

Bioactive hyaluronan fragment (hexasaccharide) detects specific hexa-binding proteins in human breast and stomach cancer: Possible role in tumorogenesis

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Hyaluronan (HA) is a component of extracellular matrix that influences cell-proliferation, migration, development, regeneration, normal tissue remodeling, tissues undergoing malignancy and tumor cell interaction. The widespread occurrence of HA binding proteins, their involvement in tissue organization and the control of cellular behavior are well documented. The low molecular mass HA fragments can also induce a variety of biological events, including chemokine gene expression, transcription factor expression and angiogenesis. It is believed that these fragments are more potent in cellular activities than high molecular mass HA. In this study, we isolated the various fragments by gel permeation chromatography of hyaluronidase digested HA and characterized by fluoro assisted carbohydrate electrophoresis (FACE) and matrix assisted laser desorption ionization analysis (MALDI). Detection and distribution of cellular receptors in invasive tumor tissues for HA polymer and HA fragments were determined both by Western blot and histochemistry. The study demonstrated the overexpression of HA-hexa binding protein in human tumors of breast and stomach and its involvement in tumorogenesis.

Keywords: Hyaluronan, CD44, Breast cancer, Stomach cancer, Fibroadenoma, Tumorogenesis

Hyaluronan (hyaluronic acid, HA), a a straight chain, non-sulphated glycosaminoglycan is a component of the extracellular matrix and is composed of repeating units of the disaccharide β (1-3)-D-glucuronic acid and β (1-4)-N-acetyl-D-glucosamine. It is known to have various diverse functions. It promotes cell motility, regulates cell-cell interaction and cell-matrix adhesion, proliferation and suppresses differentiation of cell. It not only participates in vertebrates fundamental processes such as embryological development and morphogenesis^{1,2}, but is also involved in wound healing^{3,4}, wound repair, regeneration and inflammation of tissues⁵⁻⁷. HA levels

increase in response to severe stress and in tumor progression and invasion^{8,9}.

At the cellular level, HA is degraded progressively by the hyaluronidase enzyme family that generates polymers of decreasing sizes. Despite their exceedingly simple primary structure, HA fragments have extraordinarily wide-ranging and often opposing biological functions. Biologically active low molecular size HA fragments appear to function as endogenous "danger signals", while even smaller fragments can ameliorate these effects^{10,11}. Hexasaccharides, for example, inhibit endothelial cells proliferation¹², while tetrasaccharides are anti-apoptotic and inducers of heat-shock proteins¹³. It is assumed that the small HA fragments are generated by hyaluronidase. However, it is not known, nor it is established whether the enzymes of HA synthesis and degradation are involved in maintaining proper polymer sizes and concentration. Maintenance of small oligosaccharides, such as hexa and tetrasaccharides *in vivo* is still open to questions.

HA fragments can bind to HA-binding proteins or hyaladherins^{12,14,15}. Some HA binding proteins

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Abbreviations: 2AA, 2-anthranilic acid; bHA, biotinylated hyaluronic acid; ECM, extracellular matrix; FACE, fluoro assisted carbohydrate electrophoresis; GAG, glycosaminoglycan; HABP, hyaluronic acid binding proteins; HOBP, hyaluronic acid oligo binding protein; HEXABP, hexa binding protein; HPO9, streptavidin peroxidase conjugate; MALDI, matrix assisted laser desorption ionization.

interact with HA within the extracellular matrix are referred as matrix hyaladherins, whereas others interact with the plasma membrane of the cells are known as cell surface matrix receptors. This binding has an array of functions from intracellular effects, such as regulators of the cell cycle, or as splicing factors¹⁶ to endothelial cell migration^{12,17}. Extracellular effects are provided by binding to cell surface receptors, such as RHAMM and CD44 or to extracellular proteoglycans, such as aggrecan and versican. Variations occur in the minimum size of HA oligosaccharides that bind to HA-binding proteins. The HA chain takes on various secondary and tertiary structures that are in part dependent on polymer size. Specific lengths of HA fragments also stabilize or organize arrays of hyaladherins by supporting or inhibiting various combinations of such proteins¹⁰.

In this study, we have isolated and characterized both hyaluronan oligosaccharides and hexasaccharide after hyaluronidase digestion by gel permeation chromatography and characterized by FACE and MALDI analysis. Here we provide the evidence for the presence of specific receptor for HA-hexasaccharide, namely hexa binding protein (HEXABP) which differs from CD44 in advanced human tumors of breast and stomach.

Materials and Methods

Hyaluronic acid (Across Organics), Sephadex G-50, bovine testicular hyaluronidase (type-IS), N-acetyl-D-glucosamine, D-glucuronic acid, EDC [1,ethyl 3-(3-dimethyl amino propyl) carbodimide hydrochloride), MES buffer (2-N-morpholinoethane sulfonic acid) were procured from Sigma chemicals Co, St. Louis, MO, USA. Anti-human CD44 (anti-human cell adhesion molecule, H-CAM) antibody was purchased from Fisher Scientific, Pittsburgh, PA, USA. Biotin LC hydrazide (EZ-Link), PBS-A (calcium and magnesium free) and DMSO (dimethyl sulfoxide) were purchased from Himedia, Mumbai. PVDF [immobilon-p] was obtained from Millipore, Massachusetts, USA and biotinylated goat anti-mouse IgGs was purchased from Vector, Burlingame, CA, USA. Protein A agarose and HPO-9 (streptavidin peroxidase) were obtained from Zymed, San Francisco, CA, USA. Protease inhibitors kit was purchased from InVitrogen and Sigma Chemicals Co., St. Louis, MO, USA and ECL kit from Amersham Bioscience, USA.

Tissue homogenization

Fresh samples of malignant and benign tissues of breast, stomach and appendicitis were collected from Bharath Cancer Hospital, Mysore in cold PBS and stored at -20°C . Before extraction of proteins, the samples were resuspended in lysis buffer and then homogenized (1:4, w/v) using a glass-teflon homogenizer at 4°C . The lysate was centrifuged at 10,000 rpm for 45 min at 4°C . An aliquot of the supernatant was assayed for protein at 280 nm in a UV-Shimadzu spectrophotometer.

Preparation of hyaluronan oligosaccharides

100 mg of hyaluronic acid was dissolved in 50 ml of 0.05 M sodium acetate buffer (pH 5.0) containing 0.15 M NaCl. The dissolved HA was incubated with 1000 units of bovine testicular enzyme hyaluronidase (type-IS, dissolved in same buffer) for 24 h at 37°C . The reaction was terminated by boiling for 15 min and centrifuged at 10,000 rpm for 20 min. The supernatant was taken and passed through 0.45 micron filters. It was lyophilized and then redissolved in minimum amount of triple-distilled water and stored at -20°C until further use.

HA oligosaccharides assay

The carbazole assay was used to determine the glucuronic acid content of HA-derived oligosaccharide fragments using D-glucuronic acid as standard¹⁹. The reducing end N-acetylglucosamine was assayed using N-acetyl glucosamine as standard²⁰. The value obtained by the glucuronic acid (U) divided with value of N-acetylglucosamine (A) gave the approximate size of the HA oligosaccharide.

Gel permeation chromatography

It was essentially according to the modified procedure of Banerjee and Toole²¹. In brief, G-50 (DNA grade) was swollen in double-distilled water for 12 h at 4°C . The gel was then packed into a column of bed volume 85×1.75 cm and equilibrated with 0.2 M ammonium acetate buffer, pH 5.0. The flow rate was adjusted to 10 ml/h. 50 mg of digested HA sample was loaded to sephadex G-50 column and the fractions were eluted in the same buffer. 1 ml fractions were collected each time and screened for glucuronic acid¹⁸ and N-acetyl glucosamine¹⁹, after which the peak fractions were pooled as fractions I and II. They were lyophilized and stored at -20°C until further use. The U/A ratio were calculated and further analysis was done by FACE to confirm the sizes of the oligosaccharides.

Characterization of HA-oligosaccharides and HA-hexasaccharides by FACE and MALDI

After purification, characterization of HA oligosaccharides was essential to ensure purity and homogeneity prior to experimental study. So, fragmentation was checked by fluoroassisted carbohydrate electrophoresis (FACE) essentially according to Sayfried *et al*²². Both the fractions I and II were derivatized with 2-anthranilic acid (2AA) by reductive amination at the reducing end sugar. A 5 ml stock solution of 2AA (150 mg) and sodium cyanoborohydride (225 mg) dissolved in 2% (v/v) acetic acid in methanol was prepared and added to 500 µg of above-mentioned samples at a 3:1 (v/v) ratio in microcentrifuge tubes. Samples were heated at 80°C for 45 min to complete the reaction. Excess 2AA was removed by running samples over a Sephadex G-10 desalting column.

Purified 2AA labeled oligosaccharides were diluted with water and 50% (v/v) glycerol to give a final concentration of 16% (v/v) glycerol. Native polyacrylamide of 20% (w/v) containing 0.72% (w/v) methylene bisacrylamide gels (without stacking gel) was made in running buffer composed of 0.1 M Tris-borate (pH 8.4) containing 1 mM EDTA. Approximately equimolar amounts of each labeled oligosaccharides were loaded on to 20% native PAGE and ran at 250 V for 1 h, after which the gel was viewed under a UV transilluminator. For further experimental studies, which required a pure HA hexasaccharides, fraction II was subjected to MALDI analysis which was essentially carried out according to Sayfried *et al*²² by negative ion reflectron mode using 2,5-dihydroxybenzoic acid (DHB) as the MALDI matrix.

Preparation of biotinylated hyaluronic acid fragments

500 µg of the HA oligosaccharide fragments from fraction I (obtained from G-50) was dissolved in 500 µl of 0.2 M MES buffer, pH 5.5. To this solution, 1 mM biotin-LC-hydrazide (dissolved in DMSO) and 10 mM EDC were added. The reaction mixture was incubated at 4°C for 16 h and dialyzed against PBS-A for 36 h at 4°C. The dialyzed bHA-oligo was stored in glycerol at -20°C.

Overlay and pull down experiment to identify tumor receptors

Soluble protein extracts were subjected to 10% SDS-PAGE. Western blot was carried out according to the modified procedure of Towbin *et al*²³. The gel was transblotted to PVDF membrane at 200 mA for 45 min at 4°C. The membrane was blocked using

blocking buffer composed of 5% fat-free milk powder, 1% bovine serum albumin dissolved in TTBS. The blot was then incubated with biotinylated HA polymer, fraction I or fraction II probe at 4°C overnight, followed by washing with Tris buffer saline containing 0.1% Tween 20 and incubated with HPO9 (streptavidin peroxidase) for 1 h. The complex was detected using an ECL detection kit.

In pull down experiments, protein extracts were cleared by protein-A Sepharose for 1 h. After centrifugation at 10000 rpm for 30 min at 4°C, biotinylated HA hexa (F-II) probe was added and incubated overnight at 4°C. Hexabinding proteins from the reactions were pulled down with protein-A Sepharose beads, washed extensively with lysis buffer containing 0.1% triton, boiled in sample buffer and subjected to 10% SDS-PAGE. Western blot were performed as mentioned in overlay experiments. Protein extracts were also pulled down with anti-HCAM monoclonal antibody. Extracts from tumor samples (250 ug) were precleared with 30 ul protein-A Sepharose, and 5 ug anti-HCAM mAb was added to the supernatant and incubated overnight at 4°C. Next day, 50 ul protein-A was added and incubated for 1 h and centrifuged at 10000 rpm for 30 min at 4°C.

The supernatant and the beads were separated. The beads were boiled in sample buffer and ran for SDS-PAGE and transblotted to PVDF membrane and reacted with anti-HCAM antibody, incubated overnight at 4°C. The blot was washed in TTBS, reacted with b-goat anti-mouse secondary antibody (1:5000) for 1 h, washed and reacted with HPO9 (1:20000) for 1 h and developed with ECL to detect CD44 complex. The supernatant fraction after anti-HCAM mAb pulled down was further precipitated with ammonium sulfate. The precipitate was further dissolved in 2% SDS. Dissolved precipitate was subjected to SDS-PAGE under reducing condition and transblotted to PVDF membrane and reacted with b-hexasaccharide overnight, followed by treatment with HPO9 and developed with ECL to detect the hexa binding proteins.

Histochemical localization of binding proteins using bHA polymer and bhexasaccharide probe

It was essentially according to the modified procedure of Boregowda *et al*²⁴. Freshly excised tissue samples were collected from patients undergoing either biopsy or radical surgery at Bharath Cancer Hospital, Mysore, India. The tissues were fixed in 10% neutral buffered formalin and processed

for paraffin embedding and sectioning to give 3-4 micron sections. In all cases, independent pathological diagnosis was made in the pathological laboratory. Approvals of the Hospital and the University Ethical Committee were obtained for this project.

Tissue sections were pre-digested with bovine testicular hyaluronidase (1 mg/ml) in 0.1 M sodium acetate buffer (pH 5.3) containing 0.15 M NaCl for 1 h at 37°C and the expression of HABPs antigen was analyzed by indirect immunoperoxidase staining. Paraffin sections of tumor tissues were deparaffinized in xylene and were rehydrated by passing through 100, 90, 70 and 30% ethyl alcohol for 2 min in each and finally in PBS. Thereafter, the sections were treated with 3.0% H₂O₂ in methanol for 30 min at room temperature to quench the endogenous peroxidase activity to avoid non-specific reactions and then with blocking buffer for 1h at room temperature. The sections were washed with PBS and incubated with 1 µg/ml dilution of biotinylated probe(s) in PBS-BSA overnight at 4°C.

Next day, the slides were washed with PBS and then sections were treated with HPO-9 (streptavidin peroxidase) 1:3000 dilution for 1 h in humidified chamber, followed by washing with PBS. The color reaction was developed by treating the sections with the chromogen substrate, diaminobenzidine hydrochloride (DAB) and the reaction was terminated by rinsing the sections with distilled water. The sections were dehydrated through 30, 70, 90 and 100% ethyl alcohol and then treated with isopropyl alcohol for 30 min twice. To completely dehydrate, the sections were kept in xylene for 20 min thrice and then mounted with DPX mounting media. Control samples did not receive the hyaluronidase redigestion step or incubation with labeled probes. The stained slides were observed under compound microscope (Olympus BX40) and photographs of sections were taken at 20X and 40X magnification using Nikon camera attached to the microscope.

Results

The polymeric HA was digested with hyaluronidase type IS to produce different chain length HA oligosaccharides and partially purified by gel permeation chromatography to obtain HA hexasaccharide and a mixture of various size HA oligosaccharides. The column profile showed 7 major peaks (Fig. 1A). Peaks 1-6 were pooled and named as fraction I (F-I) and peak 7 as fraction II (F-II). Both

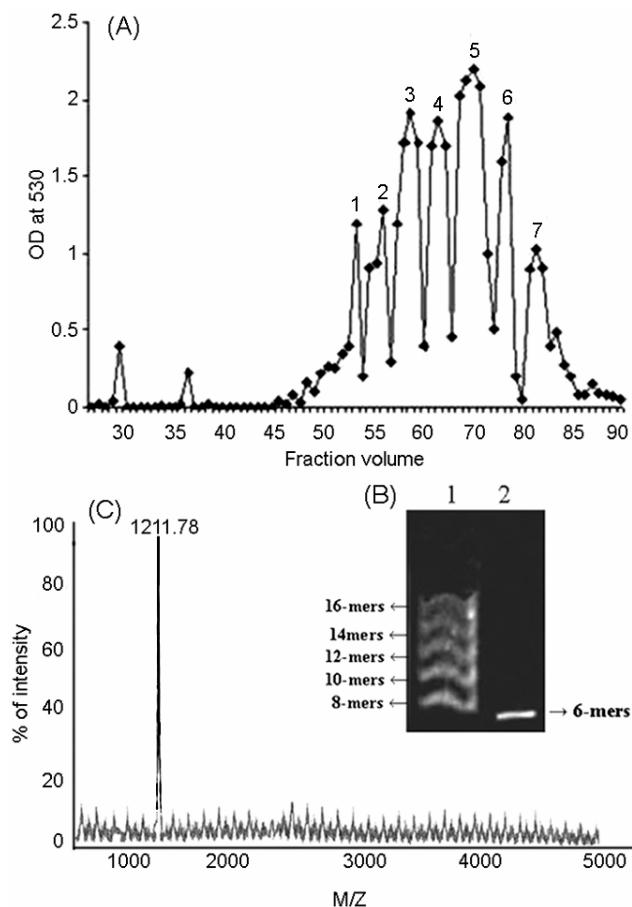


Fig. 1—(A): Partial purification of digested HA on sephadex G-50 column [50 mg of HA oligosaccharide mixture was loaded on sephadex G-50 column (85 x 1.75 cm) and eluted with 0.2 M ammonium acetate pH 5.0 with the flow rate was adjusted to 10 ml/h. The glucuronic acid content was estimated by carbazole method. The Peaks 1 to 6 were pooled as fraction I (F-I) and peak 7 as fraction II (F-II)]; (B): FACE analysis for G-50 fraction I and II [G-50 fractions I and II were derivatized with 2-AA, and run at 200 V on 20% tris-borate native PAGE. The fragmentation was viewed under the UV light. The fractions I containing HA 8 to 16-mers (lane 1) and fraction II (lane 2) comprised of a single specific length of HA oligosaccharide (HA 6-mers); and (C): MALDI spectra of purified fraction II from sephadex G-50 column chromatography [MALDI spectra for the G-50 eluted fraction II by negative ion reflectron mode using 2, 5 DHB as the MALDI matrix. It showed a single peak at 1211.78 Da]

fractions I and II were derivatized with anthranilic acid and subjected to native-PAGE and the gel viewed under UV-Transilluminator (Fig. 1B). F-I showed HA oligo fragments of 8 to 16-mers (lane 1), whereas F-II was hexasaccharide (lane 2). The F-II was further characterized using MALDI-TOF. The MALDI analysis showed a pure HA hexasaccharide with the molecular mass of 1211.78 Da (Fig. 1C). The fractions I and II were biotinylated separately for further analysis.

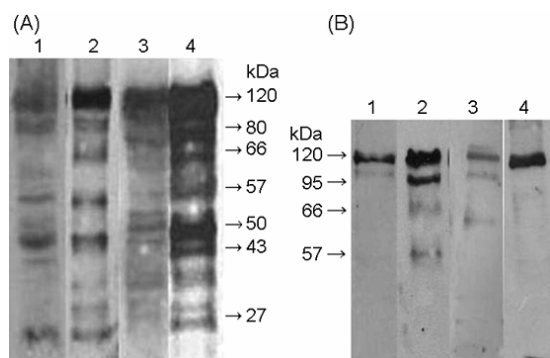


Fig. 2—(A): Overlay analysis to detect HA polymer binding proteins using bHA as a probe [Western blot analysis was performed to detect the HABPs from breast cancer g- II, stomach cancer g-II (lanes 2 and 4), fibroadenoma and appendicitis (lanes 1 and 3) by transblotting 50 μ g crude protein tissue extracts and incubating the blot overnight at 4°C with bHA polymer, then reacted with HPO9 and developed with ECL. bHA probe detected multiple hyaluronic acid binding proteins and their significant increase in the breast and stomach cancer tissue protein extracts than the fibroadenoma and appendicitis tissue protein extracts]; and (B): Overlay analysis to detect HA oligo binding proteins using G-50 FI bHA oligo as a probe [Western blot analysis was performed to detect the HOBBPs from breast cancer g-II, stomach cancer g-II (lanes 2 and 4), fibroadenoma and appendicitis (lanes 1 and 3) by transblotting 50 μ g crude protein tissue extracts and incubating the blot overnight at 4°C with bHA oligo probe (G-50 FI), reacted with HPO9 and developed with ECL. The results showed multiple binding proteins in breast cancer and expression of 120 kDa in all the tissue extracts, but significantly less in fibroadenoma and appendicitis. Scion image analysis showed that the expression of 120 kDa protein between lanes 1 and 2 was 64% and between lanes 3 and 4 was 66%]

Western blot analysis was performed by overlaying with biotinylated probes to detect the expression of binding proteins using HA polymer, HA oligosaccharide and HA hexasaccharide in the different extracted tissue protein samples. bHA Polymer recognized moderate reactions with multiple smear bands, mostly ranging from 50 to 120 kDa in fibroadenoma and appendicitis (lanes 1 and 3), whereas stomach cancer grade II (lane 4) showed very strong reactions at 120, 80, 57, 50, 43 and 27 kDa. Even though 120 kDa band was the major band in breast cancer grade II (lane 2), minor bands were also found at 80, 66, 57, 50 and 27 kDa (Fig. 2A).

HA oligo probe (F-I) recognized proteins with molecular mass of mainly 120 and 95 kDa and a minor band corresponding to 57 kDa in breast cancer grade II (Fig. 2B, lane 2), while in stomach cancer grade II expressed a band corresponding to 120 kDa only (Fig. 2B, lane 4). Even though an inflammatory tissue fibroadenoma, showed a band corresponding to 120 kDa (Fig. 2B, lane 1), but in appendicitis

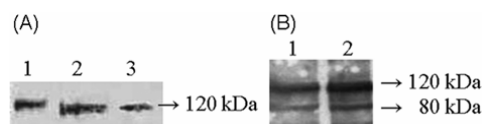


Fig. 3—(A): Overlay analysis to detect HA hexasaccharide (HA6-mers) binding proteins using bHA hexasaccharide as a probe [The bHA hexa overlay interactions with protein extracts of stomach cancer g-II, breast cancer g-II and fibroadenoma (lanes 1, 2 and 3) by resolved on 10% SDS-PAGE and were transblotted to a PVDF membrane. The data showed 120 kDa in all tissue extracts and the expression was more in breast cancer]; and (B): bHA hexa pull down for the detection of HA hexa binding protein [250 μ g crude cancer tissue protein extracts of stomach and breast were pulled down with biotinylated hexasaccharide overnight at 4°C and precipitated with ammonium sulfate. The pellet dissolved in 2% SDS, resolved on 10% SDS-PAGE under reducing conditions and transblotted to PVDF membrane. The membrane was reacted with streptavidin peroxidase conjugate and detected with ECL. The data showed the expression of 120 and 80 kDa and their expression was more in breast cancer (lane 2)]

almost no reaction was observed with b-oligo probe (Fig. 2B, lane 3).

However, protein extracts from stomach cancer, breast cancer, fibroadenoma and appendicitis when probed with b-hexa (Fig. 3A), only 120 kDa protein was detected very strongly in stomach and breast (lanes 1 and 2) while in fibroadenoma (lane 3) showed light reaction and appendicitis (result not shown) did not show any reaction. Incubation of protein extract with bHA hexasaccharide and pulled down with protein A Sepharose beads showed proteins of molecular mass at 120 kDa and a minor band at 80 kDa in both cancer stomach and cancer breast (Fig. 3B).

Because we detected 120 kDa and a near 80 kDa proteins after hexa probe pulled down both from stomach and breast cancer, including fibroadenoma tissue extracts, we performed Western blot analysis with anti-CD44 (HCAM) monoclonal antibody overlay to confirm whether the b-HA hexasaccharide detected proteins may be a member of CD44 family (data not shown for fibroadenoma). It detected 80 and 95 kDa protein as a CD44 protein in both stomach and breast cancers (Fig. 4A, lanes 1 and 2). Hence HA hexasaccharide is detecting 120 and 80 kDa protein (CD44), CD44 immuno pull down assay was carried out by incubating both stomach and breast cancer protein extracts with anti-CD44 mAb overnight to confirm the 120 kDa protein was a specific binding protein for HA hexasaccharide. The CD44 immune complex was pulled down with protein A Sepharose beads.

The immune complex beads were boiled in sample buffer, whereas the supernatant was precipitated with

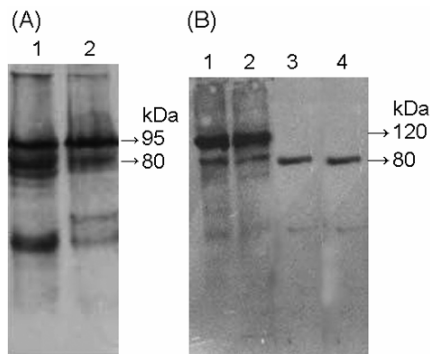


Fig. 4—(A): Detection of CD44 using anti CD44 (H-CAM) monoclonal antibody [50 μ g of cancer tissue protein extracts from stomach and breast tissues were transblotted to PVDF membrane and reacted with anti CD44 mAb overnight at 4°C. The next day, it was reacted with goat anti mouse HRP conjugated antibody and the reaction was detected using ECL. The expression of CD44 protein was more in breast cancer tissue protein extract (lane 2); (B): Immunoprecipitation of CD44 with anti CD44 (HCAM) mAb and detection of HA hexa binding protein (HEXABP) in immune complex-free supernatant: 250 μ g cancer tissue protein extracts of stomach and breast were pre-cleared with protein-A sepharose beads and the supernatants were incubated over night at 4°C with anti CD44 (HCAM) mAb. The CD44 immune complexes were pulled down by using protein-A sepharose beads and the beads were washed with lytic buffer. The supernatant was precipitated with saturated ammonium sulfate and dissolved in 2% SDS. Both the beads and precipitated supernatant were boiled with reducing sample buffer, clarified, resolved on a 10% SDS-PAGE and transblotted. The CD44 immune complexes were reacted with the antibody goat anti-mouse HRP conjugate and supernatant reacted with bHA hexasaccharide probe overnight at 4°C followed by HPO9 reaction and detected by ECL. CD44 immune complex showed a single band at 80 kDa in both stomach and breast cancers (lanes 1 and 2), whereas bHA hexasaccharide shown 120 kDa as a major band in CD44 immune complex-free supernatant of stomach and breast cancer tissue extracts (lanes 3 and 4)]

(NH₄)₂SO₄ dissolved in 2% SDS and boiled with sample buffer. Both supernatant and boiled beads supernatant were transblotted to PVDF membrane as mentioned in the ‘Materials and Methods’ section. The CD44 immune complex showed a single protein at 80 kDa (Fig. 4B, lanes 3 and 4), whereas the supernatant reacted with b-HA hexasaccharide gave a very strong protein of 120 kDa (Fig. 4B, lanes 1 and 2), however, a band near 85 kDa showed negligible reaction (Fig. 4B, lanes 1 and 2).

Breast cancer tissue sections stained with H & E showed solid tumor areas with desmoplastic stroma. Migrating tumor cell clumps were found between the dense fibrous stroma (Fig. 5A). The stomach cancer tissue section showed nodular tumors located mostly in the antrum and the tumor cells were seen infiltrated into all layers, including adjacent omentum (Fig. 5B). bHA Staining showed uniform distribution of HABP throughout the tumor sections of both breast and stomach cancer at the surface and stromal region intensively (Fig. 5C and D). bHA Hexasaccharide showed staining mostly in the nuclear region of tumor cells, light stain in the cytoplasm and in stromal region (Fig. 5E and F).

Discussion

The present study was conducted with the understanding that hyaluronan polymer (HA) and its bioactive fragments (HA-hexasaccharide) were directly involved in tumorigenesis. It is known that HA-oligosaccharides above decasaccharide have diverse biological functions both *in vivo* and

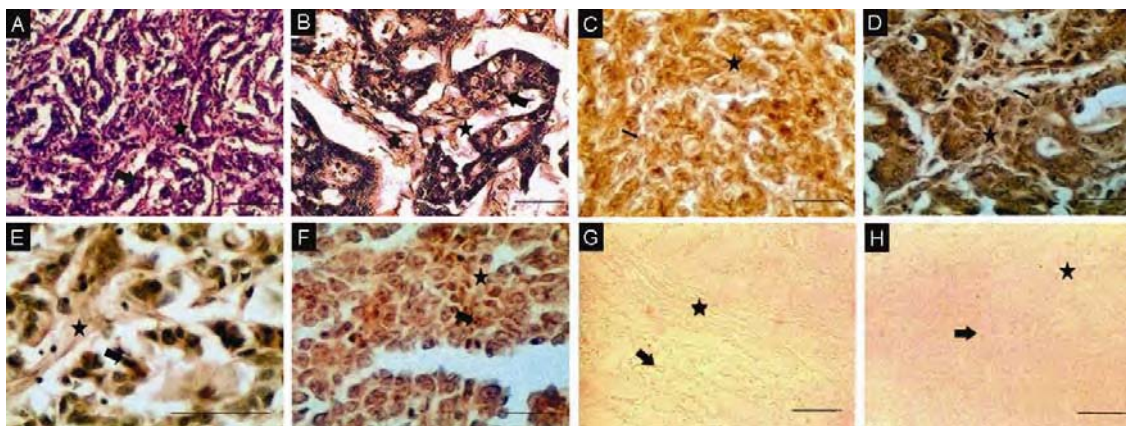


Fig. 5—Immunohistochemical detection of HA and HA Hexasaccharides distribution in paraffin embedded breast and stomach cancer sections [Breast and stomach cancer tissue sections stained with H & E (A and B). Breast and stomach cancer tissue sections stained with bHA showing stain at surface of the tumor cells and stroma (C and D), asterisk shows the stromal components and arrow shows membrane stain. Breast and stomach cancer tissue sections stained with bhexasaccharide showing intense stain at nuclei and moderate stain at the surface of the tumor cells (E and F), arrow shows the stain in the nucleus. Figs G and H are control, stained without probe bHA or bhexasaccharide and no stain was observed. (Scale bar 50 μ m)]

*in vitro*²⁵. This study mainly addressed whether the HA hexasaccharides recognized multiple binding proteins in malignant, benign and inflammatory tissues that may or may not be homologous to known HA binding proteins (hyaladherins).

HA exists as a high molecular mass polymer consisting of multiple disaccharide units and under physiological conditions fragmented HA can be generated by endogenous hyaluronidase, acid hyaluronidase, oxygen-derived free radicals or *de novo* synthesis of small molecular weight oligosaccharide²⁶. Low molecular weight HA oligosaccharides have been shown to induce inflammatory cytokine expression²⁷, enhance Fas-induced apoptotic cell death²⁸ and activate integrin expression in colon carcinoma cells²⁹. HA 8 to 16-mers induce angiogenesis³⁰, HA tetrasaccharides induce heat-shock protein¹² and fragmented HA can also inhibit tumor growth and tumor invasion^{31,32}. HA hexasaccharides, but not tetrasaccharides are known to induce even MMP13 in articular chondrocyte system³³. Thus, it is known that HA fragments can and may induce multiple events in cell adhesion, migration and possibly tumor invasion.

In this report, HA-oligosaccharides of various sizes from hexasaccharide (HA-6mers) to HA-16mers in length were purified by digestion of HA-polymer with testicular hyaluronidase and characterized by FACE and MALDI-TOF analysis. The oligosaccharides were derivatized with 2-AA to get the correct masses. Further the HA-polymer, HA-oligomers and HA-hexasaccharides were conjugated with biotin and used to detect the possible binding proteins from advanced cancers and benign tissues.

During purification of HA oligosaccharides by G-50 column, several peaks were observed. The peaks were divided into two major fractions based on their U/A ratio (Fig. 1A). To characterize the size of oligosaccharide(s) in both the fractions, FACE analysis was done after derivatization with 2-AA (Fig. 1B). The 2AA was used for labeling because it is a small molecular weight (137.1 Da), highly fluorescent compound which enhances high performance in gel electrophoreses and signal in MALDI^{34,35}. Also, it doesn't disrupt the biological activity during fluorescent assay³⁶. The fluorescent oligosaccharides gave a linear separation during gel electrophoreses (Fig. 1B). The purified HA-hexasaccharides were derivatized and subjected to MALDI analysis. As 2-AA enhances the signal in MALDI, a good spectrum was achieved for HA-hexasaccharide with a

molecular mass of 1211.78 Da (Fig. 1C). Therefore, these techniques provided a clear separation and purification of both HA oligosaccharide mixture 8-16mers and hexasaccharides for further experiments.

Multiple observations have provided the evidence that HA polymer from the range of 10^4 - 10^7 Da molecular sizes react with number of HA receptors. The most common transmembrane receptor studied is CD44s³⁷ and the line of evidence implicating CD44s is probably ligand size-specific. The minimum size is documented to be octasaccharides and probably it is a monovalent binding. However, interaction of hexasaccharide with a specific binding protein in freshly excised cancer tissues is an open question. We described the affinity not only of HA polymer overlay interactions with multiple HA binding proteins from 120 to 27 kDa (Fig. 2A), but also of oligosaccharides (16 mers) where it interacted with only 120 and 95 kDa proteins in breast and in stomach only 120 kDa protein was observed (Fig. 2B).

The discrepancy in the detection of multiple proteins might be due to the origin of tissue or tissue specificity. It is demonstrated earlier that HA production is increased in invasive and metastatic human breast and stomach cancers compared to pre-malignant lesions²⁴ and CD44 has a high affinity to the HA. To define the system of HA interaction with binding proteins, anti-CD44 (anti-human cell adhesion molecule) antibody was used as a control to determine its specific interactions with both breast cancer and stomach cancer g-II tissues extract. As usual, both 95 and 80 kDa proteins were detected, showing the specificity of antibody interaction with specific molecular sizes of protein. These two proteins (95 and 80 kDa) were CD44 specific. However, HA oligomers interaction with breast and stomach cancer showed specific proteins mainly at 120 kDa, while the hexasaccharide interacted only with 120 kDa. This indicated the specificity of the HA-hexasaccharide affinity towards specific cancer protein, designated as HEXABP.

Further experiments like pulled down with b-HA polymer, oligomers and hexasaccharide or cross-reacted with HCAM antibody of cancer tissues extracts of breast and stomach showed significant differences in the protein bindings (data not shown). The hexasaccharide once again showed only the 120 kDa protein. When the tumor tissues extract were pulled down with HCAM antibody and the supernatants were tested, the CD44 was negative,

while hexa binding showed very strong reaction, indicating hexa binding protein was distinct and might be different from CD44. To confirm if this is a specific protein for both cancer grade II tissues and probably an isomer of HCAM, further experiments are underway to examine the nature and a probable homology with hyaladherin group by MALDI-TOF MS.

Because of the nature of hexasaccharide identifying a specific protein both in breast and in stomach cancer, further immunohistochemical localization was performed. Freshly excised cancer tissues were fixed in paraformaldehyde and the sections were reacted with b-HA polymer and b-hexasaccharide probe. Significant differences were found in distribution of b-HA vs the b-hexasaccharide. HA polymer distribution showed throughout the tumor section, including the stromal regions. On the other hand, hexasaccharide was localized only in the nuclear area. It indicated that hexasaccharide was bound to specific protein in the nuclei which might be recognized as HEXABP and might be homologous to Hyaladherins.

In conclusion, the study suggested that HEXABP might be involved in tumor progression and showed that hexasaccharides were produced endogenously by the multiple enzymes including endo and hexosaminidase. Hexasaccharides binds either at the nuclear protein or in the plasma membrane might be effecting endothelial cell migration, proliferation and preventing cancer cell invasion¹².

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