and specific marker for detecting bladder cancer patients regardless of the tumor grade (Lokeshwar et al., 1998; Lokeshwar, et al., 2000). HA also forms a halo around tumor cell that protects them against immune surveillance (Hobarth et al., 1992).

Some malignant cells exhibit membrane bound activities stimulating HA synthesis in adjacent fibroblasts (Knudson et al., 1984). HA in carcinogenesis plays a critical role in dynamic structural changes within extracellular matrix during development and tissue remodelling as well as maintenance of mechanical properties and homeostasis of many tissues (Toole et al., 2002).

The elevated hyaluronan production by mouse mammary carcinoma (Kimata et al., 1983) and melanoma cells (Zhang et al., 1995) correlates with metastatic capacity. Besides, the interaction of several types of malignant tumors, cells with stromal components induces hyaluronan secretion (Knudson et al., 1984; Asplund et al., 1993). Thus, hyaluronan accumulation occurs at the interface of invasive tumors and host tissues in various tumor types (Knudson et al., 1989) Small fragments of HA (3-25 disaccharide units) are angiogenic. HA fragments induce endothelial cell proliferation and migration with lumen formation (Banerjee and Toole, 1992) Thus, a regulated degradation of HA in tumor tissues is significant for both tumor metastasis and angiogenesis. Stimulated HA synthesis by cancer cells or stromal fibroblasts may
force gaps through connective tissue, creating space for the invading cancer cells (Knudson et al., 1989). Further, HA has been proposed to regulate cell locomotion and cyto differentiation that occur during these phenomena. HA is likely to mediate these effects by sustained attachment to hyaluronan synthase across the plasma membrane (Toole, 2001; Tammi et al., 2002).

Hyaluronan receptors have been widely implicated in tumorigenesis. HABPs such as CD44 (a trans membrane glycoprotein receptor) and TSG-6 are present on cell surface (Aruffo et al., 1990). Recent evidences suggests that CD44 mediated events can enhance or inhibit tumor progression in different types of tumor. CD44 is a widely distributed cell surface glycoprotein that is encoded by a single gene but expressed as numerous isoforms as a result of alternative splicing. It is evidenced that CD44 gene, particularly the 80-90 kDa protein, plays a role in metastasis of certain human tumor cell lines. It was shown that variant isoforms of CD44 are required for metastatic behavior of rat pancreatic adenocarcinoma cells.

HA and RHAMM interactions have also been implicated in tumor cell behavior both in vitro and in vivo. RHAMM is also involved in the ras and erk signaling pathways and associated with the cytoskeleton. HA-RHAMM interactions induces transient phosphorylation of P125 FAK with respect to turnover of focal adhesions in ras-transformed cell, thus leading to initiation of locomotion (Hall et al., 1995) Suppression of this interaction inhibits cell locomotion and proliferation in vitro and leads to inhibition of tumor growth in vivo, whereas, over-expression of RHAMM leads to enhanced tumor growth and metastasis (Hall et al., 1995). It has been shown that over expression of cell surface RHAMM causes fibroblast to become tumorigenic (Hall et al., 1995). CDC37 and P-32 mediate novel intracellular hyaluronan functions. CDC37 is required in G2/M phase as well as G1 phase and exhibits characteristic properties of a hyaluronan-binding protein. P32 is a 68 kDa homodimer (34-kDa subunit) hyaluronan binding protein from rat kidney tissue. It is highly phosphorylated in transformed fibroblasts compared to normal fibroblasts.

On the role and presence of extracellular hyaluronan, evidences are growing in the cytoplasm and in nuclei of cells in a number of tissues (Margolis et al., 1976;
Ripellino et al., 1989). During mitosis, both HA and HABP molecules completely fill the cytoplasm and surround the chromosomes during their arrangement at the metaphase plate and during separation of the chromosomes in anaphase. This pattern is highly similar to the distribution of laminin during mitosis (Chaly et al., 1984).

Hyaluronan is associated with chromatin and might somehow play a role in chromosome condensation in some cells. With all these data, discovery of several IHABPs, including CDC 37 (Grammatikakis et al., 1995), RHAMM/IHABP (Entwistle et al., 1996), P32 (Deb et al., 1996) and IHABP4 (Huang et al., 2000), has raised an interesting question. Intracellular hyaluronan, like extracellular hyaluronan, plays an important role in regulating cell behavior through interactions with IHABP. In this section, experiments were carried out to find out the expression of HABPs in different cancer tissue samples.

**Materials and Methods**

**Chemicals:**

Hyaluronan (Na salt human umbilical cord) was procured from Sigma, USA. Streptavidin-horseradish peroxidase conjugated (HPO9) 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 purchased were from Sigma, USA.

**Sample collection:**

The study group consists of various malignant samples collected from patients undergone either biopsy or radical surgery at Bharath Cancer Hospital and KRH Hospital, Mysore, India. In all cases independant pathological investigation were made in local hospitals by qualified pathologists. Patients were examined by two or more pathologists for the confirmation of diagnosis and grades of malignant tumors for the collection of tissues or blood. Malignant tumors were classified into well differentiated (Grade I), moderately differentiated (Grade II) and poorly differentiated (Grade III) based on the level of differentiation. The tissue / blood samples were used
for various biochemical analysis as mentioned below. Wherever it is necessary, the tissues / blood samples were processed and this information is provided in that particular chapter/ section. All experiments were repeated six times.

2.1A) Extraction of protein from human cancer tissues
Fresh tissues from benign and malignant samples were collected from the hospitals in cold PBS buffer was stored at −20°C. Before extraction of the proteins, the samples were resuspended in lytic buffer and then homogenized in a glass-teflon homogenizer at 4°C. The lysate was centrifuged at 10,000rpm for 45min. The pellet was discarded and the resulting supernatant was used for further analysis.

2.1A.a) 4X Lytic buffer
50mM Tris buffer, 150mM sodium chloride and 0.1% sodium lauryl sulphate were dissolved in dd water and pH was adjusted to 8.0 with 1N HCl. It also contains 1mM PMSF [Phenyl methyl sulfonyl fluoride] that is dissolved in ethanol.

2.1A.b) Phosphate buffered saline (PBS)

120 mM NaCl
10 mM NaH₂PO₄
40 mM K₂HPO₄
They were dissolved in 1000 ml of dd water and pH of the buffer was adjusted to 7.3.

2.2 A) Preparation of biotinylated hyaluronic acid (bHA) (Banerjee and Toole, 1992)

2.2A.a) Phosphate buffered saline (PBS) (As mentioned earlier, 2.1A.b)

2.2A.b) MES buffer (2-N-Morpholono ethane sulfonic acid)

0.1 MES dissolved in dd water and pH was adjusted to 5.5

2.2A.c) Hyaluronic acid (Na salt from human umbilical cord)

2.2A.d) Sulfo-NH-S-LC biotin EZ-Link (Biotin LC hydrozide)

2.2A.e) EDC [1, ethyl 3-(dimethyl amino propyl) carbodimide hydrochloride]

2.2A.f) Dimethyl sulfoxide
50mg of hyaluronic acid was dissolved in 10ml of filtered PBS 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 16 hrs at 4°C. This was then dialysed against PBS for 36hrs at 4°C. Finally the dialysed bHA was stored in glycerol at -20°C.

2.3A) SDS-PAGE (Laemmli, 1970)

2.3A.a) Resolving gel buffer

1.5M Tris buffer
0.8% SDS
The above reagents were dissolved in dd water and the pH was adjusted to 8.8 with 1N HCl.

2.3A.b) Acrylamide and bis-acrylamide solution:

30% acryl amide
0.8% bis-acrylamide
Stock solution was prepared in double distilled water and then filtered through Whatman #1 filter paper.

2.3A.c) Stacking gel buffer

0.5M Tris
0.8% SDS
The above reagents were dissolved in dd water and the pH was adjusted to 6.8 with 1N HCl.

2.3A.d) Ammonium per sulfate (APS)

10% APS
Solution was freshly prepared in double distilled water.

2.3 A.e) TEMED
2.3 A.f) Tank buffer
0.014M Tris
0.19M glycine
0.25% SDS
The above reagents were dissolved in double distilled water and the pH was adjusted to 8.3.

2.3 A.g) 4X Sample buffer
0.25M Tris, pH 8.0
10% Sodium dodecyl sulfate
10% glycerol
0.1% bromophenol blue
The above reagents were dissolved in Tris buffer and are used so as to give a final concentration of 1X with the sample. They were dissolved in double distilled water and the pH was adjusted to 8.0 with 1N HCl.

2.4 A) Western blotting (Pia Nyberg et al., 2003)
Vertical wet immunoblotting [Western blotting] apparatus was purchased from Genei.

2.4A.a) Poly Vinyl Difluoridine (PVDF) [Immobilon-p] membrane was purchased from Millipore, Germany.

2.4A.b) Transfer buffer
0.192M glycine
0.025M Tris
0.01% Sodium lauryl sulphate
10% methanol
The above reagents were dissolved in double distilled water and pH was adjusted to 8.3 [if necessary] with saturated glycine.

2.4A.c) Tween-Tris buffer saline [TTBS]
50mM Tris
150mM Sodium Chloride
0.1% Tween
The above reagents were dissolved in double distilled water and pH was adjusted to 8.0 with 1N HCl.

2.4A.d) Blocking buffer
5% fat free milk powder and 1% bovine serum albumin were dissolved in TTBS.
The blocking buffer was directly used to block any non-specific reaction.

2.4 A.e) Enhanced chemiluminescence (ECL)
Super signal west pico chemiluminescence substrate was used. Working solution was prepared by mixing equal parts of the stable peroxide solution and the luminol/enhancer solution.

2.4A.f) Developer

2.4A.g) Fixative

Detection of tissue hyaluronan binding proteins using bHA probe by overlay experiment:
Equivalent amounts of protein (100µg) from benign appendicitis and malignant tumor tissues of ovary, cervix, stomach, colon, breast, lymphoma and buccal mucosa were denatured with sample buffer and boiled for 5min. The protein samples were separated under reducing conditions on 10% SDS-PAGE 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 of bHA probe for 1hr at room temperature and overnight at 4°C. The membrane was washed with TTBS. The membrane was treated with HPO-9 [1:20000, Strepta avidin peroxidase] for 1hr. Wash the membrane couple of times with TTBS buffer. The immunoreactive proteins were visualized with ECL western blotting detection system.

Detection of tissue hyaluronan binding proteins using bHA probe and cold HA by competition experiment:
To confirm these proteins which are reacting with bHA are really HABPs or not, HA competition experiment was carried out. In this experiment 100µg protein from cancer
tissues samples were denatured with sample buffer and boiled for 5min. The protein samples loaded on two identical lanes and were separated under reducing conditions on 10%SDS-PAGE at 25mA constant current 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 to 2 strips .To one strip, the membrane was incubated with cold HA (500 μg/ml) for 3hr at room temperature and then the probe bHA (1:100) was added to it and incubated over night at 4°C. To another strip (1:100), only bHA was added and incubated over night at 4°C. Routine western blot analysis was carried out in both the strips as mentioned above. After which they were detected using an ECL plus western blotting detection system.

Detection of tissue hyaluronan binding proteins using bHA probe by pull down assay:
250 μg of the protein extract from different cancer samples was mixed with 100 μg of bHA probe for 1hr at room temperature. Then saturated (NH₄)₂SO₄ was added to final concentration of 50% after which the sample were mixed thoroughly and incubated over night at 4°C. They were then centrifuged and the pellet was dissolved in 1% SDS. 1X SDS sample buffer was added to each of the samples which were then run on 10% SDS-PAGE at 25mA constant current 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. Then membrane incubated with streptavidin peroxidase (1:20,000 dilutions) for 1 hr at room temperature. The immunoreactive proteins were visualized with ECL western blotting detection system.

Results
The expression of HABPs as detected by bHA was analyzed by western blotting analysis method in different cancer tissues samples. Proteins from ovary, cervix, stomach and colon cancer tissue samples were resolved in 10% SDS-PAGE and transblotted on to PVDF membrane and, they were screened for HABPs by reacting with probe bHA. These results showed multiple proteins which range from 90 to 20 kDa HABPs. These results are presented in Fig. 2.1.
One more experiment was carried out with other cancer tissue samples in the same way as mentioned above. The cancer tissue samples used were lymphoma, breast and buccal mucosa and compared with appendicitis tissue sample. Here also multiple bands were observed like in the Fig 2.1. More expression was observed in cancer tissue samples when compared to normal appendicitis tissue sample. These results are depicted in Fig. 2.2.

Whether these proteins in cancer tissue samples studied as recognized by bHA are HABPs or not, the HA competition experiment was carried out. In this experiment 100 μg proteins from stomach cancer tissue samples were run on 10% SDS-PAGE under reducing conditions in two identical lanes. The experiment was done in duplicates. These proteins were transblotted to PVDF membrane and separated in two strips. To one strip, bHA (1:100) was added and incubated over night at 4°C. To another strip, the membrane was incubated with cold HA (500 μg/ml) for 3hr at room temperature. bHA (1:100) was added later. This was incubated over night at 4°C; Routine western blot analysis was carried out as mentioned earlier. After which they were detected using an ECL plus western blotting detection system. In this experiment , all HABPs are reacting with bHA probe (Lane 1,2), whereas, the ones which were reacted with cold HA prior to the addition of bHA, no bands were observed (Lane 3,4). This was due to the reaction that was taking place between cold HA that was not labelled and HABPs. These results are given in Fig.2.3.

10 μg of bHA probe was added to 250μg of protein from colon and stomach cancer tissue samples and the reaction mixture was incubated for 1hr at 4°C. Saturated (NH₄)₂SO₄ was added to give a final concentration of 50%. The sample were mixed thoroughly and incubated overnight at 4°C followed by centrifugation. The pellet so obtained was dissolved in 1% SDS and loaded in SDS sample buffer and ran on 10% SDS-PAGE. Then, the proteins were transblotted onto PVDF membrane, which were detected by streptavidin peroxidise and ECL kit method. The result showed HABPs which were reacting with bHA were pulled down with ammonium sulphate and they were also showing the similar trend of expression like that of Fig. 2.1 and 2.2 These results are shown in Fig 2.4.
Fig. 2.1
Western blotting analysis of HABPs expression in cancer tissue samples using probe bHA

Lane 1: ca.ovary  Lane 2: ca.cervix  
Lane 3: ca.stomach  Lane 4: ca.colon  
Lane 5: molecular weight markers

HABPs expression was detected by western blot analysis in different cancer tissue samples. Proteins were resolved on a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with probe bHA.
Fig. 2.2

Western blotting analysis of HABPs expression in appendicitis and cancer tissue samples using probe bHA

Lane 1: appendicitis    Lane 2: ca.lymphoma
Lane 3: ca. breast    Lane 4: ca. buccal mucosa

HABPs expression was detected by western blot analysis in appendicitis and in different cancer tissue samples. Proteins were resolved on a 10% SDS-PAGE followed by transblotting on PVDF membrane and reacted with probe bHA.
Western blotting analysis of HABPs expression in stomach cancer tissue samples using probe bHA and competition assay using cold HA.

Lane 1 to 4 ca. stomach
Lane 1 & 2 showed the blot with bHA
Lane 3 & 4 showed the blot competed with cold HA and bHA

HABPs expression was detected by western blot analysis in stomach cancer tissue sample (done in duplicates). The proteins were resolved on a 10% SDS-PAGE followed by transblotting on PVDF membrane and reacted with probe bHA (Lanes 1 & 2) and reacted with cold HA first and probe bHA was added later (Lanes 3 & 4). These were detected by streptavidin peroxidise and ECL kit method.
Western blotting analysis of HABPs expression in stomach and colon cancer tissue samples using probe bHA by pull down experiment.

Lane 1: ca. colon       Lane 2: ca. stomach

bHA probe was added to the protein from stomach and colon cancer tissue samples and the reaction mixture was incubated for 1hr at 4°C. Saturated (NH₄)₂SO₄ was added, the sample were mixed and incubated overnight at 4°C followed by centrifugation. The pellet so obtained was resolved on 10% SDS-PAGE and followed by transblotted onto a PVDF membrane, which were detected by streptavidin peroxidise and ECL kit method.
SECTION – B:

Screening of hyaluronic acid binding proteins (HABPs) in different cancer serum samples

Introduction

HA promotes the detachment process that permits cells to migrate. (Turley and Torrance, 1985). HA takes up a large volume of water in ECM 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 (Knudson et al., 1989; Toole, 1981).

HA is a prominent factor in serum whenever rapid tissue growth occurs particularly during embryogenesis and tumour growth and its spread. (Knudson et al., 1989; Toole, 1990; Laurent and Fraser, 1992). From the concentration of hyaluronan in blood and its turnover rate as measured by labeled tracers, it was estimated that a total amount of 10-100 mg turned over in the circulation of an adult human every day (Fraser and Laurent, 1989). There is some evidence that a minor part of the circulating hyaluronan is metabolized in the kidneys and the spleen (Laurent and Fraser, 1986, 1991). Major part was taken up by the liver. Lymph nodes have a high capacity (Fraser et al., 1988) for uptake and degradation of the polysaccharide which probably makes them a more important catabolic site than the liver endothelial cells.

Levels of HA on the surface of tumor cells often correlate with cancer aggressiveness (Zhang et al., 1995) Investigations of various human tumours such as hepatocellular carcinoma (Kojima et al., 1975), malignant mesothelimoa (Roboz et al., 1985), several types of lung cancer (Horai et al., 1981) and human pancreatic carcimona (Fries et al., 1994) have demonstrated a high concentration of HA in tumor tissues. Studies have also shown that HA levels were increased in the serum of patients with various malignant tumors such as multiple myeloma (Dahl et al., 1999), breast cancer (Delpech et al., 1990), bladder cancer (Hautmann et al., 2001) and malignant epithelial ovarian tumours (Hiltunen et al., 2002).
Analysis of human cancer serum has revealed the presence of high amounts of hyaluronan (HA) when compared to human normal serum. It has also been shown that HA is significantly elevated in the serum of patients with metastatic disease compared to the serum of patients without metastatic disease. (Delpech et al., 1990).

HA deposition is up regulated in most malignancies. While high molecular mass HA is found in most normal biological processes, much lower weight material is readily detected in cancers (West et al., 1985; Lokeshwar et al., 1997), where it facilitates tumor cell motility and invasion. Most of the effects of HA are mainly caused by the interaction with its HABPs. Intercellular HA not only plays a vital role during cancer progression but also has diverse and interesting biological functions both in vitro and in vivo such as induction of angiogenesis (Slevin et al., 2002), regulation of macrophage inflammatory responses (Noble et al., 1996) and suppression of tumour growth possibly by disrupting endogenous CD44 HA-receptor binding to its ligand (Toole, 2004). In addition to these cellular activities, HA fragments of defined length are widely used to characterize protein–HA interactions.

Hyaluronan binding proteins have been grouped together as a family termed hyaladherins further subdivided in matrix and cell-surface HABPs. Specific hyaluronan and HABPs interactions affect cell behaviour both clearance and turnover of hyaluronan involving hyaluronan receptor-mediated endocytosis. Pericellular matrix assembly and retention on many cells especially chondrocytes were mediated by hyaluronan receptors in coordination with other matrix HABPs. Hyaluronan can also have an independent and direct effect on cell to cell adhesion as well as migration again mediated by specific cell-surface hyaluronan receptors. This is especially apparent in tumor cells where metastatic potential is correlated with hyaluronan receptor expression. As migrating cells encounter new environments enriched in matrix hyaladherins, the capacity for matrix assembly may terminate cell migration (Knudson and Knudson, 1993).

Functional complexity is thought to arise from the interaction of HA with a large number of specific HABPs (Day and Prestwich, 2002), which can form structurally diverse complexes (Day and Sheehan, 2001). The majority of these HABPs belong to a super family of proteins that share a common -100 amino acid domain termed Link
module that mediates the interaction with HA which have been proposed to be related to C-type lectin modules owing to their similar structural topology and function (Blundell et al., 2003).

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 (Toole, 1991; Entwistle et al., 1996). Most of the malignant solid tumors contain elevated levels of hyaluronan products (Knudson et al., 1989) which are correlated with poor differentiation of cells (Auvinen et al., 1997). Enrichment of hyaluronan in tumors is due to the increased production of hyaluronidase enzymes by tumour cells themselves. So malignant cells secrete or present membrane bound activities stimulating HA synthesis (Knudson et al., 1984). Recent evidences suggest that CD44 mediated events can enhance (Sy et al., 1991) or inhibit (Takahashi et al., 1995) tumor progression depending on the size of the oligosaccharides.

Most cell adhesion molecules exist in two forms: a membrane interested form, detectable on the cell surface and a soluble form detectable in the serum and in biological fluids. The soluble forms of several adhesion molecules including ICAM-1 (sICAM-1), E-cadherin (sE-cadherin) and CD44 proteins (sCD44), have emerged as tumour markers (Seth et al., 1991; Tarin and Matsumura, 1994). The adverse prognostic value of elevated circulating concentration of sICAM-1, sE-cadherin and sCD44 has been underlined in several types of neoplasia including adenocarcinomas of various origins, tumours of the central nervous system and haematological malignancies (Banks et al., 1993; Hyodo et al., 1993; Guo et al., 1994; Ristamaki et al., 1994). In the same way, elevated concentrations of soluble adhesion molecules in biological fluids in contact with neoplastic cells have proven to be of diagnostic and prognostic value in certain cancers such as those of the bladder (Jackson et al., 1993) and stomach (Matsumura et al., 1992). Thus, these HABPs 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.
Materials and Methods

Chemicals:
Hyaluronan (Na salt human umbilical cord) was procured from Sigma, USA. Streptavidin-horseradish peroxidase conjugated (HPO9) 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 purchased were from Sigma, USA.

Sample collection:
This is done as mentioned in the chapter II, section A.

2.1B) Extraction of protein from normal and cancer human blood serum:
Blood samples were collected from normal and cancer patients from local accessible cancer hospitals. Blood samples were collected in tubes with or without anticoagulant (EDTA) for the separation of serum. Sample was taken from each patient prior to any treatment like radiation or chemotherapy. After keeping them at room temperature for one hour, these were centrifuged at 2000rpm for 30 min. The serum so obtained was stored at -80°C in liquid nitrogen for further studies. The tumor sections from patients were graded using TNM grading system. Both blood and serum samples were homogenized in 4x lytic buffer containing 50mMTris (Ph 8.0), 80mM EDTA, 1mM PMSF, 4mM Benzamidine-HCl and 2% Triton X100 plus protease cocktails. The homogenate was centrifuged at 10,000rpm for 30 min at 4°C after which the supernatant was stored at either at -80 or at -20°C until further analysis was carried out. The amount of protein was estimated using Bradford method using Bovine serum albumin as standard (Bradford, 1976).

2.1B.a) 4X Lytic buffer
50mM Tris
80mM EDTA,
1mM PMSF, (Phenyl methyl sulfonyl fluoride)
4mM Benzamidine-HCl
2% Triton X100
The above reagents were dissolved in double distilled water and pH was adjusted to 8.0 with 1N HCl.

2.2B) Preparation of biotinylated hyaluronic acid (bHA) (As mentioned in section A)

2.3B) SDS-PAGE (As mentioned in section A, 2.3A)

2.4B) Western blotting (As mentioned in section A 2.4A)

2.5B) Preparation of hyaluronan oligosaccharides:

2.5B.a) 0.05M sodium acetate buffer containing 0.15M sodium chloride (pH 5.0)

2.5B.b) Bovine testicular hyaluronidase enzyme type Is

100mg of hyaluronic acid was dissolved in 50ml of 0.05M sodium acetate buffer containing 0.15M sodium chloride. 1000 units of bovine testicular hyaluronidase enzyme type Is, dissolved in the same buffer was incubated for 16hrs at 37°C. The reaction was terminated by boiling for 15 min. It was then centrifuged at 10,000rpm for 20 min and the supernatant was passed through 0.45 micron filter. It was lyophilized and redissolved in minimum amount of water.

2.6B) HA oligosaccharides assay:

2.6B.a) Carbazole
0.125% in 95% ethanol

2.6B.b) N-acetylglucosamine
1mg per ml was prepared in distilled water.

2.6B.c) Glucuronic acid
1mg per ml was prepared in distilled water.

The carbazole assay was used to determine the glucuronic acid content of macromolecular HA derived oligosaccharides using D-glucuronic acid as the standard (Bitter and Muir., 1962). The reducing N-acetylglucosamine was checked using N-acetylglucose as the standard. This information was used along with its glucuronic acid content to determine the average degree polymerization of the HA oligosaccharide preparation. The value obtained by the glucuronic acid (U) will be
divided by value of N-acetylglucosamine (V), the value obtained will give approximate size of HA-oligosaccharides.

2.7B) Biotinylation of HA-oligosaccharides:

2.7B.a) MES buffer (2-N-Morpholono ethane sulfonic acid)

2.7B.b) Hyaluronic acid (Na salt from human umbilical cord)

2.7B.c) Sulfo-NH-S-LC biotin EZ-Link

2.7 B.d) EDC [1, ethyl 3-(3-dimethyl amino propyl carbodiimide hydrochloride)

500µg of the HA oligosaccharide digest mixture was taken in 0.1M MES buffer (pH 5.5). To this, 10mM of freshly prepared biotin L.C. hydrazide and 100mM of EDC were added to get a final concentration of 1mM and 10mM respectively. The reaction mixture was incubated at room temperature overnight with continuous stirring followed by dialysis for 24hr against PBS. The reaction mixture was then centrifuged and stored in glycerol at -20°C, until further use.

Scion Image analysis information:

Scion image is a program for processing and analysis of images. It allows for better visualization and provides tools for the quantitative analysis of the picture. The program does that by counting the dark pixels that are appearing in a designated area of a picture taken from the blot.

Detection of serum hyaluronan binding proteins using bHA probe by overlay experiment:

100µg protein extract from normal and cancer serum samples 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 incubated 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 serum hyaluronan binding proteins using bHA probe and cold HA by 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 normal and cancer serum samples were run on 10% SDS-PAGE 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 membranes was incubated with cold HA (500 μg/ml) for 3hr at room temperature and then the probe bHA (1:100) was mixed with cold or unlabelled HA (500 μg/ml) and then spreaded on the membrane and incubated over night at 4°C, to other strip (1:100) bHA was added and over night at 4°C. Routin western blot analysis was carried out as mentioned above. After which they were detected using an ECL plus western blotting detection system.

Detection of tissue hyaluronan binding proteins using bHA probe by pull down assay:

250 μg of the protein extract was mixed with 100 μg of bHA probe for 1hr at room temperature. Then saturated (NH₄)₂SO₄ was added to final concentration of 50% after which the sample were mixed thoroughly and incubated over night at 4°C. They were then centrifuged and the pellet was dissolved in 1% SDS. 1x SDS sample buffer was added to each of the samples which were then run on 10% SDS-PAGE 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 streptavidin peroxidase (1:20,000 dilutions) for 1 hr at room temperature. Protein complexes were detected using an ECL kit.

Detection of serum hyaluronan binding protein using bHA-oligo probe by Western blot:

Serum samples were ran 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 bHA-oligo probe (1:1000 dilution) overnight at 4°C. The blot was then 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.

**Determination of the expression of HABPs using bHA by ELISA:**
Serum extracts of normal and different grades of stomach were taken individually and mixed with coupling buffer. One microgram of protein was put in the each well and in control wells only coupling buffer was added. The proteins were kept at 4°C overnight for binding onto the wells of the 96 well plates. Unbound protein was washed off with PBS-T. bHA was added and incubated for 3hrs at room temperature. This was followed by incubation with streptavidin peroxidase (1: 20000) for 1hr each at room temperature. In between each step the plates were washed with PBS-T. The reaction was developed using ABTS and the absorbance was measured at 410nm.

**Results**
Normal and cancer blood samples from stomach and colon cancer patients were screened for HABPs, HABPs expression was done by western blot analysis in normal and cancer blood samples. Proteins were resolved on a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with probe bHA. The levels of HA binding proteins were elevated and over expressed in the blood of cancer patients compared with that of normal individuals. All of the cancer blood samples showed the over expression of HABPs including 57kDa. Compared to the normal serum, the expression of HABPs in cancer blood was higher. Image analysis of the 57kDa protein in normal and stomach and colon cancer blood samples are also given. These results are presented in Fig. 2.5.

Normal and cancer serum samples from tongue, ovary and cervix were screened for HABPs, HABPs expression was done by western blot analysis in normal and cancer serum samples. Proteins were resolved on a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with probe bHA. The levels of HA binding proteins
were elevated and over expressed in the serum of cancer patients compared with that of normal individuals. All of the cancer serum samples showed the over expression of HABPs including 57kDa. Compared to the normal serum, the expression of HABPs in cancer serum was higher. Image analysis of the 57kDa protein in normal and cancer serum samples were also given. These results were presented in Fig. 2.6.

This experiment was done in order to find out the serum HABPs levels in different cancers of various grades and compared with normal serum. 100μg proteins were resolved on a 10% SDS-PAGE followed by transblotting and reacted with probe bHA. The blot showed the expression level of the HABPs in the serum of normal and different cancers of various grades. In all cancer serums, the expression was more when compared to normal serum. When compared within grades, grade III showed more expression. A significant increase in the expression level of the 57kDa HABP can be seen in cancer samples as the tumour progresses from grade I to grade III. In normal serum, very low expression was observed. The result showed the expression of 57kDa HABP in normal (Lane1,2), stomach GI,II,III (Lane3,7,10), breast GII,III (Lane 6,9), colon GII,III (Lane8,11)and cervix GII,III (Lane5,12) cancer serum samples and increase in the level of 57kDa protein during tumour progression. Image analysis of the 57kDa molecular weight protein in normal and cancer serum samples were also given. These results are presented in Fig.2.7.
**Fig. 2.5**

Western blotting analysis of HABPs expression in normal and cancer blood samples using probe bHA

Lane 1: normal          Lane 2: Ca. stomach
Lane 3: Ca. colon       Lane 4: Mol. Wt. Markers

**Scion Image analysis of the 57kDa HABP**

HABPs expression was detected by western blot analysis in normal and cancer blood samples. Proteins were resolved on 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with probe bHA. The levels of HA binding proteins were elevated in blood of both stomach and colon cancer patients. All cancer blood samples showed the over expression of a HABPs including 57kDa.
Western blotting analysis of HABPs expression in normal and cancer serum samples using probe bHA

Lane 1: normal           Lane 2: ca.tongue
Lane 3: ca. ovary       Lane4: ca.cervix

Scion Image analysis of the 57kDa HABP

HABPs expression was detected by western blot analysis in normal and cancer serum samples. Proteins were resolved on a 10% SDS-PAGE and transblotted on to PVDF membrane and reacted with probe bHA. The levels of HA binding proteins were elevated in the serum of tongue, ovary and cervix cancer patients. All cancer serum samples showed the over expression of a HABPs including 57kDa.
Western blotting analysis of HABPs expression in normal and different cancer serums and also in different grades samples using probe bHA

Lane 1, 2: normal    Lane 4: ca. tongue, GII
Lane 3, 7 & 10: ca. stomach, GI, GII & G III
Lane 6 & 9: ca. breast, GII & G III
Lane 8 & 11: ca. colon, GII & G III
Lane 5 & 12: ca. cervix, GII & GIII

Scion Image analysis of 57kDa band (HABP)

Proteins were resolved on a 10% SDS-PAGE, followed by transblotting and reacted with probe bHA. The blot showed the expression level of the HABPs in the serum of normal and different cancers. A significant increase in the expression level of the 57kDa HABP can be seen in cancer samples as the tumor progresses from grade I to grade III. In normal serum, very low expression was observed.
The results of Fig. 2.7 are expressed in bar diagram a depicted in Fig.2.8 Mean and pixel values were used with a standard deviation of 1044.4.

In order to confirm the proteins which are reacting with HA are really HABPs or not, HA competition experiment was carried out. In this experiment 100 μg protein from normal and different grades of stomach cancer serum samples were run on 10% SDS-PAGE under reducing conditions in two identical lanes from each sample. These proteins were transblotted to PVDF membrane and separated in two strips. To one strip, bHA (1:100) was added and incubated over night at 4°C, To another strip, the membrane was incubated with cold HA (500 μg/ml) for 3hr at room temperature. Then the probe bHA (1:100, that was previously mixed with cold or unlabelled HA (500 μg/ml) was added and then spread it on the membrane. This was incubated over night at 4°C, Routine western blot analysis was carried out as mentioned earlier. After which they were detected using an ECL plus western blotting detection system. In these serum samples, all HABPs are reacting with bHA probe (Lane 1,2 and 3), whereas, the ones which were reacted with cold HA prior to the addition of bHA, no bands were observed. This was due to the reaction that was taking place between cold HA that was not labeled and HABPs. These results are shown in Fig.2.9 Image analyses was also carried out.

10 μg of bHA probe was added to 250μg of protein from normal and different grades of stomach cancer serum samples and the reaction mixture was incubated for 1hr at 4°C. Saturated (NH₄)₂SO₄ was added to give a final concentration of 50%. The sample were mixed thoroughly and incubated overnight at 4°C followed by centrifugation. The pellet so obtained was dissolved in 1% SDS and loaded in SDS sample buffer and ran on 10% SDS-PAGE. Then proteins were transblotted onto PVDF membrane which were detected by streptavidin peroxidise and ECL kit method. , Lane 1 shows normal serum, Lane, 2,3 show stomach,GII,GIII cancer serum. These results are presented in Fig.2.10 Image analysis was also carried out .The result showed HABPs pulled down with probe bHA in normal and cancer serum samples, thus corresponding to the overlay experiments.
Fig. 2.8
The expression of 57kDa HABP in normal serum and also in different cancers of different grades

Lane 1, 2: normal  
Lane 4: ca. tongue GII  
Lane 3, 7 and 10: ca. stomach, GI, G II and G III  
Lane 8 and 11: ca. colon GII and G III  
Lane 5 and 12: ca. cervix GII and G III  
Lane 6 and 9: ca. breasts GII and G III

The data presents the differential expression of the 57kDa HABP in the serum of normal and in different cancers and in different grades as quantified by western blot analysis. Mean and pixel values were used with a standard deviation.
**Fig. 2.9**

Western blotting analysis of HABPs expression in the serum of normal and in different grades of stomach cancer samples using probe bHA and competition assay using cold HA.

Lane 1, 2, 3 showed the blot with bHA
Lane 4, 5, 6 showed the blot competed with cold HA and bHA
Lane 1, 4, normal Lane 2, 5, ca stomach, GII
Lane 3, 6, ca- stomach, GIII

**Scion Image analysis of the 57kDa HABP**

Proteins were resolved on a 10% SDS-PAGE, followed by transblotting and reacted with probe bHA and also with cold HA. The blot showed the expression of the HABPs in the serum of normal and in different grades of stomach cancers with bHA, significant increase in the expression level of the 57kDa HABP can be seen in cancer samples as the tumour progresses from grade II to grade III. In normal serum, very low expression was observed. No bands were found when competed with cold HA.
Western blotting analysis of pull down HABPs using probe bHA in normal and different grades of stomach cancer serum

Lane1, normal
Lane2, ca. stomach, GII
Lane3, ca. stomach, GIII

Scion Image analysis of the 57kDa HABP

bHA probe was added to protein from normal and different grades of stomach cancer serum samples and the reaction mixture was incubated for 1hr at 4°C. Saturated (NH₄)₂SO₄ was added the sample 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 proteins were transblotted onto PVDF membrane, which were detected by streptavidin peroxidise and ECL kit method.
Proteins from normal and stomach cancer samples were resolved on a 10% SDS-PAGE followed by transblotted on to PVDF membrane and reacted with probe bHA-oligo. The HA-oligo binding protein expression was analyzed in normal and different grades of stomach cancer serum samples. Increased level of expression of the 57kDa HA-oligo binding protein was seen in cancer serum, however, the expression was in more grade III stomach cancer serum samples than in normal serum. Very low expression was found in normal serum. Image analysis of the 57kDa molecular weight protein in normal and cancer serum samples. These results are presented in Fig.2.11. Image analysis is also carried out.

**Expression of HABPs using bHA by ELISA:**
Serum extracts from normal and different grades of stomach cancer were mixed with coupling buffer. One microgram of the protein was plated in each well, with only coupling buffer in control wells. The plate was incubated overnight at 4°C followed by washing with PBS-T. bHA was added and incubated for 3hrs at room temperature followed by incubation with streptavidin peroxidase (1: 5000) for 1hr each at room temperature. In between each step the plates were washed with PBS-T. The reaction was developed using ABTS and the absorbance was measured at 410nm. The result showed over expression of HABPs in cancer serum during tumor progression compare with normal serum. These results are depicted in Fig.2.12.

**Discussion**
In section A, the expression of HABPs in the tissues of benign (appendicitis) and in cancers of ovary, cervix, stomach, colon, lymphoma, breast and buccal mucosa were studied by overlay experiment using bHA (Fig.2.1, 2.2). The competition experiment using bHA and cold HA in normal and in different grades of stomach cancer (Fig.2.3), pull down experiment using bHA and ammonium sulphate in normal, colon and stomach cancers (Fig.2.4) were also studied. In all these tissues, multiple bands of proteins were observed which range from 90 to 20 kDa HABPs. The over expression of these proteins in cancers was found when compared with benign appendicitis. Similar trend was also observed in tissues by earlier workers. Enough evidences has been documented the significance of elevated levels of HA and its receptors expression during human tumour progression, tumour migration and proliferation. (Zhang, *et al.*, 1995; Knudson, *et al.*, 1989).
Fig. 2.11
Western blotting analysis of HA-oligoBP expression in normal and cancer serum samples using probe bHA-oligo

Lane 1, normal  Lane 2, ca. stomach, GII
Lane 3, ca. stomach, GIII

Scion Image analysis of the 57kDa HABP

Proteins were resolved on a 10% SDS-PAGE, followed by transblotted to PVDF membrane and reacted with probe bHA-oligo. The HA-oligo binding protein expression was analyzed in normal and different grades of stomach cancer serum samples. Increased level of expression of the 57kDa HA-oligo binding protein was seen in cancer serum, however, more expression 57kDa HABP was observed in grade III stomach cancer serum samples than in normal serum, very low expression was found in normal serum.
Fig. 2.12

Expression of HABPs using bHA by ELISA

Lane 1, normal  Lane 2, ca. stomach, GI
Lane 3, ca. stomach, GII  Lane 4, ca. stomach, G III

Extracts of normal and different grades of stomach cancer serum were mixed with coupling buffer. 1µg of the protein was plated in each well, with only coupling buffer in control wells, they were incubated overnight at 4°C followed by washing with PBS-T. bHA was added and incubated for 3hrs at R.T followed by incubation with streptavidin peroxidase (1:20000) for 1hr each at room temperature. Then reaction was developed using ABTS and the absorbance was measured at 410nm. The result showed over expression of HABPs in cancer serum during tumour progression as compare with normal serum.
In section B, the expression of HABPs in the blood (Fig.2.5) and in the serum of normal and in different cancers of different grades were studied by overlay experiment using bHA (Fig.2.6 to 2.8). The competition experiment using bHA and cold HA (Fig.2.9), pull down experiment using bHA and ammonium sulphate (Fig 2.10), overlay experiment using bHAoligo (Fig2.11) and ELISA experiments using bHA (Fig.2.12) were also studied. In all these experiments, studies were consistent and showed the over-expression and distribution of HABPs in serum. HABPs were increasing in the serum of different cancers and also increasing with the grades when compared to normal serum. Previous studies showed the importance of hyaluronan expression during tumour progression. It explains the positive association of stromal hyaluronan expression with invasive nature of tumors irrespective of their origin. Their studies explains the expression of hyaluronan in tumour cells and in intratumoral stromal areas in different types of tumour tissues with different origin and reveals the possible role of hyaluronan involvement with progressive malignant behavior. Their results showed the down-regulation of hyaluronan expression in tumor epithelial cells during later stages of all types of malignancies irrespective of their origin. The highly differentiated tumors expressed increased amount of hyaluronan in both tumor epithelia and the intratumoral areas (Boregowda et al., 2006).

In the present investigation, western blot analysis of HABPs in the serum as detected by b-HA probe recognizes two proteins. One is a major band with 57kDa and the other one is a minor one with 30kDa. The HABPs expression of 57 and 30kDa was seen in almost all types of cancer as well as in normal serum but these proteins were over expressed in cancer serum.

These HABPs 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. Assessment of circulating tumor cells as a surrogate marker of cancer status is promising because, unlike tumor tissue, blood can be sampled repeatedly with limited invasive procedures (Pantel et al., 1999; Hoon et al., 2000; Cristofanilli et al., 2004; Voit et al., 2005).
A malignant tumor is continuously shedding tumor cells (Liotta et al., 1976). This shedding is related to tumor angiogenesis (Culloch et al., 1995; Fox et al., 1997), vascular invasion (Choy and Culloch, 1996), tumor size and proliferation rate (Fox et al., 1997). Isolated tumor cells found in lymph nodes, blood or bone marrow are the result of this continuous shedding from the primary tumor and most of them will never evolve to macroscopic metastases. This is suggested by finding that tumor cells are detected in the bone marrow of patients with gastrointestinal malignancies (Jauch et al., 1996; Lindemann et al., 1992). Although recent studies have shown the importance of circulating tumor cells in blood (Pantel et al., 1999, Hoon et al., 2000, Cristofanilli et al., 2004), the clinical utility of molecular detection has had controversy because there is no consistent correlation with disease outcome. Development of sensitive and specific marker assay is needed to evaluate circulating tumor cells as a surrogate marker of disease progression. The investigation demonstrated the existence of tissue multiple HA binding proteins with multiple isomers as detected by bHA. They were over expression in tissues of cancers. The tissue HA may be secreted to the circulatory system and binding to HABPs present in the serum. They may be used as a serum biomarker.

Molecular early detection tools can be divided into nucleic acid–based and protein-based markers. Hu et al., (2002) reviewed many of the nucleic acid–based markers which include loss of heterozygosity, microsatellite instability, p53 mutations, abnormal promoter hypermethylation and mitochondrial DNA mutations. Telomerase activity has also been studied using a PCR-based assay (Califano et al., 1996) More recently such feasibility studies show promise but none of these markers have been validated in large trials. Furthermore, most of these methods do not fulfill the ideal characteristics of a tumor marker because they are relatively expensive to carry out, require considerable expertise and are not widely available. Unlike nucleic acid–based techniques, protein-based early detection tools detect posttranscriptional and posttranslational changes that may take place as a result of tumorigenesis (Franzmann et al., 2007). 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 (Wight and Merrilees, 2004).
HA and its receptors (hyaluronan binding proteins) are involved in matrix regulation, cell proliferation, migration and malignant tumor 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.

CD44 represent a family of transmembrane glycoproteins. They are found at the cell surface in many tissues (Higashikawa et al.,1996). CD44 is also present as a soluble form in extracellular fluids (sCD44). sCD44 has been measured and partially characterized in human plasma (Lucas et al.,1989). CD44 acts as a principal receptor for hyaluronate and is involved in cell-cell or cell-extracellular matrix interactions (Miyake et al.,1990). In a first report, sCD44 has been proposed to be a valuable indicator of tumour growth in gastric and colorectal cancer (Guo et al.,1994). High serum levels of soluble CD44 compared to healthy donors were found in patients with ovarian cancer (Zeimet et al.,1997). Increased levels of soluble CD44 were detected in the serum of some patients with primary and metastatic malignant melanoma (Schaid er et al.,1997). Migration of a highly metastatic melanoma cell line on immobilized hyaluronic acid was associated with increased CD44 turnover and shedding of the molecule (Goebeler et al.,1996). Previous studies demonstrated that sCD44 standard levels were significantly higher in colorectal cancer and chronic inflammatory bowel disease than in normal individuals (Weg et al.,1998). Furthermore, a number of clinical studies have provided evidence that up-regulation of certain CD44 isoforms in tumour cells is related to adverse clinical outcome (Mayer et al, 1993; Gunthert et al.,1991; Stauder et al.,1995). However, the results concerning the prognostic value of the immune histochemically assessed cellular expression of CD44 variant isoforms have been controversial in breast, colorectal and ovarian cancer (Mulder et al.,1994; Cannistra et al.,1995; Friedrichs et al.,1995; Kaufmann et al.,1995; Koretz et al.,1995; Uhl-Steidl et al.,1995).

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, there can be a significant effect on clinical outcomes. Further studies are necessary to
characterize the distribution and behaviour of the soluble adhesion molecules in the circulation and to determine whether soluble molecules in the circulation can provide a marker for cancer.

The growing evidence of the presence of intracellular hyaluronan and its interaction with intracellular hyaladherins such as CDC37, IHABP4 and further the subsequent loss of hyaluronan interaction with its receptor during late malignancy lead to study the distribution of new HABP in multiple cancer serum of different grades. In all the different experiments carried out in cancer serum either by bHA or cold HA or with pull down or bHA oligo or with ELISA indicate the over expression of protein of 57kDa, which corresponds to the molecular size of the proteins recognized by the mAb H11B2C2 (CHAPTER- 3). Even though bHA was recognizing other HABPs and the ELISA assay showed over expression of HABPs including 57kDa peptide during tumour progression, the investigation was focused on specific 57kDa peptide.