Cerebrospinal Fluid Hyaluronan and Neurofibromatosis Type 2

Prasanth S. Ariyannur¹ · Narendranath Vikkath¹ · Ashok B. Pillai²

Received: 25 February 2018 / Accepted: 15 August 2018 / Published online: 25 August 2018
© Springer Nature B.V. 2018

Abstract

Neurofibromatosis type 2 (NF-2) is associated with mainly three types of recurrent benign tumors restricted to the central nervous system: schwannoma, meningioma and ependymoma. The absence of the protein NF2/Merlin causes an uninterrupted cell proliferation cascade originating from an abnormal interaction between an extracellular mucopolysaccharide, hyaluronan (HA), and schwann cell surface CD44 receptor, which has been identified as one of the central causative factors for schwannoma. Most tumors in NF-2 have a predilection to originate from either arachnoid cap cells or schwann cells of the cisternal portion of nerve rootlets that share a continuous exposure to cerebrospinal fluid (CSF). We hypothesize that the CSF HA may play a role in tumorigenesis in NF-2. In a prospective analysis over a period of one year, the levels of medium to low molecular weight HA (LMW HA) was estimated in the CSF of three subjects with central schwannomas and compared against that of age-sex matched controls, using Cetyltrimethylammonium bromide coupled turbidimetric assay and found to be seventeen-fold higher in the schwannoma subjects compared to the controls. HA was observed to be actively secreted by cultured schwannoma cells isolated from tumor tissues commensurate with their proliferation rate. On cell viability index analysis to compare the cell proliferation of astrocytoma cells with LMW HA vs. oligomeric HA (OHA), we found a decrease in cell proliferation of up to 30% with OHA. The study provides initial evidence that CSF HA may have a central role in the tumorigenesis of schwannoma in NF-2.

Keywords Neurofibromatosis type 2 · NF2 · Merlin · Hyaluronan · CD44 · Cerebrospinal fluid

Introduction

Type 2 Neurofibromatosis (NF-2) is an inherited tumor syndrome of central nervous system (CNS), predisposed by germline mutations of the gene *NF2* [1]. NF-2 is notorious for the occurrence of multiple benign tumors in the brain and spinal cord leading to progressive disability and poor quality of life [2]. The worldwide incidence of the disease is estimated to be 1 in 25,000–50,000 depending on the geographical region and ethnicity [3, 4]. However, it has not been completely studied within the Indian population. The tumors seen in NF-2 are

restricted to schwann cells of cranial and spinal nerves, arachnoid cap cells and ependymal cells giving rise to schwannoma, meningioma and ependymoma respectively; no tumors were found to occur in parenchyma of brain or spinal cord. A high incidence of somatic biallelic mutations of *NF2* gene is described in the sporadic forms of these tumors as well [4, 5].

The exact mechanism of tumorigenesis and progression in NF-2 remains largely unknown. The current knowledge on the tumor suppressive effect of the *NF2* gene product, NF2/Merlin, is based on its role in maintaining the stability of cell adherence junctions and regulating contact-dependent cell growth and proliferation in multicellular organisms [6, 7]. Merlin belongs to a superfamily of proteins called FERM proteins having a common N-terminal domain. Under normal conditions, intracellular Merlin and other FERM proteins in adult schwann cells regulate a proliferation signal cascade initiated by a transmembrane receptor known as CD44. Merlin switches other ERM proteins' binding to CD44 [8, 9] and controls the cell proliferation of schwann cells [10–12]. CD44 is a multi-subunit cell surface receptor for the extracellular matrix (ECM) mucopolysaccharide hyaluronan (HA) [13, 14]. The absence of functional NF2/Merlin protein has a significant role in pathogenesis of benign tumors of schwann cells [15].

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12307-018-0216-2>) contains supplementary material, which is available to authorized users.

✉ Ashok B. Pillai
brashok@aims.amrita.edu

¹ Department of Biochemistry, Molecular Oncology Diagnostics Division, Amrita Institute of Medical Sciences, Amrita Vishwa Vidyapeetham, Kochi 682041, India

² Department of Neurosurgery, Amrita Institute of Medical Sciences, Amrita Vishwa Vidyapeetham, Ponekkara P.O, Kochi 682041, India

The ECM HA binding to CD44 is identified as a primary mechanism in initiating the tumorigenic microenvironment in many other tumors as well [16, 17]. HA is an extracellular mucopolysaccharide synthesized and secreted by normal cells during embryogenesis, tissue remodeling, and repair [14]. Prior to mitosis, HA promotes detachment and imparts mobility to the newly formed cells [18]. While HA around most normal cells degrades, cancer cells retain HA thanks to the action of secreted hyaluronidase (HYAL) enzyme. During the last seventy years, several studies conducted on the tumorigenic role of HA [19] have shown the involvement of HA in invasive and malignant tumors such as CNS gliomas [20], hematologic and other cancers [16] and less association with benign tumors. HA was also suggested as one of the providers of a non-genetic microenvironmental cue for initiation, persistence and progression of cancer [21]. The proliferative role of CD44 has been extensively studied with several targeted chemotherapeutic agents [17]. Abnormal ECM HA-CD44 interaction transcribed to intracellular proliferation pathways, that activate Rac1 and Ras pathway [22], anti-apoptotic pathways such as P13K, MAPK and GTPase signaling streams [13, 23, 24], have not shown to be directly associated with Merlin.

From a clinico-pathologic point of view, a common factor in the multiple benign tumor types of NF-2 is that the Schwann, ependymal and arachnoid cap cells are in constant contact with the cerebrospinal fluid (CSF). Since Merlin is involved in the regulation of HA-CD44 induced proliferative function, we hypothesize that an abnormal interaction of CSF HA and CD44 in these cells could trigger an induction or recurrence of cell proliferation and tumorigenesis in NF-2.

Materials & Methods

Subjects

Schwannoma tumor tissues and CSF samples were obtained from three syndromic NF-2 cases. Evaluation and approval by the Institutional Ethics Committee were obtained prior to the study. The subjects were on regular clinical follow-up through the Amrita Phakomatosis Clinic at Amrita Institute of Medical Sciences. Tissues were obtained during an elective debulking spinal or intracranial surgery as clinically indicated. These three cases were known to have bilateral vestibular and extensive spinal schwannomas and have a well-documented family history (see Table 1). CSF was specifically collected at the time of dural opening/cisternal dissection prior to tumor surgery. The control CSF was obtained from age- and sex-matched normal healthy individuals who underwent surgery for unrelated cause and CSF was obtained as part of routine spinal anesthesia. Detailed written informed consent was obtained prior to the surgery from both cases and control subjects.

Materials

A 200 kDa low molecular weight HA (LMW HA) sample and a 5 kDa oligosaccharide HA (OHA), containing approximately ten monosaccharide units in pure synthesized form were obtained from LifeCore Bioscience, Minneapolis, USA. A different form of HA of 140 kDa (extracted from rooster comb) was obtained from Qingdao Haitao Biochemical Co. Ltd. Qindao, Shandong, China. C6 rat astrocytoma cells were obtained from ATCC laboratories (Manassas Virginia USA). Cetyltrimethylammonium bromide (CTAB), Sodium Chloride, Fetal Bovine Serum (FBS), poly-D-lysine, protease inhibitor cocktail and phosphate buffered saline (PBS) were analytical grade and obtained from Sigma Aldrich (St. Louis, MO USA). Dulbecco's Modified Eagle Media (DMEM), OptiMEM, Hanks Balanced Salt Solution (HBSS), L-glutamine, penicillin-streptomycin antibiotic cocktail, human recombinant insulin and Prestoblu[®] cell viability assay kits were obtained from Life Technologies, PA USA. Collagenase III was obtained from Worthington Biochemical Corp. NJ USA.

Quantification of HA

HA is a linear unbranched acid mucopolysaccharide consisting of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid. To estimate HA, we used a previously described turbidimetric assay method [25] with modifications to suit culture broths [26]. The assay is based on the extent of turbidity formed by HA when incubated with CTAB, a cation surface active agent [27]. Briefly, in each well of a 96-well assay plate, 100 μ l CTAB was added to 200 μ l HA containing standards or solutions prepared in 0.2 M NaCl. The plate was kept for equilibration for 20 min at room temperature. The turbidity was measured as absorbance at 405 nm in a spectrophotometer (Biotek[®]). The standards were made of different molecular weights of HA and assayed in triplicates. The sterile CSF samples collected intraoperatively from patients (cisternal or lumbar puncture) were transported to the laboratory in wet ice, where they were stored at -20°C until assayed.

Cell Culture

Human schwannoma cells were obtained from schwannoma tumor tissue of NF-2 subjects during an elective debulking surgery. The tissue was stored in sterile ice-cold PBS and transported to the tissue culture lab. The preparation of the tissue and primary schwannoma cell culture was done as described previously [15, 28] with minor modifications. Upon arrival in the tissue culture laboratory, the tissue was rinsed with Hanks Balanced Salt Solution and minced into small pieces of less than 1 mm³ using a sterile surgical blade in a glass petri dish under aseptic conditions. The tissue was

Table 1 Subjects and clinical features

Subject ID	Age of Onset	Clinical features
AK-349867	15 years	Presented with bilateral acoustic schwannoma with ependymoma detected on localized symptoms and imaging. Subsequently, frontal meningioma, brachial and facial peripheral schwannomas over a period of 10 years. Elder brother and father had similar occurrences of tumors.
RI- 670025	13 years	Presented with bilateral acoustic schwannomas, and subsequently temporal and basal meningiomas and multiple spinal intradural schwannomas and peripheral nerve sheath tumors in limbs and fingers. No family history.
SS- 1495098	53 years	Presented with bilateral acoustic schwannoma with left sided mass effect and obstructive hydrocephalus. No peripheral lesions. No family history.

Details of the three subjects who were clinically identified to have neurofibromatosis type-2, according to empirical diagnostic criteria. The age of onset is the age at which they presented for the first time

disaggregated into single cells by incubating with DMEM containing 0.05% collagenase III, L-glutamine, 20% FBS, 100 U/ml penicillin-streptomycin and 4 mg/L human recombinant insulin at 37 °C for 30 min. The cells were pelleted by spinning the tissue preparation at 200 × g for 5 min, resuspended in the above media without collagenase III, seeded evenly in a 25 cm² flask (BD Biosciences), which was previously coated with poly-D-lysine and washed. The cells were incubated at 37 °C in a sterile humidified chamber containing 5% CO₂/O₂. The schwannoma cells were cultured for 6 weeks with multiple changes of media. Media was replaced when its color changed. Cell-free media was also incubated as control during the period to rule out microbial contamination. Further culturing for >6 weeks showed morphological signs of senescence. The culture broth was taken every two weeks to estimate the level of HA using CTAB turbidimetric assay (CTA) method as described above. C6 rat astrocytoma cells were used previously to study the effect of HA on cell proliferation [29]. We initiated and maintained a monolayer culture of C6 astrocytoma cells in 25 cm² culture flasks containing DMEM, 2% FBS in a sterile humidified incubator containing 5% CO₂/O₂. Each passage would last for typically 72 h, attaining complete confluence and sub-cultured. After each passage, we estimated the HA levels in the cell culture broth to ascertain the levels of HA secreted.

Cell Viability Assay

To study the effect of OHA on cell proliferation, we incubated C6 rat astrocytoma cells with LMW HA or OHA in three different cell densities as it was described to be different according to earlier studies [12]. Once reaching confluence, cell density was counted and seeded into separate wells of 96-well culture plates in three different cell densities, 40 × 10³, 10 × 10³ and 4 × 10³ cells/well. The cell proliferation was assessed using PrestoBlue Cell viability assay (Life Technologies Inc. PA USA). Prestoblue is a resazurin based solution that functions as a cell viability indicator using the reducing power of cells to quantitatively measure the proliferation of cells. It is a

live cell assay thought to be more sensitive than metabolic or other bioluminescence-based end-point vital cell assays [30]. As compared to end-point assays, a live cell assay would be able to differentiate between active cell proliferation and inactive suspended but viable state of a cell. As described in subsequent sections, this is very important when cell growth is manipulated by different cell density and under the influence of certain external growth regulating agents. According to the manufacturer's method, 10 µl Prestoblue was directly added with fresh media into a confluent 96-well culture plate containing C6 astrocytoma cells (HA treated or control) and incubated for 1 h at 37 °C. The optical density of each well was measured at two different wavelengths (570 nm and 600 nm) in a spectrophotometer (Biotek®). The value is normalized by subtracting A₆₀₀ from A₅₇₀ in the experimental wells and is called the Cell Viability Index (CVI).

Results

Patient Cohort

Three subjects who presented with bilateral acoustic schwannoma and/or had a history of craniospinal multiple schwannoma exclusively within the central nervous system (CNS) at the time of onset of clinical symptoms and during the initial clinical visit were included in the study (See Table 1 for details). These subjects were identified on routine screening of subjects for similar neuro-cutaneous tumors at the Amrita Phakomatoses Clinic during the study period of one year from September 2014 to September 2015. These subjects were also clinically confirmed to have NF-2 according to the NIH, Manchester as well as National Neurofibromatosis Foundation (NNFF) criteria [31].

Performance Characteristics of CTA

The CTA reaction formed a turbid complex within five minutes of incubation of CTAB with two different forms of

LMW HA, one with 140 kDa and another with 200 kDa. However, turbidity was not observed with OHA (5 kDa) even at a concentration of 1 mg/ml and after one hour of incubation at room temperature (Fig. 1a). With LMW HA, we found that the assay is sensitive to detect up to 0.02 mg/ml (approx. 100 μ M for 200 kDa HA), (Fig. 1b). This is in the detection range as observed in previous CTA studies [25]. The detection limits and sensitivity of CTA in our studies were found to be similar to that of advanced methods such as size-exclusion column chromatography Multi-Angle Laser Light Scattering Detection method (SEC-MALLS) [32].

Comparison of HA Levels in CSF

The concentration of HA in CSF of the study as well as control subjects using CTA is given in Table 2. CSF of one subject with multiple spinal and peripheral schwannomas had a very high value of HA level, approximately 150 times than that of the mean values of the control. Apart from that, at least 17-fold increase in the mean values of HA levels was observed in study CSF compared to control (mean value of 1.395 ± 0.474 mg/ml in NF-2 CSF vs. 0.0815 ± 0.038 mg/ml in Normal CSF).

Secretion of HA by Cultured Human Schwannoma Cells Vs. C6 Astrocytoma Cells

Culturing of commercially available schwann cells required special growth inducers such as Forskolin/hereregulin (as detailed in the discussion) and growth factors. Similarly, primary schwann cells culture also require specific growth factors and/or growth inducers as these cells are extremely slow growing and time consuming. In our experiments, specific proliferative

agents could not be added into the schwannoma tumor cell culture as that might not replicate the innate condition and confound the results. Instead, we used freshly isolated and cultured human schwannoma cells and did not use commercially available schwannoma cell lines or primary schwann cells to check the HA secretion. Human schwannoma cells exhibited steady but slow growth and took up to 6 weeks to attain 80% confluence with three changes of culture media, approximately 2 weeks' culture cycle per change. After 6 weeks, the cells showed signs of senescence, such as cytoplasmic granulations and reduced or erratic cellular extensions. Due to slow growth, a parallel cell-free culture media was also incubated during the same period to assess microbial contamination as microbial cells are also known to synthesize HA. The culture broth was sampled at the second and fourth week and assessed for HA levels (see Fig. 2a).

During the first fortnight, HA was found to be increased by about 50% of that of the cell-free media and during the second fortnight period (2nd to 4th week), the increase in HA concentration was twice that of cell-free media and of the 2nd week. The media was sterile during the entire period. The HA concentration of culture broth of C6 astrocytoma cells doubled every 72 h (Fig. 2b). Compared to rapidly proliferative C6 rat astrocytoma cells (Fig. 2b), the doubling time of HA concentration in the culture broth was found to be longer in slow growing primary human schwannoma cells (Fig. 2a) (72 h vs. 4 weeks). However, we show that concentration of HA in the culture broth of both the cell cultures at the end of the experiment was similar, irrespective of their distinct growth rate. This was suggestive of a consistent synthesis and secretion of HA by both the tumor cells during their proliferation and that the rate of synthesis and secretion of HA was dependent on the rate of cell proliferation in both these cell types.

Fig. 1 Performance characteristics of HA CTA. Graphs showing absorbance (at 405 nm) of products of CTA with different forms of HA at various concentrations. Left side graph (Fig. 1a) shows the difference in absorbance of 200 kDa HA (rhomboid dots) compared to that of 5 kDa OHA (triangular dots). Right side graph (Fig. 1b) shows the detection limit of 200 kDa HA. The details of the assay and detection are given in the 'Materials and Methods' section. Each assay point is the average of

triplicates and error bar depicts SD. The error bars of absorbance of oHA are shown but not visible due to very small variance. Correlation coefficient (R^2 values) is given on the lateral side of the graph. A linear correlation of approx. 0.99 is achieved with LMW HA at the tested concentration range. For 200 kDa HA, the linear correlation of absorbance was present up to 0.02 mg/ml, which amounts to 100 μ M

Table 2 Comparison of HA levels in the CSF of NF-2 vs. matched Controls

Name	Age	CSF HA conc. (mg/ml)	Mean $\pm$ s.d
NF2			
RI	20	1.06	1.395 $\pm$ 0.474
SS	60	1.73	
AK	23	12.6*	
Control			
Kv	24	0.078	0.0815 $\pm$ 0.038
Ew	20	0.052	
Pr	22	0.136	
Xx	62	0.06	

The mean values of the HA levels in CSF samples of NF2 vs. age-sex matched control subjects are compared. The starred value found in one subject had multiple spinal schwannomatosis at the time of CSF collection and repeated assay showed similar values. This value was not taken for calculation of mean and standard deviation

Effect of LMW HA and OHA in Cell Proliferation of C6 Rat Astrocytoma Cells

Because of the extremely slow growth rate in an isolated in vitro culture in a most innate condition, human primary schwannoma cells could not be tested for the effects of externally added HA. The cell proliferation of C6 astrocytoma cells was determined using CVI, a live cell assay. To differentiate actively proliferating cells and inactive suspended, but viable cells, the CVI assay had to be standardized against cells incubated with PBS, where the cells cannot survive. When the cells were incubated with only PBS, very low CVI was obtained, irrespective of the seeding cell-density (see comparative CVI in supplementary figures), showing the specificity of the live cell viability assay. When cultured at higher seed density (40×10^3 cells/well), cells were overcrowded limiting

the nutrient resources and detached off the culture plate before 72-h incubation period, CVI was same as that of PBS. Thus, the CVI was lowest making it infeasible to ascertain the effect in different conditions (irrespective of the presence of HA) (see supplementary fig. S1). When cells were cultured at the lowest seed density (4×10^3 cells/well), the CVI values of negative control (PBS incubated set) were moderately high, with values almost similar to that of CM + OHA (Fig. S3). This could be due to the survival ability of highly proliferative C6 astrocytoma cells in nutrient-free harsh conditions up to 72-h period in viable suspended state. Moreover, the presence of LMW HA/OHA might have mimicked those harsh nutrient-free conditions even in the presence of a complete media. When cells were seeded in two different cell densities and incubated with serum-free media, there was no significant change in cell proliferation with the addition of HA. This was because, the cells remained in a viable suspended state without active cell division in the serum free media, demonstrating that the effect of HA oligomers is specific to the conditions where the cells are actively dividing.

Out of the three different seed cell densities, wells containing lower cell densities showed significant difference in CVI upon incubation with HA (either LMW HA or OHA) for 72 h. The CVI was found to be significantly decreased by approximately 30% (p value <0.01 , Student's t test) when OHA was incubated in lower cell densities (those wells seeded with 10×10^3 , 4×10^3 per well) (see Fig. 3).

Discussion

This is the first report of very high levels (approximately 17-fold) of HA in CSF of subjects with CNS schwannoma. CSF HA levels were assessed earlier in a broad group of patients who belonged to CNS inflammatory disorders, tumors and conditions of obstructions of CSF flow [33], with none

Fig. 2 Comparison of HA secretion by Human Schwannoma cells vs. C6 astrocytoma cells. A: Human schwannoma cells were isolated and cultured, and HA was assayed using CTA. The values are the average of triplicates, error bar shows standard deviation and stars represent statistically significant increase at 4th week (p value <0.001 , Student's t test). B: C6 astrocytoma cells were cultured as described in the 'Materials

and Methods' section. HA was assayed using CTA at complete confluence of 72 h. Of note, the mean concentration of HA in the culture broth at the end of experiments was 0.97 ± 0.075 mg/ml for schwannoma cultured plate and 0.72 ± 0.08 mg/ml for C6 astrocytoma cell cultured plates

Fig. 3 Effect of LMW HA and OHA in proliferation of C6 astrocytoma cells. Bar graph depicting the cell viability index (CVI) of C6 astrocytoma cells at two different seed cell densities (10×10^3 or 4×10^3 per well) when incubated with 100 $\mu\text{g/ml}$ of either 200 kDa LMW HA (solid grey) or 5 kDa oHA (horizontal stripes) for 72 h. The CVI values are the average of triplicate cell culture assays, error bars are the standard deviation of triplicates. Single star (*) and double star (**) depicts a

statistically significant decrease in CVI ($p < 0.01$, Student's *t* test) found when OHA incubated with any of the given cell densities. About 32 and 28% decrease in CVI was observed at 10×10^3 per well and 4×10^3 per well initial seed density, respectively. LMW HA showed significant decrease in cell density only at 10×10^3 per well. More details are described in the 'Materials and Methods' section and [supplementary figures](#)

collected from central schwannoma cases. Furthermore, the HA levels in the normal CSF samples of our study (mean value of ~ 82 mg/L) were over 1000-fold higher than that described in the previous study (mean value 50–70 $\mu\text{g/L}$). This may partly be due to the difference in sample preparation and assay method. A two-step process including an initial gel chromatographic purification of HA followed by radioimmunoassay in the previous study [34] may have resulted in the loss of a significant amount of HA in the initial step. The current method is a one-step assay that does not require a purification step and devoid of any intermediate molecule (such as an antibody for radioimmunoassay) for detection. The higher mean value of CSF HA in our study, thus, suggests that the current method might be more sensitive than radioimmunoassay [25].

In continuation to previous studies on the pathogenesis of schwannomas linked to the tumorigenic landscaping role of HA [16, 21] and deranged HA-CD44 interaction in NF-2 [8, 9, 35, 36], we have identified for the first time that cultured primary human schwannoma cells from NF-2 secrete LMW HA commensurate to cell proliferation. Unlike schwannoma cell lines in previous studies showing higher affinity for HA [35], we cultured cells from schwannoma tumor sections and identified an increased secretion of HA. Previous studies have shown that forskolin and β -heregulin (hereafter refer as For/Her) have synergistic effect on activating cell proliferation via increasing cyclic AMP levels in the cells and mitogen activated kinase kinase (MEK) and extracellular signal regulated kinase (ERK) pathway, respectively, when primary schwannoma

cells are cultured in serum free media [37]. But a recent study has shown that the inductive effect of ERK/MEK as well as phospho ERK/MEK was observed only in cultured primary schwann cells and immortalized schwannoma cell lines. The expression of these signals was very high in cultured primary schwannoma cells and had no change irrespective of the presence of For/Her in the culture media (depicted in Fig. 3 of reference [38]). This shows that schwannoma cells obtained from fresh tumor tissues (which we adopted in the current experiment) would have high level of MEK/ERK activation innately, hence it was not required to add an inducing agent such as For/Her into the culture media.

Our primary intention of growing schwannoma cells in an in vitro culture environment was to explore whether isolated primary schwannoma cells can secrete HA, or not. HA, being a large extracellular anchor molecule for many matrix and functional proteins and polysaccharides, could be secreted by adjacent stromal supportive cells such as fibroblast cells, tumor infiltrating immunocytes etc. as a general tissue reactionary effect. Also, apart from the identification of an optimal condition for schwannoma cell proliferation, the purpose of culturing schwannoma cells in vitro was to analyze their self-propagation potential via secreted HA, under normal physiological conditions. Moreover, we were better able to identify the difference in the rate of HA secretion between benign schwannoma cells vs. malignant astrocytoma cells (doubling time of 2 weeks vs. 72 h) without the presence of active cell proliferator agents such as For/Her or other growth factors. Perhaps, this might also explain a potentially critical role of

other stromal cells in perpetuating the cell proliferation. In order to explore the direct anti-proliferative effect of OHA or any other inhibitory agents in schwannoma, it should be done in a mixed-cell culture system. With the innate condition, our data demonstrate for the first time that human schwannoma cells secrete LMW HA. The implications of a large elevation in CSF HA in NF-2 in this context suggest an important role played by HA in the central schwannoma tumorigenesis, irrespective of whether it is cause or effect. The exact mechanism needs to be evaluated further in primary human schwannoma cells, and other central tumor cell types, such as meningioma, and ependymoma cells.

Several methods of inhibition of endogenous HA-CD44 interaction have been previously reported. In the context of the tumor suppressive effect of NF2/Merlin protein, it was identified that the inhibitors of HA-CD44 binding (either HA or soluble CD44) can significantly reduce the cell proliferation in Merlin deficient (*NF2*^{-/-}) rat schwannoma cell lines [12]. Perturbation of HA-CD44 interaction was suggested in other studies as well [39] and has been identified as a potential target for cancer therapy [23]. It has also been found that the tumor suppressor action of Merlin is to counter the cell proliferative trigger by HA-CD44 interaction [40]. Furthermore, medium molecular weight HA MMW HA (molecular weight > 800–1000 kDa) was found to have a dominant role in fibrocyte growth than LMW HA (< 250–500 kDa) [41]. Cancer cells, especially glioma cells, are found to secrete predominantly MMW HA [16, 29]. However, previous studies showed that the proliferative effect of endogenous MMW HA could not be enhanced by externally supplied MMW HA, though it can be suppressed by externally added OHA [29]. Replacing multivalent endogenous HA with monovalent low affinity oligosaccharides has been shown earlier in TA3/St murine mammary carcinoma and HCT 116 human colon carcinoma cells [39]. Although one previous study had shown the inhibitory effect of OHA in soft-agar colony formation of C6 astrocytoma cells using a tetrazolium dye (MTT) based end-point cell viability assays, it was not demonstrated in monolayer cultures [29]. In the current study, we exhibited this effect with a live cell proliferation assay on a monolayer culture using Prestoblue based method, which was more sensitive than the end-point cell viability assays. This is the first study that demonstrates the inhibitory effect of OHA and LMW HA in the proliferation of C6 astrocytoma cells in monolayer cultures.

We analyzed the effect of HA in three different cell densities as earlier studies have shown that different seed densities in cell culture would determine the proliferative effect of HA in the absence of a functional Merlin protein [12]. One of the implications of this study is that, with higher cell density (10×10^3 /well), the effect of OHA was lesser compared to lower cell density (4×10^3 /well). Perhaps this shows that the effect of OHA might be lower in malignant tumors with rapid

cell proliferation and high cell density than in benign tumors, such as schwannomas, where the cell proliferation and cell density might only be moderate. Specific inhibitors of HA-CD44 interaction, such as OHA, may thus be better suited for low cell density tumors such as schwannoma and meningioma rather than glioma. Confirmation of this finding might involve incubating different HA with cultured primary schwannoma cells at different cell densities or in animal models of multiple tumor types. From a clinical point of view, this finding of significant elevation of CSF HA combined with the observation that most NF-2 tumors develop in relation to CSF space, specific inhibitors (OHA or other agents such as HA binding agents) can be tested via intrathecal delivery for its therapeutic potential in dampening disease progression.

Conclusion

Significant increase in CSF HA levels in NF-2 subjects suggest that ECM HA might be a prominent proliferative trigger for the tumorigenesis of NF-2 tumors; further comparative studies on human CSF samples from different CNS conditions are required to explore whether its potential as a novel marker for recurrence of NF-2 related tumors. Schwannoma cells secrete HA commensurate to its slow growth. The role of HA in schwannoma cell proliferation and the inhibitory effect of OHA are dependent upon the cell density and growth of the cells. Further studies to explore the possibilities of an extracellular inhibitory molecule based on the action of HA in schwannoma cells and other cell types of NF-2 are warranted.

Acknowledgements The authors wish to acknowledge Mrs. Gisha Girish, Mrs. Sikha, and Mrs. Anuja for their contributions in the coordination of Amrita Phakomatosis Clinic, Dr. Jeyakumar Rengasamy and Dr. Krishnakumar Menon at Amrita Center for Nanosciences and Molecular Medicine (ACNSMM) for facilitating the procurement of hyaluronan crude extracts and providing C6 astrocytoma cell lines.

Author Contributions PSA designed and conducted the experiment, compiled, analyzed and interpreted the data and wrote the manuscript. NV collected the samples, cultured cells and assisted PSA in compiling and analyzing the data. ABP initiated the concept, reviewed data and edited manuscript.

Funding Information The study was funded by Indian Council of Medical Research (54/18/2011-HUM/BMS) to ABP.

Compliance with Ethical Standards

Disclosure of Conflict of Interest The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Informed Consent An oral and written informed consent was obtained from all the human subjects participated in the study before collecting the samples.

Ethical Approval Institutional Ethics Committee of Amrita Institute of Medical Sciences convened on the 11th of January 2014 reviewed the project protocol with data sheets and consent forms presented by the co-investigator and granted ethical clearance on official letter dated 13th of Jan 2014.

References

- Rouleau G, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B (1993) Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 363(6429):515–521. <https://doi.org/10.1038/363515a0>
- Evans DG (2009) Neurofibromatosis type 2 (NF2): a clinical and molecular review. *Orphanet J Rare Diseases* 4:16. <https://doi.org/10.1186/1750-1172-4-16>
- Evans DGR, Moran A, King A, Saeed S, Gurusinge N, Ramsden R (2005) Incidence of vestibular schwannoma and neurofibromatosis 2 in the north west of England over a 10-year period: higher incidence than previously thought. *Otol Neurotol* 26(1):93–97
- Baser ME, Contributors to the International NFMD (2006) The distribution of constitutional and somatic mutations in the neurofibromatosis 2 gene. *Hum Mutat* 27(4):297–306. <https://doi.org/10.1002/humu.20317>
- Bianchi A, Hara T, Ramesh V, Gao J, Klein-Szanto A, Morin F, Menon A, Trofatter J, Gusella J, Seizinger B (1994) Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types. *Nat Genet* 6(2):185–192. <https://doi.org/10.1038/ng0294-185>
- Stamenkovic I, Yu Q (2010) Merlin, a “magic” linker between the extracellular cues and intracellular signaling pathways that regulate cell motility, proliferation, and survival. *Curr Protein Pept Sci* 11(6):471–484
- Schroeder RD, Angelo LS, Kurzrock R (2014) NF2/merlin in hereditary neurofibromatosis 2 versus cancer: biologic mechanisms and clinical associations. *Oncotarget* 5(1):67–77. <https://doi.org/10.18632/oncotarget.1557>
- Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 126(2):391–401. <https://doi.org/10.1083/jcb.126.2.391>
- Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S, Tsukita S (1998) Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J Cell Biol* 140(4):885–895. <https://doi.org/10.1083/jcb.140.4.885>
- Curto M, McClatchey A (2008) Nf2/Merlin: a coordinator of receptor signalling and intercellular contact. *Br J Cancer* 98(2):256–262. <https://doi.org/10.1038/sj.bjc.6604002>
- McClatchey A, Giovannini M (2005) Membrane organization and tumorigenesis—the NF2 tumor suppressor, Merlin. *Genes Dev* 19(19):2265–2277. <https://doi.org/10.1101/gad.1335605>
- Morrison H, Sherman L, Legg J, Banine F, Isacke C, Haipek C, Gutmann D, Ponta H, Herrlich P (2001) The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 15(8):968–980. <https://doi.org/10.1101/gad.189601>
- Ponta H, Sherman L, Herrlich P (2003) CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 4(1):33–45. <https://doi.org/10.1038/nrm1004>
- Joy RA, Vikkath N, Ariyannur PS (2018) Metabolism and mechanisms of action of hyaluronan in human biology. *Drug Metabolism and Personalized Therapy* 33(1):15–32. <https://doi.org/10.1515/dmpt-2017-0031>
- James M, Lelke J, Maccollin M, Plotkin S, Stemmer-Rachamimov A, Ramesh V, Gusella J (2008) Modeling NF2 with human arachnoidal and meningioma cell culture systems: NF2 silencing reflects the benign character of tumor growth. *Neurobiol Dis* 29(2):278–292. <https://doi.org/10.1016/j.nbd.2007.09.002>
- Toole B (2004) Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* 4(7):528–539. <https://doi.org/10.1038/nrc1391>
- Zöller M (2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 11(4):254–267. <https://doi.org/10.1038/nrc3023>
- Toole BP (2001) Hyaluronan in morphogenesis. *Semin Cell Dev Biol* 12(2):79–87. <https://doi.org/10.1006/scdb.2000.0244>
- Stem R (2009) Hyaluronan in cancer biology. *Hyaluronan in Cancer Biology*, vol 1, 1st edn. Academic Press, Elsevier Inc, San Diego
- Koochekpour S, Pilkington G, Merzak A (1995) Hyaluronic acid/CD44H interaction induces cell detachment and stimulates migration and invasion of human glioma cells in vitro. *Int J Cancer* 63(3):450–454. <https://doi.org/10.1002/ijc.2910630325>
- Weaver V, Gilbert P (2004) Watch thy neighbor: cancer is a communal affair. *J Cell Sci* 117(Pt 8):1287–1290. <https://doi.org/10.1242/jcs.01137>
- Bourguignon L, Zhu H, Zhou B, Diedrich F, Singleton P, Hung M (2001) Hyaluronan promotes CD44v3-Vav2 interaction with Grb2-p185(HER2) and induces Rac1 and Ras signaling during ovarian tumor cell migration and growth. *J Biol Chem* 276(52):48679–48692. <https://doi.org/10.1074/jbc.M106759200>
- Misra S, Heldin P, Hascall V, Karamanos N, Skandalis S, Markwald R, Ghatak S (2011) Hyaluronan-CD44 interactions as potential targets for cancer therapy. *FEBS J* 278(9):1429–1443. <https://doi.org/10.1111/j.1742-4658.2011.08071.x>
- Turley EA, Noble PW, Bourguignon LY (2002) Signaling properties of hyaluronan receptors. *J Biol Chem* 277(7):4589–4592. <https://doi.org/10.1074/jbc.R100038200>
- Chen Y-H, Wang Q (2009) Establishment of CTAB Turbidimetric method to determine hyaluronic acid content in fermentation broth. *Carbohydr Polym* 78(1):178–181. <https://doi.org/10.1016/j.carbpol.2009.04.037>
- Song J-M, Im J-H, Kang J-H, Kang D-J (2009) A simple method for hyaluronic acid quantification in culture broth. *Carbohydr Polym* 78(3):633–634. <https://doi.org/10.1016/j.carbpol.2009.04.033>
- Di Ferrante N (1956) Turbidimetric measurement of acid mucopolysaccharides and hyaluronidase activity. *J Biol Chem* 220(1):303–306
- Kaewkhaw R, Scutt AM, Haycock JW (2012) Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. *Nat Protoc* 7(11):1996–2004. <https://doi.org/10.1038/nprot.2012.118>
- Ward J, Huang L, Guo H, Ghatak S, Toole B (2003) Perturbation of hyaluronan interactions inhibits malignant properties of glioma cells. *Am J Pathol* 162(5):1403–1409. [https://doi.org/10.1016/s0002-9440\(10\)64273-3](https://doi.org/10.1016/s0002-9440(10)64273-3)
- Boncler M, Rozalski M, Krajewska U, Podsedek A, Watala C (2014) Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *J Pharmacol Toxicol Methods* 69(1):9–16. <https://doi.org/10.1016/j.vascn.2013.09.003>

31. Baser ME, Friedman JM, Joe H, Shenton A, Wallace AJ, Ramsden RT, Evans DGR (2011) Empirical development of improved diagnostic criteria for neurofibromatosis 2. *Genetics In Medicine* 13: 576. <https://doi.org/10.1097/GIM.0b013e318211faa9>
32. Baggenstoss BA, Weigel PH (2006) Size exclusion chromatography-multiangle laser light scattering analysis of hyaluronan size distributions made by membrane-bound hyaluronan synthase. *Anal Biochem* 352(2):243–251. <https://doi.org/10.1016/j.ab.2006.01.019>
33. Laurent UBG, Laurent TC, Hellsing LK, Persson L, Hartman M, Lilja K (1996) Hyaluronan in human cerebrospinal fluid. *Acta Neurol Scand* 94:194–206. <https://doi.org/10.1111/j.1600-0404.1996.tb07052.x>
34. Laurent UB, Tengblad A (1980) Determination of hyaluronate in biological samples by a specific radioassay technique. *Anal Biochem* 109(2):386–394
35. Sherman L, Skroch-Angel P, Moll J, Schwechheimer K, Ponta H, Herrlich P, Hofmann M (1995) Schwann cell tumors express characteristic patterns of CD44 splice variants. *J Neuro-Oncol* 26(3): 171–184. <https://doi.org/10.1007/bf01052620>
36. Sherman L, Xu H, Geist R, Saporito-Irwin S, Howells N, Ponta H, Herrlich P, Gutmann D (1997) Interdomain binding mediates tumor growth suppression by the NF2 gene product. *Oncogene* 15(20): 2505–2509. <https://doi.org/10.1038/sj.onc.1201418>
37. Schworer CM, Masker KK, Wood GC, Carey DJ (2003) Microarray analysis of gene expression in proliferating Schwann cells: synergistic response of a specific subset of genes to the mitogenic action of heregulin plus forskolin. *J Neurosci Res* 73(4): 456–464. <https://doi.org/10.1002/jnr.10679>
38. Neff BA, Voss SG, Schmitt WR, Driscoll CL, Link MJ, Beatty CW, Kita H (2012) Inhibition of MEK pathway in vestibular schwannoma cell culture. *Laryngoscope* 122(10):2269–2278. <https://doi.org/10.1002/lary.23472>
39. Ghatak S, Misra S, Toole B (2002) Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. *J Biol Chem* 277(41):38013–38020. <https://doi.org/10.1074/jbc.M202404200>
40. Bai Y, Liu YJ, Wang H, Xu Y, Stamenkovic I, Yu Q (2007) Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressor activity of merlin. *Oncogene* 26(6): 836–850. <https://doi.org/10.1038/sj.onc.1209849>
41. Maharjan AS, Pilling D, Gomer RH (2011) High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. *PLoS One* 6(10):e26078. <https://doi.org/10.1371/journal.pone.0026078>