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# Detection of $H_{11}$ antigen in human tumor progression

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## ABSTRACT

The development and progression of human tumors is accompanied by various cellular biochemical and genetic alterations. These events include tumor cells interaction with extracellular matrix molecules including hyaluronan and hyaluronan binding protein (HA-HABP). Hyaluronan is a large polysaccharide associated with pericellular matrix of proliferating, migrating cells, regulation of hyaluronan expression during cervical ripening. Its implication in malignant transformation, tumor progression and with the degree of differentiation in various invasive tumors has well accepted. It has been well known the role of HA receptors in tumor growth and metastasis in various cancer tissues. 

## KEYWORDS

Hyaluronan (HA);
H11 antigen;
Hyaluronan-binding proteins (HABP);
Cervical cancer.

## INTRODUCTION

As mentioned earlier, Hyaluronan (HA) is ubiquitously present in the extracellular matrices (ECMs) of animals, plays important roles in ECM organization and cell behavior through binding to hyaluronan-binding proteins (HABPs). The family of HA binding proteins are termed as hyaladherins[14], 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, fibrinogen, hyaluronectin 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 extra cellular matrix component hyalurona (HA) are involved in a broad spectrum of biological processes i.e., organogenesis[6], development of embryonic structures[7], migration of normal cell[16] and activation of the immune response[5] and trans membrane proteins such as CD44 and RHAMM family. The extra cellular hyaladherins, although present in smaller proportions, may participate in cartilage matrix assembly and maintenance of matrix integrity[10]. Cellular hyaladherins have been detected in several cell types from a wide variety of tissues[14,17].

The diverse physiological functions of HA in cervical softening and ripening remain to be elucidated. In other cell systems, HA plays a structural role and mediates signaling events via interactions with cell surface receptors, such as RHAMM and CD44[15]. CD44 and RHAMM are established signal-transducing receptors that influence cell proliferation, survival and motility, and are known to be relevant to cancer. Other cell-surface

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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[12,13,15]. Thus the interactions of the extra cellular matrix component hyaluronic acid and its cellular receptors CD44 and RHAMM/IHABP4/CDC37 have been linked to tumor progression and metastasis formation.

Both RHAMM and CD44 mediate hyaluronan signaling and participate in growth factor regulated signaling. However, they are likely to regulate signaling 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 signaling has not yet been reported. Therefore, this study focuses upon the signaling cascades that RHAMM and CD44 regulate.

The linear over-expression of $H_{11}B_2C_2$ antigen (HABP) was observed by biochemical analysis in benign and cervical cancer tumors with different grades.

## MATERIALS AND METHODS

Regular laboratory chemicals and reagents of analytical grade were purchased from Merck and Ranbaxy, India. Media and glasswares for mammalian cell culture were purchased from Gibco, BRL, Germany, Nunclon, Germany and Millipore, Germany. The secondary antibody was purchased from Genei, Bangalore, India.

### Extraction

**Lysis buffer:** It contains 50mM Tris buffer, 150mM sodium chloride, 1% NP-40, 0.1% Sodium lauryl sulphate, 1mM PMSF, (100mM Stock dissolved in ethanol).

### Electrophoresis

**Ammonium per sulphate (APS):** Always fresh 10%APS solution in doubled distilled water was prepared.

**TEMED**

1 **Acryamide -bis-acryamide solution:** The stock solution 30% acryamide and 0.8% bis- acryamide were prepared in double distilled water and then filtered through whatman #1filter paper.
2 **Resolving gel buffer:** It contains 1.5M Tris buffer, 0.8% Sodium lauryl sulphate. These reagents were dissolved in double distilled water and the pH was adjusted to 8.8 with 1N HCl.
3 **Stacking gel buffer:** It contains 0.5M Tris buffer, 0.8% Sodium lauryl sulphate. These reagents were dissolved in double distilled water and the pH was adjusted to 6.8 with 1N HCl.
4 **4X Sample buffer:** It contains 10% sodium lauryl sulphate, 0.1% bromophenol blue and 10% Glycerol. These reagents were dissolved in 0.25M tris buffer of pH 6.8 and are used so as to give a final concentration of 1x with the sample.
5 **Tank buffer:** It contains 0.19 M Glycine, 0.014M Tris buffer. These reagents were dissolved in double distilled water and the pH was adjusted to 8.3[if necessary] with saturated glycine and finally 0.2%SDS was added to the tank buffer.

### Western blotting

1 **Western blot apparatus:** Vertical wet Immunoblotting [Western blotting] apparatus was purchased from Genei Bangalore, India.
2 **PVDF:** Immobilon-P was purchased from Millipore Germany.

### mAb $H_{11}B_2C_2$

1 **Transfer buffer:** It contains 0.192M Glycine, 0.125M Tris, 0.01% sodium lauryl Sulphate, 10% methanol. The above reagents were dissolved in double distilled water and the pH was adjusted to 8.3[if necessary] with saturated Glycine.
2 **Tween-Tris buffer saline [T-TBS]:** It contains 0.05M Tris, 150mM sodium chloride, 0.1%Tween.These reagents were dissolved in double distilled water and the pH was adjusted to 8.0 with 1N HCl.
3 **Blocking buffer:** It contains 5%fat free milk powder.1% serum albumin [Bovine], These were dis-

solved in T-TBS. The blocking buffer was directly used to block any non-specific reaction.

4 **T-TBS/BSA buffer:** It contains 0.1% serum albumin [Bovine], The albumin was dissolved in T-TBS buffer. This was used for the dilution of different antibodies used during Western blotting.

5 **DAB Chromogen substrate solution:** It contains 3,3'-diaminobenzedine[DAB] [Acros, USA] and hydrogen peroxide. The working solution was prepared by mixing 300 µl of 4% DAB stock solution, 4µl of 30% Hydrogen peroxide in 15ml of 0.1M Tris-buffer [pH7.4] and filtered through whatman #1 filter paper.

6 **ECL [Enhanced chemiluminescence]:** Super signal west pico chemiluminescent substrate from pierce [USA] was used. Working solution was prepared by mixing equal parts of the stable peroxide solution and the luminol/enhancer solution. 0.125ml-working solution per $cm^2$ of membrane was used.

**Developer and fixative**

**(1) Production of monoclonal antibody [mAb $H_{11}B_2C_2$]**

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[4]. Hybridomas producing IVd4 antibody were selected, whose interaction with antigen was competed out by hyaluronic acid and hyaluronan oligomers[4]. 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. Further more the hybridoma was selected in HAT and HT media in DMEM. One of the clones $H_{11}B_2C_2$ was selected. The antibody production in human serum of any blood groups did not affect $H_{11}B_2C_2$ antibody in recognizing the human antigen expressed in tissues derived from malignant tumors. The clones 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.

**(2) Preparation of biotinylated hyaluronic acid (bHA)[4]**

50mg of hyaluronic acid was dissolved in 10ml of filtered PBS-A buffer (Ca and Mg free). The dissolved hyaluronan solution was dialyzed against 0.1M MES buffer PH 5.5 for 16hrs at 4°C. Later hyaluronan solution was mixed with 50mM Sulfo-NHS-LC-Biotin dissolved in DMSO, 50mM EDC for 16hrs at 4°C and then dialyzed against PBS-A for 36hrs at 4°C. Finally the dialyzed bHA was stored in glycerol at -20°C.

Extraction of protein from benign and malignant human tumor tissues: Fresh tissues from benign and malignant cervical cancer samples were collected from the hospitals in cold PBS were stored at -20°C. Before extraction the samples were resuspended in lytic buffer and then homogenized (1:4w/v) in lysis buffer in a glass-Teflon homogenizer. The lysate was centrifuged at 10000rpm for 45min and an aliquot of the supernatant was assayed for protein concentration using Bradford method.

**(3) Detection of $H_{11}$ antigen in benign and malignant human tumor tissue using mAb $H_{11}B_2C_2$**

After extraction, equal amount of protein (50µg) from each of the tissue sample extract were taken and electrophoresed on a 10% SDS-PAGE[11] at 25mA constant current and electro transferred to a PVDF (Immobilon-p, Millipore) 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 Tween-TBS buffer. The membrane was incubated with mAb $H_{11}B_2C_2$, (1:100 to 1:500 dilutions) for 1hr at room temperature and overnight at 4°C. Next day the membrane was washed with Tween-TBS. The membrane was then incubated with secondary antibody (goat anti mouse IgG biotin conjugated 1:1500 dilution) for 1hr at room temperature. The membrane was washed with Tween-TBS. The membrane was treated with HPO-9 (Strepta avidin peroxidase, Sigma 1:3000 dilution) for 1hr at room temperature. After extensive washing with Tween-TBS, the immuno-reactive proteins were visualized with ECL western blotting detec-

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tion reagents (Pierce) or with DAB.

### (4) Detection of $H_{11}$ antigens in benign and malignant human tumor tissue using a specific probe bHA

After extraction, equal amount of protein (50μg) from each of the tissue sample extract were taken and electrophoresed on a 10% SDS-PAGE[11] at 25mA constant current and electro transferred to a PVDF (Immobilon-p, Millipore) 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 Tween-TBS buffer. The membrane was incubated with b.HA (1:50 dilution) for 1hr at room temperature and overnight at 4°C. Next day the membrane was washed with Tween-TBS. The membrane was treated with HPO-9 (1:3000 dilutions) for 1hr at room temperature. After extensive washing with Tween-TBS, the immuno-reactive proteins were visualized with ECL western blotting detection reagents (Pierce) or with DAB.

## RESULTS

### $H_{11}$ antigen (HABP) expression by western blot

The expression of $H_{11}$ antigen (HABP) as detected by mAb$H_{11}B_2C_2$ was analyzed by western blotting method in different cancer tissues. They were also compared with benign cervical and cervical cancer tissues samples of different grades. Proteins were resolved in 10% SDS-PAGE. The benign samples screened for $H_{11}$ antigen (HABP) are from chronic cervix and fibroadenoma. The cancer samples are from breast, rectum, cervix, colon, larynx, cheek, stomach, esophagus, tongue, pancreas, ovary, and cervix (G-I, II & III). Grade I, II and III are poorly differentiated, moderately differentiated and well differentiated cervical cancers respectively. The expression of two proteins of mol.wt 55-57 KD and 30KD was observed in all samples. Graphs showing the intensity of 57KD protein in each lane of the blots were done using Scion Image Analysis Software.

The $H_{11}$ antigen (HABP) expression as detected by mAb $H_{11}B_2C_2$ after western blotting from breast, ovary, rectum, cervix, colon and cheek cancer tissue samples was shown in figure 1. The H11 antigen(HABP) expression as detected by mAb $H_{11}B_2C_2$ after western blotting from larynx, stomach, esophagus, cervix, tongue and pancreas cancer tissue samples was shown in figure 2. The $H_{11}$ antigen(HABP) expression as detected by mAb $H_{11}B_2C_2$ after western blotting from stomach, larynx, esophagus, cervix (benign), cervix and fibroadenoma cancer tissue samples was shown in figure 3. The cervical cancer shows strong expression and broad bands at 55- 57 KD. and 30 KD. Compared to other cancer tissue samples. The $H_{11}$ antigen (HABP) expression as shown figure 1 and 1 were developed with DAB after western blotting, whereas, the $H_{11}$ antigen (HABP) expression as shown in figure 3 was developed with ECL after western blotting for higher sensitivity.

The H11 antigen(HABP) expression as detected by mAb $H_{11}B_2C_2$ after western blotting in different grades of cervix, breast, colon and rectum cancer tissue samples was shown in figure 4. A gradual increase in the expression of mAb $H_{11}B_2C_2$ reactive $H_{11}$ antigen (HABP) was observed from grade I to grade III of cervical cancer samples when developed with ECL for higher sensitivity.

Figure 5 shows the $H_{11}$ antigen expression in grade I and grade III cervical cancer tissue samples as detected by bHA when developed with ECL for higher sensitivity. They were compared with benign cervix samples. All the grades of cancer samples were showing strong reaction with bHA when compared with benign samples.

Figure 6 shows $H_{11}$ antigen (HABP) expression in different samples of grade III cervical cancer tissue samples as detected by mAb $H_{11}B_2C_2$ It shows strong expression and broad band at 55- 57 KD.

Figure 7 presents $H_{11}$ antigen (HABP) expression in different grades of cervical cancer tissue samples and benign samples as detected by bHA. It shows gradual increase of $H_{11}$ antigen (HABP) in different grades of cervical cancer tissue samples when compared with benign samples. This was developed with ECL for higher sensitivity.

The expression of $H_{11}$ antigen in all grades III cervical cancer tissue samples as detected by bHA was given in figure 7. All the samples showing very strong expression of $H_{11}$(HABP) The reaction was developed

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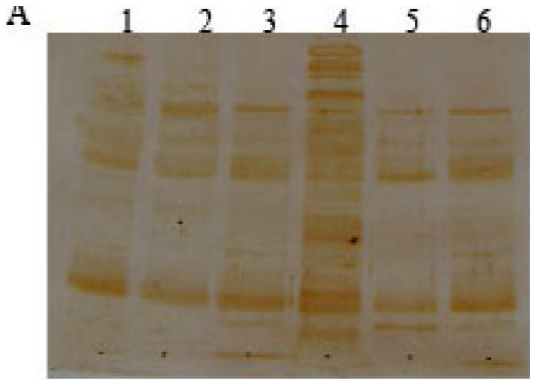


Figure 1 : $H_{11}$ antigen expression in different cancers as detected by mAb $H_{11}B_2C_2$ after western blotting. It was developed with DAB. Lane 1: Breast; Lane 2: Ovary; Lane 3: Rectum; Lane 4: Cervix; Lane 5: Colon; Lane 6: Cheek

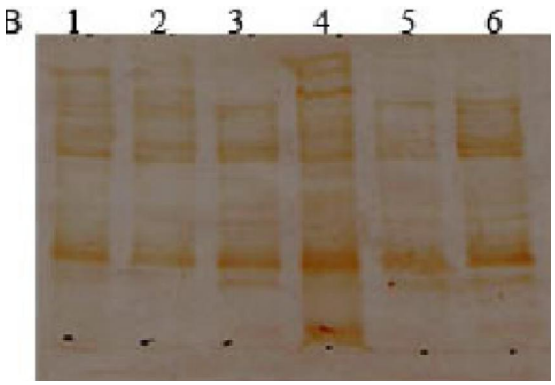


Figure 2 : $H_{11}$ antigen expressions in different cancers as detected by mAb $H_{11}B_2C_2$ after western blotting. It was developed with DAB. Lane 1: Larynx; Lane 2: Stomach; Lane 3: Esophagus; Lane 4: Cervix; Lane 5: Tongue; Lane 6: Pancreas

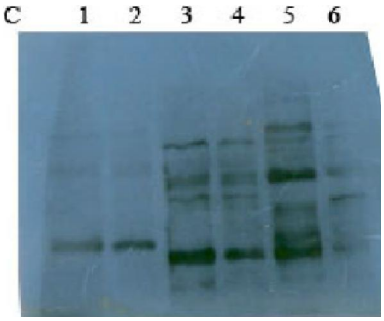


Figure 3 : $H_{11}$ antigen expression in different cancers as detected by mAb $H_{11}B_2C_2$ after western blotting. It was developed with DAB. Lane 1: Stomach; Lane 2: Larynx; Lane 3: Esophagus; Lane 4: Cervix(Benign); Lane 5: Cervix(Cancer); Lane 6: Fibroadenoma

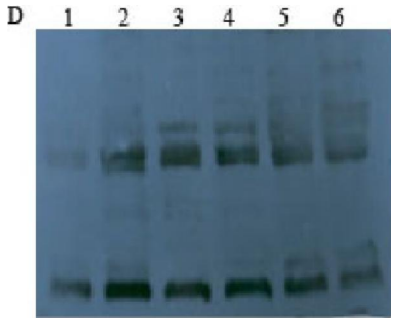


Figure 4 : $H_{11}$ antigen expression in different cancers as detected by mAb $H_{11}B_2C_2$ after western blotting. It was developed with ECL. Lane 1: Cervix(Gr 1) ; Lane 2: Cervix(Gr 2); Lane 3: Cervix(Gr 3); Lane 4: Breast; Lane 5: Rectum; Lane 6: Colon

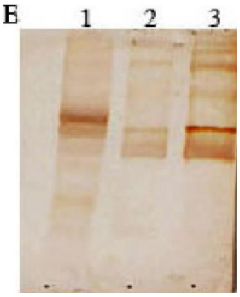


Figure 5 : $H_{11}$ antigen expression in different grades of Cervical cancers and also in benign Cervix as detected by b.HA after western blotting. It was developed with DAB. Lane 1: Cervical Cancer (Gr 1) ; Lane 2: Benign Cervix(Gr 2); Lane 3: Cervical Cancer (Gr 3)

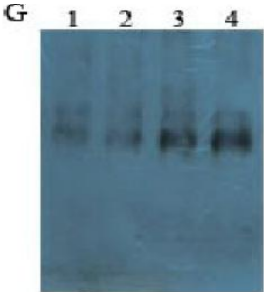


Figure 6 : $H_{11}$ antigen expression in different grades of Cervical cancers as detected by b.HA after western blotting. It was developed with ECL. Lane 1&2: Cervical Cancer (Gr 1) ; Lane 3: Cervical Cancer (Gr 2); Lane 4: Cervical Cancer (Gr 3)

with ECL for higher sensitivity.

Among all the cancer tissues, cervical cancer tissue samples were selected to carryout the research program because it is the most common form of invasive carcinoma of cervix in women in developing countries and also the excessive availability of the cervical cancer tissues. It is the leading cause of cancer related deaths in women in the world. In such tissue sample the $H_{11}B_2C_2$ (HABP) expression was analyzed in different grades of cervical cancer tissue samples. There was a

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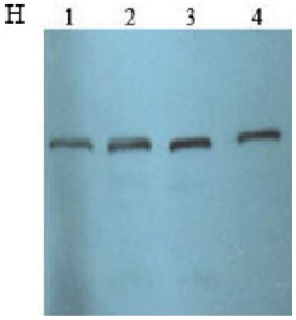


**Figure 7 : $H_{11}$ antigen expression by western blotting in grade 3 of Cervical cancers as detected by b.HA. Lane 1, 2 and 3: Grade 3 of Cervical Cancer**

linear over-expression of $H_{11}B_2C_2$ antigen (HABP) as the tumor progressed from G-I, G-II and G-III.

## DISCUSSION

The importance of hyaluronan expression during tumor progression was investigated. It explains the positive association of stromal hyaluronan expression with invasive nature of tumors irrespective of their origin. The study explains the differential expression of hyaluronan in well differentiated tumors in contrast to the poorly differentiated[3]. The growing evidence of the presence of intracellular hyaluronan and its interaction with intracellular hyaladherins such as CDC37, IHABP4[8,9] and further the subsequent loss of hyaluronan interaction with its receptor during late malignancy led to study the distribution of $H_{11}$ antigen (HABP) in multiple cancer tissues.

Human cervical cancer smears and paraffin tissues section of different types of cancers were screened by immunohistochemical technique for the presence of $H_{11}$ antigen (HABP). High expression of $H_{11}$ antigen (HABP) was seen in all human cervical cancer tissues. The distribution of $H_{11}$ antigen (HABP) was mainly on the tumor cell surface and intracellular localization. The adjacent normal areas showed low expression of $H_{11}$ antigen (HABP).

Current study presented the nature of $H_{11}B_2C_2$ antibody and its affinity towards its antigen in different types of benign and cancer tissues. Western blot analysis indicated that $H_{11}B_2C_2$ antibody recognizes mainly two proteins of molecular weight 55-57KD and 30KD irrespective of the origin of tissue. The $H_{11}$ antigen (HABP) expression of molecular weight 55-57 and 30KD was seen in almost all types of cancer and by Scion Image Analysis it is evident. A significant increase in the $H_{11}$ antigen (HABP) expression was seen as the tumor progressed from benign to malignant. The over-expression of $H_{11}$ antigen (HABP) in the progression of malignant cervical tumor was well differentiated to poorly differentiated independent of their histological origin.

The 55-57KD and 30KD proteins, which were observed in western blot, were probably a HABP. It was shown by using a specific probe b. HA for the detection of HABP, which recognizes all types of hyaladherins. The results indicated that it is a HABP. However further studies are necessary to show that 57KD protein could be a common marker. Activation of 57KD antigen may therefore represent a critical step in tumor progression and correspondingly may play an important role in the malignant transformation of human cells. Presently, limited information is available to provide the mechanism involved but the cumulative data suggests over-expression of $H_{11}$ antigen (HABP) in tumor cells and it is an important parameter and a clinical diagnostic marker for all progressive human tumors.

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