International Journal of Multidisciplinary and Current Research ISSN: 2321-3124
Review Article Available at: http://ijmcr.com

Diagnostic usefulness of Hyaluronan & Hyaluronan Synthases-A Review

Dr.Swaminathan.S[1*], King David Edward.T[2], Abirami.M.J[3]and Dr. Oviya Senthilraj[4]

[1]Director of Laboratory Services & Consultant Biochemist,[2]Bio-Statistician, [3]Dietician,[4]Corporate Manager, Techmed Health Centre & Diagnostic Pvt Ltd, No. 01 Siva Building, Krishna Street , Off North Usman Road, T.Nagar, Chennai, India

Received 15 Oct 2018, Accepted 20 Dec 2018, Available online 24 Dec 2018, **Vol.6 (Nov/Dec 2018 issue)**

Abstract

The first isolation of HA was in the year 1934, but research on HA and its clinical significance in health and disease started only in the year 1970. Both HA and HASs play a significant role in many physiological and pathological processes. The cellular HA synthesis are coordinated collectively by HASs. HA has wide range of applications in clinical practice. HAS1 is expressed in malignancies like bladder and prostate cancers as well as in myeloma and malignantmesothelioma. Studies have predicted the involvement of HA in endothelial cell proliferation migration, new vessels formation and leucocytes recruitment. HA was found to influence stroma cell recruitment, tumor angiogenesis and epithelial – mesenchymal transition. Measurement of HASs may help to find out tumor aggressiveness towards targeting appropriate therapies. Measurement of both HA and CD44 will be very useful in the evaluation of breast cancer. Many different assays based on ELISA and PCR are now available for reliable quantification of HA. It also play a key role in bone metabolism and its latest application is for breast cancer screening and prognostic evaluation. It has also therapeutic value in multidrug resistance. Assays for HA and HASs have also been discussed in this review article. The contents of this review article will be very useful for future researchers to evaluate a simple, reliable and cost saving methods for measuring HA and HASs in human blood and to include them as metabolites for cancer screening.

Keywords: *HA, HASs, CD44, Isoenzymes, Saccharides, Cancer, Breast*

Introduction

Hyaluronan (HA) is an anionic non-sulfated glycosylaminoglycan which was found to be widely distributed throughout human body mainly in connective, epithelial and neural tissues. It is biosynthesized by a group of isoenzymes Hyaluronan Synthases (HASs). Its main role involves cell proliferation and migration and also plays a role in the progression of malignant tumors, particularly in breast tissue. The clinical significance of HA was studied in the post 1970. HA plays a significant role in wound repair, inflammation, granulation and skin and fetal wound healing. This review article gives a condensed summary of the research findings during the last two decades on the clinical usefulness of HA and HASs.

The first isolation of HA was in the year 1934 by Meyer and Palmer. They showed that the original HA contained hexauronic acid, an amino sugar without sulfoesters and proposed the name hyaluronic acid (Hyaluronan, HA). The material used to isolate was vitreous of eye. The number of repeating disaccharide molecule in HA may exceed 30,000. From 1966 to 1996, a total of 6300 papers have been published about HA. The outcome of these research publications have established that HA is present in every tissue in vertebrates and it is now widely used in various clinical applications as intra – articular matrix supplement and in eye surgery. HA plays a significant role in modulating cell migration and differentiation during embryogenesis, regulation of extracellular matrix organization, complex processes of metastasis, wound healing and inflammation. It has also been proved that HA is highly metabolically active, and cells focus much on the process of HA synthesis and catabolism. In epidermis, the half life of HA was 1 to 3 Weeks in tissues and < 1 day in cartilage [1].

Although biosynthetic pathways of HA have been studied for more than 6 decades, details regarding how HA is assembled to biosynthesis HAS is still not complete. First, extracellular HA coats are produced by HA – producing cells and secretes it into surrounding space. HAS contain multiple transmembrane domains with lipid dependent creating an intraprotein HAS – Liquid pore through which a growing HA – UDP chain is translocated continuously across the cell membrane to the exterior. The synthesis of chitin – UDP oligomers by HASs has confirmed the reducing end mechanism for sugar addiction during HA assembly by mammalian class 1

*Corresponding author's ORCID ID:0000-0002-2360-4259
DOI: https://doi.org/10.14741/ijmcr.v.6.6.19

enzymes. Hence HA biosynthesis was assumed to be initiated by the ability of HAS to use chitin – UDP oligomers as self – primers [2].

There are three isoenzymes of Hyaluronan synthase (HAS) among which isoenzyme HASI was found to be responsible for cellular hyaluronan synthesis. However, synthesis of HASI form was found to be insignificant compared to HAS2 and HAS3 as these two have higher enzymatic activities. In many cell types studied, HASI gene expression was low and hence the enzyme required a higher concentration of sugar precursors for hyaluronan synthesis. Proinflammatory factors interleukins (IL) and cytokines were required for HASI expression and activity. HASI was associated with inflammation like atherosclerosis, osteoarthritis and infectious lung disease. Further HASI was found to be expressed in malignancies like bladder and prostate cancers, multiple myeloma and malignant mesothelioma. HASI was a poor predictor of breast cancer, but correlated with high relapse rate and short overall survival. In all cell types studied HASI was found to be accumulated intracellularly. During inflammatory response, HASI was found to regulate the organization of hyaluronan in vitro leukocytes recruiting matrix. HASI was found to be an important factor associated with glycemic stress like metabolic syndrome (MS), inflammation and cancer [3].

HA, an extracellular component has been involved in many physiological and pathological processes. HA modulate several functions like cell proliferation, migration and inflammation. Its presence in tissues may have either positive or negative effects. HASs are a family of three isoenzymes, all located on the plasma membrane, responsible for the production of many polysaccharides and their activities are directly related to polysaccharides content [4].

HA was isolated in trabecular mesh work and shows actions in pathophysiology of aqueous outflow environment. HAS isoenzymes were detected in Bovine trabecular meshwork cells (BTMC) and all three were expressed at the mRNA level. HA production was found to be stimulated from BTMCs by TGF – B or PDGF – BB. HAS expression may maintain the HA content in the aqueous outflow pathway and may be useful for modulating the aqueous outflow environment [5].

HA was the only nonsulphated glycosaminoglycan present in extracellular matrix. In mammals, HA was synthesized by the three homologues of HAS1, HAS2 and HAS3. Catabolism of HA by hyaluronidase enzymes may yield either oligosaccharides or large polymers. Although HA has simple structure, it showed wide range of activities ranging from cell proliferation to tumor growth. Release of oligosaccharides from HA stimulate cytokine secretion and endothelial cell proliferation, but no data about HA presence in endothelium have been reported. But several studies have predicted the involvement of HA in endothelial cell proliferation, migration, new vessels formation and leucocytes recruitment [6].

Quantification of HA by determining its level in membrane bound enzymes will enable to understand the normal physiology of HA in inflammatory and other diseases, tumorigenesis and metastasis. HA products were more complicated compared to glycosyltransferases. Assays based on both radioactive and non radioactive are now available and studies should make use of them to isolate HA and find its clinical usefulness [7]. HA serves as a perfect environment in which cells can migrate and proliferate. Several receptors may interact with HA at cellular levels triggering multiple signal transduction responses. Hence the control of extracellular matrix (ECM) is critical in cell assembly in biology. While glycosaminoglycans are synthesized in the golgi apparatus, HA was produced at the plasma membrane by HAS – 3 using UDP – glucoronic acid and UDP – N – acetyl glycosamine as substrates. It is an energy consuming process. HA secretion will be inhibited by AMP – activated protein kinase (AMPK) using HAS2 Phosphoration at thereonine 110 [8].

In vertebrates and certain microbes, HASs catalyse polymerization of HA. HASs participate in polymer transfer out of the cell. Very good progress have been made since the first genetic identification of HAS in 1993 leading to the discovery of new enzymes and molecular details. The important findings include lipid dependence of class 1 HASs, HASs protein monomers and discovery of class II HASs. The three classes of HAS identified showed differences in protein sequences, predicted membrane topologies, potential architectures mechanisms and direction of polymerization [9].

HA and HASs ASSAYS.

Studies have proposed several assays for HA measurements based on the affinity of proteins isolated from cartilage, chondrosarcoma brains. A method based on alkaline phosphatase – linked hyaluronectin could be used to measure HA in biological fluids, tissue extracts and then characterize it by a two step procedure of reagent incubation and staining. Results obtained by this method showed good correlation to methods based on antibodies. The interassay variation was 8.5 % if assay was done at 4°c. Tissue HA could be easily done with this reagent in fetal tissue to measure HA in a wide range of biological samples. This assay could detect HA at the lowest level of amU[10].

Hyaluronase activity present in pasteurellamultocidal (Pm HAS) could be done using three enzyme coupled UV absorption to quantitative glucoronic acid and N – acetyl glucosaminetransferase activities. The assay involves measuring the activity by coupling the UDP produced from Pm HAS catalysed transfer of UDP – GlcNAc and UDP –Glc UA to a hyaluronic acid tetra saccharide primer with the oxidation of NADH. The products were then isolated by Gel Electrophoresis. This assay could be used to determine kinetic parameters inhibition constants and mechanistic aspects of the enzyme. It could also be used to quantity Pm HAS during purification of the enzyme from culture media [11].

A sensitive method has been developed for measuring HASs without the use of radioactive U5'D - Sugar precursors. This method was based on the binding of biotinylated HA binding protein (b HABP) to HA chains and the subsequent capture of b HABP – HA – HAS complexes with streptavidin agarose. The captured ceroplexes were then immunodetected by Western Blot analysis using appropriate antibodies. This assay could be used to measure a range of HASs in Vitro in cell membrane. This assay was particularly sensitive to measure active form of HAS which cannot be done using standard assays. The assay sensitivity was < 1.0 pmol[12].
In one recently emerging sensitive and rapid microtiter assay, the free carboxyl groups of hyaluronan were biotinylated in a one step reaction using biotin – hydrazide. This substance was then covalently coupled to a 96 – well microtitre plate. After the enzyme reaction was complete, residual substrate was detected with an avidin – peroxidase reaction that can be used in a standard Enzyme Linked Immunosorbent Assay (ELISA) plate reader. This method eliminated artifacts such as pH – dependent botinylated activity. This method was very sensitive for the measurement of hyaluronidase activity from cultured cells and biological samples. The interassay precision was good at 5%. This method was to be suitable for the measurement of distribution profile of plasma hyaluronidase levels in human sera. 1µL sample was sufficient for this assay. This microtiter based assay may be used as a routine clinical laboratory procedure [13].

A spectrophotometric method for the assay of hyaluronidase activity using carbocyanine dye has been described. This dye binding results in a spectral shift with an absorbance maximum at 640 nm and was proportional to the amount of hyaluronic acid. The end products of hyaluronidase activity do not cause shift in the dye spectrum. This method was able to determine hyaluronidase activity down to 0.00005 NF unit [14]. In a study, several methods were compared for the determination of HA. Three methods viz ELISA Corgenix, Echelon and R&D gave Intra assay variabilities of 11.7 ± 3.6 %, 18.9 ± 9.2 % and 12.3 ± 4.6 % respectively. The Inter assay for the above three methods were 60%, 9.5% and 34.1% respectively. The concentration of HA was over estimated by Echelon assay by 85% and under estimated by R & D and Corgenix by 34 and 32% respectively. The conclusion of this study has recommended Echelon assay as it was effective at measuring all sizes of HA tested, whereas the Corgenix and R & D were unable to detect down to 6.4 K Da HA [15].

HA and Cancer

Studies have shown that HA, a core component of ECM contribute to certain type of cancer development. Intracellular, extracellular and nuclear forms of HA has been detected in certain cancers. While intracellular HA was involved in cell signaling, nuclear HA could promote chromatin condensation facilitating mitosis. Metastasis may be due to deregulation of HAS genes which leads to abnormal biological processes. Although three HAS isoenzymes were equally involved in the process of malignancies, the exact function of their role in cell signaling remains to be elucidated. More research are required to design a novel therapeutic strategies to counter presumptive cancer – promoting effects of HAS isoenzymes[16].

HA was considered as a critical component of cancer microenvironment that was known to increase tumor progression and aggressiveness. The precursors UDP -N-acetyl glucosamine and UDP – glycoronic acid used to synthesize HA have a critical role in cancer. The catabolic products of HA, glycoronic acid and N-acetyl glucosamine acts as representative cells by acting as substrates for tumor cells [17]. Aberrant pre – m RNA splicing may lead to intra or extracellular HA synthesis by HASs leading to the initiation and progression of various types of cancer. Aberrant splicing was more influenced by intracellular HA and this is a multifactorial process. Mutations in HAS1 provide an indicator that may lead to malignancy through increased risk and predisposition. Specific gene splice variants and the splicing process itself offer potential targets for novel drug treatment strategies [18].

Oligosaccharides present in HA were reported to have suppressive effects on various malignant tumors via disruption of receptor HA interactions. However, no studies have been reported on the effects of HA oligosaccharides on bone metastasis of breast cancer. In the highly invasive breast cancer cell line MDA-MB-231, effective size of HA oligosaccharides were required to inhibit cell growth. While HA decasaccharides significantly inhibited cell growth, no effect was shown by tetrasuccharides. HAS2m RNA expression was altered after treatment with both forms of oligosaccharides. Histological analysis has revealed HA accumulation in bone metastatic lesions and was perturbed by decasaccharides. HA oligosaccharides may have suppressed progression of bone metastasis in breast cancer via interruption of endogeneous HA – CD 44 interaction and it could serves as novel therapeutic candidate to limit bone metastasis of breast cancer [19].

The Trans membrane receptor CD44 of HA was found to be implicated in various adhesions– dependent cellular processes including cell migration, tumor cell metastasis and invasion. Membrane type- 1 matrix metalloproteinase (MTI - MMP) was often expressed in invasive cancer cells. HA was found to modulate MTI-MMP expression to facilitate breast cancer cell migration. The methods available to quantity HA induced MTI-MMP expression in breast cancer are Flow Cytometry, real time PCR, Western blotting and immunoflourescent staining. MTI-MMP expression in breast cells were found to be increased after stimulation by HA – oligosaccharides followed by significant up regulation of MTI-MMP mRNA. Hence, HA oligosaccharide enhances MTI-MMP expression enhancing CD44 cleavage and cell migration. The HA oligosaccharides induced MTI –MMP expression

in breast cancer cells may be a critical step in the formation of metastatic colonies [20].

HAS2 and the key receptor for HA- CD44 expression are both poorly correlated to poor outcome in patients with basal like breast cancer. These events are brought out by deregulation of HA syntheses. Both β HAS2 and CD44 induced by TGF- β are required in the course of TGF- β induced epithelial- mesenchymal transition (EMT) processes by mammary epithelial cells. Elucidation of the regulation of HAS2 and CD44 expression may contribute to the development of better strategies to treat breast cancer patients [21].

Studies have not yet characterized the metabolism of HA in breast cancer cell lines. Studies using advanced technologies like PCR and Western blot have shown that highly invasive cell lines preferentially expressed HAS2 and isoforms, while less invasive cells expressed HAS3 isoforms. A correlation was found between elevated levels of HA synthesis, CD44 expression and cancer cell migration highlighting the pivotal role of HA metabolism in the aggressive breast cancer phenotype [22].

HA, the major polysaccharide present in the extracellular matrix of connective tissues was found to be intimately involved in the biology of cancer. HA was found to accumulate in the stroma of various human tumors and modulates intracellular signaling pathways, cell proliferation, motility and invasive properties of malignant cells. Laboratory evidences highlighted the importance of HA in tumor growth and metastasis. In human adenocarcinomas, a high stroma HA was associated with differentiated tumors and aggressive clinical behaviors. However, low HA was found in squamous cell carcinomas and malignant melanomas. HA was found to influence stromal cell recruitment, tumor angiogenesis and epithelial-mesenchymal transition. Many factors such as HA receptors, HASs, HA degrading enzymes and, hyaluronidases are involved in the modulation of cancer progression based on the tumor type. HA was also found to have therapeutic implications since it is involved in multidrug resistance [23].

In many epithelial cancers, accumulation of HA in pericellularstroma and carcinoma cells were the two unfavourable predictors of patient's prognosis. However, no study has predicted whether HA originates from carcinoma or stromal cells and whether increased expression of HAS 1-3 contributes to HA accumulation. In a study conducted on human breast cancer patients, HAS 1-3 was found to be correlated to prognostic factors and patients outcome. Both carcinoma and stroma cells showed HAS – positive. In carcinoma cells, white HAS1 and HA staining correlated with each other, in stroma cells, the staining levels of all HAS isoforms correlated with the stromal HA staining, stroma cell CD4, high relapse rates and short overall survival of the patients. Further, expression levels of stromal HAS1 and HAS2 were related to obesity, large tumor size, lymph node positivity and estrogen receptor negativity. Hence stromal HAS1 and HAS3 were found to be independent prognostic factors in the multivariate analysis. This study has established that increased levels of HAS enzymes contributed to the accumulation of HA in breast cancers and HA may be synthesized in carcinoma and stromal cells. Measurements of HAS enzymes will help to find out tumor aggressiveness towards targeting appropriate therapies[24].

The adverse effects on breast cancer prognosis were based on low mammographic breast density (MBD) and increased HA synthesis. MBD and features were found to be correlated with the expression of HA, CD44, and HAS isoforms. Very low density (VLD) breast tissue showed increased level of HA-positive carcinoma cells and stromal HA, HAS2 and HAS3. Tumor presenting masses had more HA-p carcinoma cells and stromal HAS2 and HAS3. There was a strong relationship between Row MBD and HA expression and synthesis. Further, HA around cancer cells may inhibit chemotherapy agents and antibody treatment [25].

Studies have shown interactions between HA, CD44 and Human epithelial Growth Factor Receptor-2 (HER2) in breast cancer patients and intense stromal HA staining was found to be associated with HER2 and lymph node positivity, large tumor size, poor differentiation, increased BMI, increased relapse rate and shortened overall survival. In stroma cells, CD44 positivity was found to be related to poor differentiation, post menopausal status and triple negative breast carcinoma. Further, CD44 positivity in stroma cells was associated with HER2 positivity, tumor size hormone receptor negativity and shortened overall survival. HA could be one of the factors involved in HER2 positive patients. Hence measurements of HA in breast cancer cells and CD44 in stroma cells may have clinical significance [26].

Tumor associated macrophages (TAM) are major infiltrated around solid tumor cells and accelerate tumor progression due to their immune suppressive functions. High level of HA, a prominent immunoregulator was always considered as a tumor promoter and related to poor prognosis. In breast cancer patients, M2 macrophages were highly correlated to HA expressions. Breast cancer derived HA stimulates M2 like TAM formation and showed multiple effects on macrophages, including up regulating CD204, CD206, IL-10 and TGF-β activating STAT3 signal and suppressing killing capacity. Hence targeting TAM by abrogating HA-CD44 interaction may be a potential strategy for breast cancer immunotherapy [27].

Conclusions

The review article on HA and HASs have brought out the following research findings.

- HAS1 was found to be responsible for cellular HA synthesis detecting cancers of bladder, prostate, mesothelium and breast.
- HAS1 showed association to glycemic stress like MS, inflammation and cancer, modulate cell proliferation, migration and cellular inflammation.

- Although HASs are equally involved in all types of cancer progressions the exact functions of their role still remains to be elucidated.
- HA was found to be a critical component of cancer microenvironment and increases tumor progression and aggressiveness.
- Correlation was found between increased HA levels and CD44 expression and cancer cell migration and aggressive breast cell phenotypes.
- Factors like HA receptors, synthases, degrading enzymes, hyaluronidases were found to be involved in the total modulation of cancer progression.
- HA also showed therapeutic implication in tumor detection and interventions.
- Increased HA levels may inhibit chemotherapy agents and antibody treatment.
- Measurement of HA in cancer cells and CD44 in stroma cells showed clinical significance.
- Future studies should be directed towards establishing reliable and cost effective methods for the measurement of HA and its isoenzymes, establish normal values and add these parameters to the list of cancer detection tests.

Conflict of Interest: None

Reference

[1]. Paul H. Weigel, Vincent C. Hascall, and Markku Tammi. Hyaluronan Synthases (mini review). The Journal of Biological Chemistry. Vo 272, No. 22, Issue of May 30, pp. 13997–14000. (1997)

[2]. Paul H. Weigel. Hyaluronan Synthase: The Mechanism of Initiation at the Reducing End and a Pendulum Model for Polysaccharide Translocation to the Cell Exterior. Int J Cell Biol. 367579 (2015).

[3]. Hanna Siiskonen, Sanna Oikari, Sanna Pasonen-Seppänen, and Kirsi Rilla. Hyaluronan Synthase A Mysterious Enzyme with Unexpected Functions. Front Immunol. 6: 43 (2015).

[4]. Vigetti D, Karousou E, Viola M, Passi A.Analysis of hyaluronan synthase activity. Methods Mol Biol. 1229:201-8 (2015).

[5]. T Usui, F Nakajima, R Ideta, Y Kaji, Y Suzuki, M Araie *et al*. Hyaluronan synthase in trabecular meshwork cells.British Journal of Ophthalmology 87:357-360 (2003).

[6]. Genasetti A, Vigetti D, Viola M, Karousou E, Moretto P, Rizzi M Hyaluronan and human endothelial cell behavior. Connect Tissue Res. 49(3):120-3 (2008).

[7]. Weigel PH, Padgett-McCue AJ, Baggenstoss BA.Methods for measuring Class I membrane-bound hyaluronan synthase activity. Methods Mol Biol. 1022:229-47 (2013).

[8]. Vigetti D, Viola M, Karousou E, De Luca G, Passi A. Metabolic control of hyaluronansynthases. Matrix Biol. 35:8-13 (2014).

[9]. Paul H. Weigel and Paul L. De Angelis. Hyaluronan Synthases: A Decade-plus of Novel Glycosyltransferases. The Journal of Biological Chemistry. Vol. 282, NO. 51, pp. 36777–36781 (2007)

[10]. Delpech B, Bertrand P, Maingonnat C, Girard N, Chauzy C. Hyaluronan Fundamental principles and applications in cancer. J.Intern.Med. 242(1); 41-8 (2003).

[11]. Joanne C. Krupa, David Shaya, Lianli Chi, Robert J. Linhardt, Miroslaw Cygler, Stephen G. Withers *et al*. Quantitative continuous assay for hyaluronan synthase. Anal Biochem. 361(2): 218–225 (2007).

[12]. Kyossev Z, Weigel PH.An enzyme capture assay for analysis of active hyaluronansynthases. Anal Biochem.; 371(1):62-70 (2007).

[13]. Frost GI, Stern R.A microtiter-based assay for hyaluronidase activity not requiring specialized reagents.AnalBiochem. 251(2):263-9 (1997).

[14]. Leslie C.Benchetrit, ShamL.Pahuja, Ernest D.Gray, Ronald D.Edstrom. A sensitive method for the assay of hyaluronidase activity. Analytical Biochemistry. 79, (1–2), 431-437 (1977).

[15]. Haserodt S1, Aytekin M, Dweik RA. A comparison of the sensitivity, specificity, and molecular weight accuracy of three different commercially available Hyaluronan ELISA-like assays. Glycobiology. 21(2):175-83 (2011).

[16]. Adamia S, Maxwell CA, Pilarski LM. Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer.Curr Drug Targets Cardiovasc Haematol Disord. 5(1):3-14 (2005)

[17]. Vigetti D, Passi A.Hyaluronan synthases posttranslational regulation in cancer. Adv Cancer Res. 123:95-119 (2014).

[18]. Adamia S, Pilarski PM, Belch AR, Pilarski LM. Aberrant splicing, hyaluronan synthases and intracellular hyaluronan as drivers of oncogenesis and potential drug targets. Curr Cancer Drug Targets. 13(4):347-61 (2013).

[19]. Urakawa H, Nishida Y, Knudson W, Knudson CB, Arai E, Kozawa E *et al*. Therapeutic potential of hyaluronan oligosaccharides for bone metastasis of breast cancer.JOrthop Res. 30(4):662-72 (2012).

[20]. Kung CI, Chen CY, Yang CC, Lin CY, Chen TH, Wang HS.Enhanced membrane-type 1 matrix metalloproteinase expression by hyaluronan oligosaccharides in breast cancer cells facilitates CD44 cleavage and tumor cell migration. Oncol Rep. 28(5):1808-14 (2012).

[21]. Heldin P, Basu K, Kozlova I, Porsch H.HAS2 and CD44 in breast tumorigenesis. Adv Cancer Res. 123:211-29 (2014).

[22]. Udabage L, Brownlee GR, Nilsson SK, Brown TJ.The over-expression of HAS2, Hyal-2 and CD44 is implicated in the invasiveness of breast cancer.Exp Cell Res. 15;310(1):205-17 (2005).

[23]. Sironen RK, Tammi M, Tammi R, Auvinen PK, Anttila M, Kosma VM.Hyaluronan in human malignancies. Exp Cell Res. 15;317(4):383-91 (2011).

[24]. Auvinen P, Rilla K, Tumelius R, Tammi M, Sironen R, Soini Y *et al*.Hyaluronan synthases (HAS1-3) in stromal and malignant cells correlate with breast cancer grade and predict patient survival.Breast Cancer Res Treat. 143(2):277-86 (2014).

[25]. Masarwah A, Tammi M, Sudah M, Sutela A, Oikari S, Kosma VM *et al*. The reciprocal association between mammographic breast density, hyaluronan synthesis and patient outcome. Breast Cancer Res Treat.153(3):625-34 (2015).

[26]. Auvinen P, Tammi R, Kosma VM, Sironen R, Soini Y, Mannermaa A *et al*. Increasedhyaluronan content and stromal cell CD44 associate with HER2 positivity and poor prognosis in human breast cancer.Int J Cancer. 1;132(3):531-9 (2013).

[27]. Zhang G, Guo L, Yang C, Liu Y, He Y, Du Y. A novel role of breast cancer-derived hyaluronan on inducement of M2-like tumor-associated macrophages formation.

[28]. Oncoimmunology. 29;5(6):e1172154 (2016).