

Variations in Contents of Hyaluronan in the Peritumoral Micro-Environment of Human Chondrosarcoma

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ABSTRACT: A concept consolidated in recent years is that tumor growth depends to a great extent on the micro-environment surrounding the tumor, which has a fundamental role in tumor progression and in determining the effectiveness of therapies. Our analysis focuses on chondrosarcoma, the second primary malignant bone tumor, resistant to both chemotherapeutic and radiation therapy. We quantified hyaluronan, one of the main components of the extracellular matrix, with the aim of comparing its amount in the connective tissue surrounding the tumor with intra-tumoral tissue and healthy fascia of the same anatomic district, viewed as a health control. We demonstrate that hyaluronan increased significantly in the peritumoral stroma compared with the healthy fascia, which showed an average amount according to the physical characteristics of body districts by a mean value of 26.9 $\mu\text{g/g}$. In the peritumoral stroma, the mean hyaluronan content reached 132.6 $\mu\text{g/g}$ (mean value of 63.2 $\mu\text{g/g}$). The *p*-value was less than 0.01, showing a highly significant statistical difference. Surprisingly, no significant differences were detected as a function of age, gender, or tumor grade. The levels of hyaluronan were comparable in peritumoral and tumor tissues, although there were differences depending on the state of necrosis. In addition, data on the expression of hyaluronic acid synthetase showed a decrease of about 50% in peritumoral and tumor tissues, indicating alterations in hyaluronan turnover and synthesis. This work demonstrates a variation in hyaluronan contents around the chondrosarcoma, likely correlated with the aggressiveness and resistance to chemotherapy of these tumors. Statement of Clinical Significance: Deeper knowledge about the composition of the peritumoral stroma, rich in extracellular matrix, will enhance better study and understanding of the metastatic potential of tumors and their prognostic indices. © 2018 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 37:503–509, 2019.

Keywords: hyaluronan; chondrosarcoma; peritumoral stroma; fascia; tumor micro-environment; malignant bone tumor; grade

Many authors have demonstrated that the composition of the micro-environment surrounding a tumor is closely related to the metastatic potential of the disease and to modulation of its progression.^{1–3} The peritumoral stroma contains a pool of genetically stable stromal cells, including mesenchymal supporting cells (e.g., fibroblasts and adipocytes), cells of the vascular system, and cells of the immune system, in an abundant extracellular matrix (ECM)⁴ composed of a wide range of molecules, including glycoproteins, polyglycans, polysaccharides and fibrous proteins (e.g., collagen, fibronectin, laminin).⁵ The stroma also contains several peptide factors (growth factors, chemokines, cytokines, antibodies) and metabolites, which influence the progress of the disease.⁴ One of the main components of the matrix is hyaluronan (HA), a glycosaminoglycan which plays a demonstrated fundamental role in influencing all biological activities in the ECM.⁶

Accumulation of hyaluronan near tumors has already been observed in many such diseases, such as prostate cancer⁷ and breast cancer⁸: as the tumor progresses, the stromal and malignant cells influence each other, causing modifications in the cytological and molecular composition of the stroma.⁹ Increased HA content has also been confirmed in *in vitro* studies,

showing how cultured human tumor cell lines can stimulate the synthesis of glycosaminoglycans when associated with a fibroblast co-culture.¹⁰ A recent work by Turley et al.¹¹ showed how metastatic cells acquire the ability to synthesize and process HA independently, thus creating its own HA-rich micro-environment and allowing it to survive in the circulation, metastasize to ectopic sites, and escape therapeutic intervention. The amount of HA also varies according to cell type and degree of tumor cell differentiation: High HA levels in the peritumoral stroma are associated with poorly differentiated tumors and clinical aggressiveness in human adenocarcinomas.^{12,13} The dynamic interplay between hyaluronan synthesis and degradation greatly influences the aggressiveness of malignant cells.¹³ The contribution of hyaluronan to metastasis has also been confirmed by treatment with 4-methylumbelliferone (4-MU), a hyaluronan synthase suppressor which reduces the formation of ECM by reducing proliferation, invasion, and migration.¹⁴ The effects of 4-MU as an antitumor effector on rat chondrosarcoma cells has recently been reported, highlighting the possible role of HA in chondrosarcoma metastasis for the first time.¹⁵ In addition, high HA content may be associated with resistance to chemotherapy treatment, as drugs cannot easily penetrate the tumor and membrane proteins involved in the drug transport mechanism increase their activity.^{16–18} Chondrosarcoma (CS) is a malignant bone tumor which is currently unresponsive to chemotherapy and conventional radiotherapy, with few exceptions (mesenchymal CS and dedifferentiated CS),¹⁹ so that

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surgical resection is the main therapeutic option.^{20–22} Understanding the role of HA may open up new therapeutic scenarios: the enzymes involved in its turnover and the receptors with which it interacts have recently been identified as new and effective therapeutic targets in many types of cancers²³ which are not generally responsive.

This study quantifies HA in the peritumoral area, analyzing it to ascertain whether it is altered and/or is correlated with the degree of aggressiveness of the tumor. As a healthy control, the muscular fascia of the same anatomical district of all patients was analyzed: The fasciae are identified as a containment structure for soft tissue tumors, and are essential in distinguishing benign lesions from malignant ones.^{24–26} In addition, there is a similarity of composition between fascia and peritumoral stroma, especially as regards the abundance of fibroblasts and the composition of the extracellular matrix. Pathologic processes involving inflammation and tissue fibrosis result in stiff connective tissue, and these factors are also important in cancer biology.²⁷ For all these reasons, the fasciae may be considered a good control group.

METHODS

Sample Collection

This study was approved by the Institutional Ethical Review Board (approval no. 4306/AO/17). The Institute's ethical regulations on research conducted on human tissues were followed, and written informed consent was obtained from each donor.

Twelve volunteer patients were enrolled, nine men and three women, average age 60 ± 19 . Admission criteria included verified histological diagnosis of chondrosarcoma, complete preoperative imaging, and a surgical excision of the chondrosarcoma according to tumor grade at the Department of Orthopedics and Orthopedic Oncology, University of Padova, Italy. We excluded patients with unresectable tumors, those with either local recurrent chondrosarcoma or previously treated elsewhere (i.e., already with some kind of alteration of their anatomical compartments), and patients with infectious diseases.

From each surgical specimen, $1 \times 1 \text{ cm}^2$ samples of intratumoral tissue were collected from the peritumoral micro-environment and from healthy muscular fascia in the same anatomical district (Fig. 1). Details of samples are listed in Table 1. The samples were transported to the laboratory in phosphate buffered saline within less than one hour of surgical removal and were divided into two half-sections: One was either frozen or used fresh for HA quantification, and the other was formalin-fixed for histology. If there was enough material, a third sample (frozen or kept in PBS and used fresh) was used for real-time PCR analysis to detect the expression of mRNA for hyaluronan synthase 2 (HAS2).

Isolation and Quantification of Hyaluronan

The Purple-Jelley HA assay (Biocolor Ltd.) was used to measure HA contents, as described previously.²⁸ Briefly, samples weighing typically $150 \pm 50 \text{ mg}$ were taken from all patients. The samples were then cut into small fragments with a surgical scalpel (Fig. 2), and digested overnight at 55°C in 50 mM TRIS-HCl (pH 7.6) containing Proteinase K

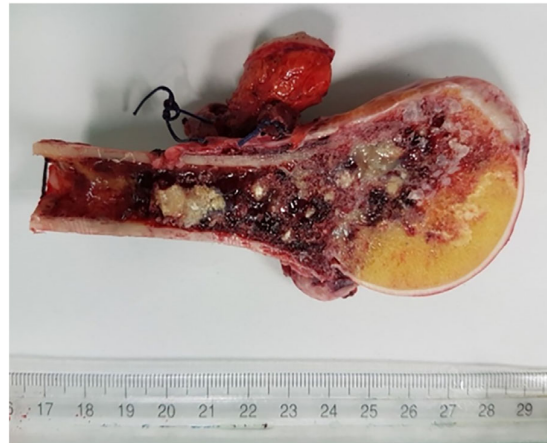


Figure 1. Grade 2 chondrosarcoma of proximal humerus: Gross pathology of surgical specimen in sagittal view shows extreme heterogeneity of tumor and pathologic fracture with soft tissue extension.

(Sigma-Aldrich S.R.L., Milan, Italy). After centrifugation at $12,000 \text{ rpm}$ for 10 min , the supernatants were mixed with GAG precipitation reagent, and the resulting residues were mixed in NaCl and cetylpyridinium chloride (CPC) in water. After these steps had been repeated and the total GAG content had been recovered, HA was isolated by the addition of $>98\%$ ethanol, and the pellets obtained after centrifuge were hydrated in $100 \mu\text{l}$ of water. This was followed by colorimetric analysis: $200 \mu\text{l}$ of Purple Dye reagent were added to $20 \mu\text{l}$ aliquots of test samples, and the absorbance value at 650 nm was read on a Mithras LB 940 spectrophotometer (Berthold Technologies, Wildbad, Germany) in 96-microwell plates. Extraction was carried out twice for each sample.

The absorbance values of the standard curve obtained with the HA standard ($200 \mu\text{g/ml}$) provided with the kit, was converted into μg HA contained in the total volume of $100 \mu\text{l}$. Lastly, the μg HA extracted from the initial weight of wet tissue was converted to the μg of HA contained in 1 g of wet tissue. The average of at least two measurements \pm standard deviation was obtained for each sample.

As control data, reference was made to our previous results from four samples of adult human skin ($176.2 \pm 12.3 \mu\text{g}$ of hyaluronan per gram of skin), thus validating the extraction method.²⁸

Table 1. Details of Sample Collection

Patient	Tumor Grade	Gender	Age	Region
1	Low grade	M	64	Hip
2	High grade	M	15	Hip
3	High grade	M	40	Hip
4	High grade	M	75	Hip
5	High grade	M	45	Hip
6	High grade	M	68	Hip
7	Low grade	F	60	Foot
8	High grade	F	98	Foot
9	Low grade	F	59	Humerus
10	High grade	M	66	Humerus
11	Low grade	M	64	Scapula
12	Low grade	M	63	Scapula

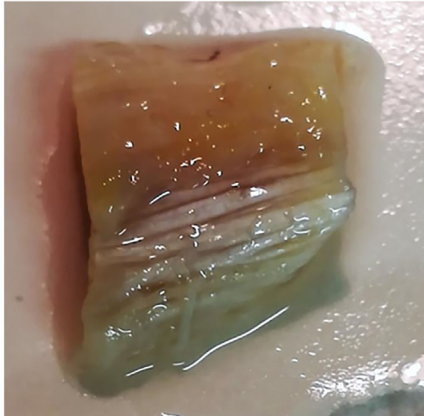


Figure 2. Fascial tissue: Macroscopic image of human fascial sample cut in a small fragment by scalpel before digestion by Proteinase K.

Immunohistochemistry: Analysis and Quantification of HABP (Hyaluronic Acid Binding Protein)

Formalin-fixed samples were dehydrated in graded ethanol, embedded in paraffin, and cut into sections 6 μm thick. Hematoxylin and eosin (H&E) staining was performed in dewaxed sections. The total HA content was then also analyzed indirectly by evaluating its positivity to Hyaluronic Acid Binding Protein (HABP). Dewaxed sections were treated with avidin and biotin solutions (20 min each at RT) to block any endogenous avidin biotin activity. Endogenous peroxidase was blocked with 0.5% H_2O_2 in PBS for 5 min at RT and then washed in PBS. Specimens were then incubated in 0.1% BSA solution for 1 h at RT, treated with biotinylated HABP, 2 $\mu\text{g}/\text{ml}$ (Amsbio), diluted in the same pre-incubation buffer, and incubated overnight at 4°C. After repeated PBS washing, samples were incubated with the secondary antibody HRP conjugated Streptavidin 1:250 for 30 min (Jackson ImmunoResearch, Cambridgeshire, UK) and washed in PBS buffer. The reaction was then developed with 3,3'-diaminobenzidine (Liquid DAB+ substrate Chromogen System kit Dako Corp, Carpinteria, CA) and the reaction terminated with distilled water. Nuclei were counterstained with ready-to-use hematoxylin (Dako Corp). Negative controls were checked with similarly treated sections, without the primary antibody, and the specificity of the immunostaining reaction was confirmed.

Images were recorded on a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) and computerized image analysis was performed with ImageJ software to quantify anti-HABP antibody positivity (HABP staining was repeated at least three times and at least 10 images for each sample were analyzed at magnification 40X).

Real-Time PCR

Briefly, total RNA was extracted from homogenized tissue specimens with the SV Total RNA Isolation System (Promega Corporation, Madison, WI) and purified. Total RNA was then transcribed to cDNA with the iScript cDNA Synthesis Kit (BioRad Laboratories, Milan, Italy). RT-PCR was carried out with a starting amount of 30 ng of cDNA in an I-Cycler iQ detection system (BioRad) with the primers listed below:

1. RPLP0 (NM_053275): F824 5'-GCA GCA TCT ACA ACC CTG AA-3'; R918 5'-CAG ACA GAC ACT GCC AAC AT-3'.

2. HAS2 (NM_005328): F1233 5'-ATC CCA TGG TTG GAG GTG TT-3'; R1485 5'-TGC CTG TCA TCA CCA AAG CT-3'.

The PCR program included a denaturation step at 95°C for 3 min, 45 cycles of three amplification steps (15 s 95°C–15 s 60°C for RPLP0, 64°C for HAS2–15 s 72°C) and melting curve (60–90°C, heating rate 0.5°C/10 s). During the exponential phase, the fluorescence signal threshold was calculated and the fraction number of PCR cycles required to reach the threshold (cycle threshold, Ct) was determined. Ct values decreased linearly with increasing input target quantity. All samples were amplified in duplicate and the specificity of the amplification was tested at the end of each run by melting curve analysis with I-Cycler 3.0 software.

The expression of HAS2, the most frequently expressed enzyme in humans for HA synthesis²⁹ was evaluated, since its variations have been found in many tumors.^{30,31} The level of expression of the enzyme has been related to that of the housekeeping gene RPLP0 (ribosomal protein lateral stalk subunit P0), chosen because it has proved to be the most stable housekeeping gene in tumor tissues.^{32,33}

Statistical Analysis

Student's *t*-test was performed to verify significant differences among healthy fascia, peritumoral tissues and intratumoral tissues of each patient, as a function of age (younger or older than 60), gender (male/female) and tumor stage (low or high grade).

RESULTS

The amount of HA in the micro-environment surrounding the tumor increased in all cases of chondrosarcoma compared with healthy fascia, mean values 63.2 and 26.9 $\mu\text{g}/\text{g}$, respectively. More specifically, the mean content of HA in healthy tissue was $42.2 \pm 11.9 \mu\text{g}$ per gram of wet tissue in the human fascia lata of the thigh, $0.75 \pm 0.25 \mu\text{g}$ per gram in that of the deltoid, and 6.0 ± 0.0 in the trapezium (Table 2). The plantar fascia showed highly divergent values, as previously reported²⁸: the mean HA in one adult patient (female, 60 y) was 11.2 $\mu\text{g}/\text{g}$, whereas in an older patient (female, 98 y) it was 44.8 μg of HA per gram of tissue.

As regards the peritumoral stroma, in some cases the difference was less evident (e.g., cases 1 and 7; Table 2) whereas in other cases HA increased to high values (e.g., cases 6 and 12; Table 2). However, in all samples statistical analysis showed that the difference between the HA amount extracted from healthy fascia and that from the peritumoral stroma was statistically significant (*p*-value < 0.01 in all 12 samples). As shown in Figure 3, this statistically significant difference was found also in the mean values subdivided by district, with the only exception of the district of the foot, in which there is more variability, as already described. In any case, the increase in HA in the peritumoral stroma of each district was at least double that of healthy reference tissue (Fig. 3).

The intratumoral tissue showed different values: In hip chondrosarcoma, HA contents decreased to 1.1 and 4.4 $\mu\text{g}/\text{g}$ in two cases (males aged 15 and 45) and

Table 2. Values of Hyaluronan Content (μg) Contained in 1 g of Wet Human Starting Tissue, in Healthy Area, Peritumoral Stroma, and Intratumoral Area of 12 Volunteer Patients, Divided According to Anatomical District

	Subject	Distance From Tumor	HA Content ($\mu\text{g/g}$)
Hip	1	HFT	38.5
		PT	46.5
	2	HFT	47.2
		PT	117.0
	3	IT	1.1
		HFT	30.5
	4	IT	115.3
		HFT	57.5
	5	PT	132.6
		IT	152.8
	6	HFT	54.4
		PT	63.1
Foot	7	IT	4.4
		HFT	25.0
		PT	110.9
8	8	IT	59.5
		HFT	11.2
		PT	20
9	9	IT	209
		HFT	44.8
		PT	92.1
10	10	IT	80.5
		HFT	0.5
		PT	16.7
Scapula	11	IT	168
		HFT	1.0
		PT	5.7
12	12	HFT	6.0
		PT	42.5
		IT	48.1
			61.9

HFT, healthy fascial tissue; PT, peritumoral stroma; IT, intratumoral area. Reported values are means of at least two repetitions for each sample.

increased to 115.3 and 152.8 $\mu\text{g/g}$ in another two cases (males, 40 and 75 y). No constant data were found in the foot region and epymisial fasciae, values being 209 and 80.5 $\mu\text{g/g}$ in the foot, 168 $\mu\text{g/g}$ in the humerus, and 61.9 $\mu\text{g/g}$ in the scapula. In any case, in most cases hyaluronan increased with increasing peritumoral stroma. No significant differences in variables at univariate analysis (p -value > 0.05) were detected, considering age (older or younger than 60), gender, or tumor grade (low or high).

Fascial tissues, the connective tissue round the tumor and intratumoral samples were seen to differ on histological evaluation. The fascia appeared as a connective tissue with few cells in an abundant extracellular matrix organized in orderly bundles (Fig. 4A). The peritumoral stroma generally showed abundant fibroblasts, homogeneously arranged (and

not grouped in little clusters) and an abundant extracellular matrix divided into bundles in a less orderly manner than healthy tissue (Fig. 4B). Lastly, the tumor tissue showed heterogeneous areas: Some were filled in cells with intensely stained and evident nuclei, whereas other areas of the same tissue showed abundant less stained matrix containing many chondrocyte-like cells (Fig. 4C).

The same samples were subjected to immunohistochemical analysis with the anti-HABP antibody, as shown in Figure 5, confirming the presence of cells surrounded by an HA-rich matrix, in all tissues, to a greater extent in the peritumoral stroma (Fig. 5B) and intratumoral tissue (Fig. 5C) with respect to the healthy tissue (Fig. 5A).

With the aim to conduct a quantification of HABP positivity, a computerized analysis by Image J software was applied in a random sample, in at least 10 slices of all three tissues: Healthy, peritumoral, and intratumoral, all free of artifacts, and with staining not saturated and constant from slide to slide. In sample 8 (chondrosarcoma of the foot) the results showed positivity of fascial tissue to the anti-HABP antibody of $16.9 \pm 8.2\%$. The percentage of positivity increased in the peritumoral stroma to $31.5 \pm 6.0\%$. Lastly, intratumoral tissue staining was generally less homogeneous, with a positivity of $24.4 \pm 10.2\%$, comparable to the connective tissue around the tumor.

For expression analysis, real-time RT-PCR was performed in triplicate on three samples, selected from different anatomical district (samples number 4, 8, 12, from hip, foot, and scapula, respectively), according to the findings of our previous work that demonstrates how the muscular fasciae have constant HA values in physiological conditions, but variable according to anatomical site.²⁸ Although it was not possible to analyze all samples, due to their small size, the most important HA-synthase was found in fascial tissue³⁴ and in both peritumoral and intratumoral tissues, although lower in expression than the housekeeping gene RPLP0. The analysis of relative expression showed that, in peritumoral tissue, the gene is hypo-expressed compared with the healthy one; the same result was also found in intratumoral tissue (Figure 6). On average, the gene was expressed by about half (0.45 and 0.6 in peritumoral stroma and intratumoral tissue) with respect to healthy fascia, independently from the anatomical district of the tumor.

DISCUSSION

Our results confirmed that hyaluronan levels increased significantly in the peritumoral stroma and tumor tissue of patients with chondrosarcoma, compared with healthy fascia. First, microscopic analysis of the tissues showed high numbers of fibroblasts in peritumoral tissue with respect to the healthy fascia of the same district. Histological findings confirmed the results already reported by Josefsson et al.,⁷ which showed how the tumor tends to recruit fibroblasts in

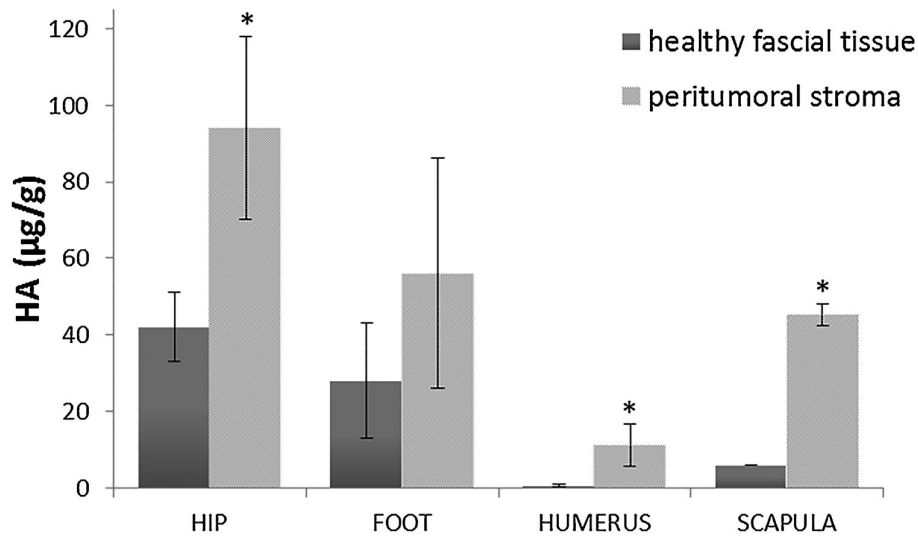


Figure 3. Mean value of HA content ($\mu\text{g/g}$) in healthy fascial tissue and peritumoral stroma of samples collected from hip, foot, humerus and scapula, respectively. * $p < 0.01$, t -test, peritumoral stroma versus healthy fascial tissue.

the peritumoral stroma by mutating their phenotype into “tumor-associated fibroblasts” (TAFs) and leading to the creation of an environment adjacent to the mass supporting tumor growth. Hyaluronan plays an active role in determining this fibroblast accumulation, as reported by Midgley et al.³⁵ but, vice versa, these fibroblasts can produce more HA under altered stimuli. This recent hypothesis³⁴ indicates the possibility of regulating the activity of fibroblast-like cells in HA production, according to the stimuli which these cells undergo. The muscular fasciae have variable HA contents, but they maintain constant values in physiological conditions according to anatomical site, as demonstrated in our previous study.²⁸ In body areas in which a greater degree of gliding is required, the amount of HA in the fascia was higher; and, likewise, it decreased where the less thick fascia adheres closely to the muscle—as happens, for example, in the epymysial fascia of the shoulder area.³⁶ These differences may make the muscular fasciae more or less efficient in acting as a barrier and containment of the tumor, depending on its biological and physical characteristics according to anatomical site.

Conversely, the variable values of HA content in intratumoral tissue and its heterogeneous areas in histological analysis may partly be explained by the fact that the tumor has a wide compositional variability and this also complicates histological diagnosis: The chondrosarcoma contains areas with different degrees of malignancy, so that some areas can still be defined as benign lesions while in others the cells are rare and only the myxoid is abundant.³⁷

To our knowledge, this is the first study demonstrating increased HA in patients with chondrosarcoma, as previously reported for other malignant lesions, in which increased HA contents in peritumoral tissue was evident and usually associated with greater aggressiveness of the pathology.^{8,38,39} In addition, real time RT-PCR results demonstrate that hyaluronan turnover is one of the aspects which is altered not only in the tumor but even, and to a greater extent, in the peritumoral area. In particular, a decrease, independent from the anatomical district of the tumor, of about 50% of the expression of HAS-2, which synthesizes high molecular weight (HMW)-HA, was found. Interestingly, HA may have different MW with differ-

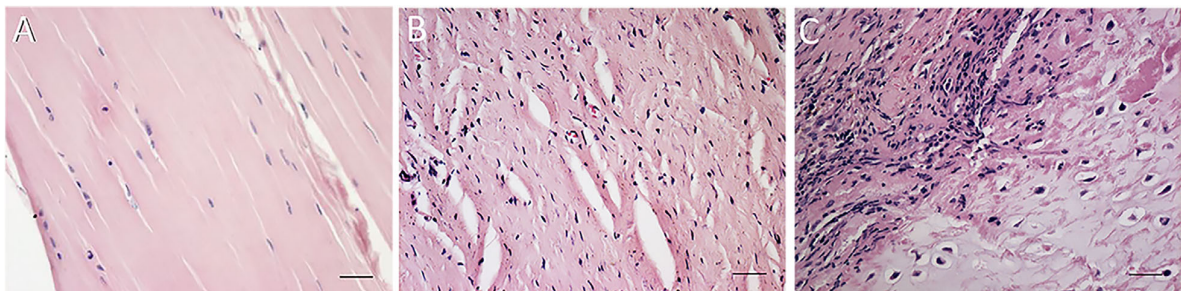


Figure 4. Hematoxylin and eosin staining of human fascial tissue (A), peritumoral stroma (B) and intratumoral tissue of chondrosarcoma (C). Scale bar: 50 μm .

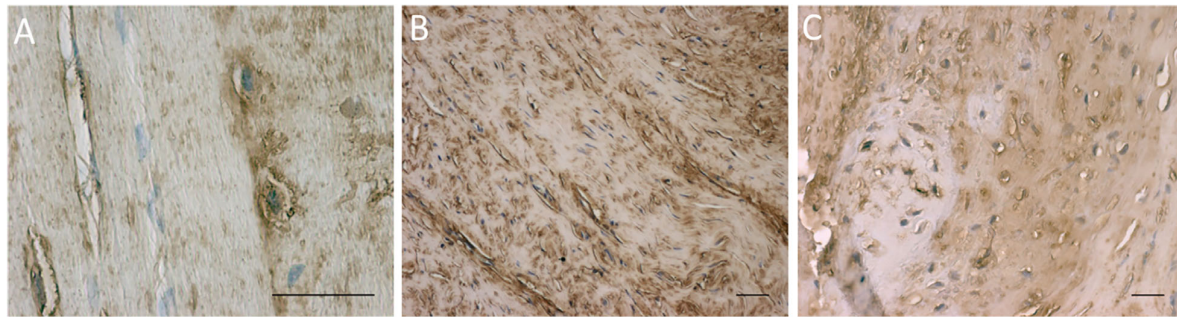


Figure 5. Sections of healthy fascia (A), peritumoral tissue of chondrosarcoma (B), and intratumoral tissue (C) after immunohistochemical staining by anti-HABP antibody. Scale bar: 50 μ m.

ent functions affecting tumor growth and progression. HMW-HA (1,000–8,000 kDa) is an inhibiting factor, whereas low molecular weight (LMW)-HA (<200 kDa) not only promotes cell proliferation but also mutations in metastatic cells, together with the production of metalloproteinases which facilitate invasion of surrounding tissues.⁴⁰ At the same time, it has been shown that treatment with HA oligosaccharides increases the sensitivity of the tumor to drugs.⁴¹ Therefore, our data indicate decreased inhibiting action on cell proliferation in chondrosarcoma, thus influencing tumor aggressiveness. A variation in the distribution of HMW-HA, caused by dysregulation of HA metabolism, may influence the capacity for invasiveness and resistance to chemotherapy of chondrosarcoma. Further studies are needed regarding analysis of the molecular weight of hyaluronan, since it may be the most discriminating factor in determining its biological effects.^{42,43}

In conclusion, increased HA in the peritumoral stroma may confirm the role of HA in chemoresistance to chondrosarcoma. Therefore, hyaluronan may become a new therapeutic target in the near future—a fact which has already emerged as regards other types of tumor, thanks to therapies affecting its production, its degradation by hyaluronidase enzymes, interaction

with its receptors, and the consequent activation of specific signal pathways.⁴⁴

AUTHORS' CONTRIBUTIONS

All the authors have made their contribution to this work and agreed with the contents of the manuscript, approving the final version. Caterina Fedè has written the work and is responsible about hyaluronan quantification and immunostaining technique; Carla Stecco is responsible about immunostaining analysis and has contributed to the writing and editing of the manuscript, and to the research design; Andrea Angelini is responsible about the sample collection from volunteers patients and has contributed to the editing of the manuscript; Chenglei Fan has contributed to the hyaluronan quantification; Elisa Belluzzi and Assunta Pozzuoli have contributed to the sample collection and to the data analysis; Pietro Ruggieri and Raffaele De Caro have worked to coordinate the work, to the data analysis and interpretation.

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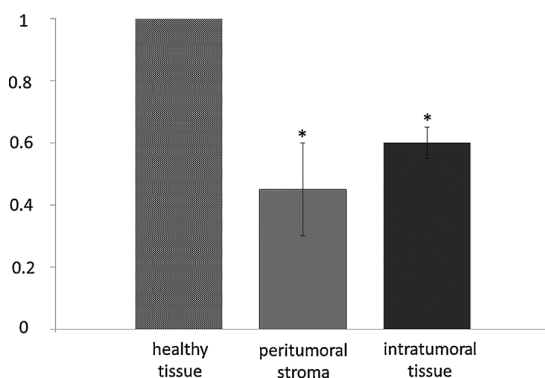


Figure 6. Relative mRNA expression of HAS-2 in patients with chondrosarcoma. Expression value in healthy fascial tissue was set at 1. Values are means of at least three experiments in triplicate. * $p < 0.01$, t -test versus healthy tissue.

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