OMICS A Journal of Integrative Biology
Volume 16, Number 3, 2012

DOI: 10.1089/omi.2011.0102



# A Structural Analysis of Glycosaminoglycans from Lethal and Nonlethal Breast Cancer Tissues: Toward a Novel Class of Theragnostics for Personalized Medicine in Oncology?

Amanda Weyers,[1] Bo Yang,[1] Dae Sung Yoon,[2] Jong-Hwan Park,[3] Fuming Zhang,[4] Kyung Bok Lee,[3] and Robert J. Linhardt[1,4,5]

## Abstract

Cancer is one of the leading noncommunicable diseases that vastly impacts both developed and developing countries. Truly innovative diagnostics that inform disease susceptibility, prognosis, and/or response to treatment (theragnostics) are seriously needed for global public health and personalized medicine for patients with cancer. This study examined the structure and content of glycosaminoglycans (GAGs) in lethal and nonlethal breast cancer tissues from six patients. The glycosaminoglycan content isolated from tissue containing lethal cancer tumors was approximately twice that of other tissues. Molecular weight analysis showed that glycosaminoglycans from cancerous tissue had a longer weight average chain length by an average of five disaccharide units, an increase of approximately 15%. Dissacharide analysis found differences in sulfation patterns between cancerous and normal tissues, as well as sulfation differences in GAG chains isolated from patients with lethal and nonlethal cancer. Specifically, cancerous tissue showed an increase in sulfation at the "6S" position of CS chains and an increase in the levels of the HS disaccharide NSCS. Patients with lethal cancer showed a decrease in HS sulfation, with lower levels of "6S" and higher levels of the unsulfated "0S" disaccharide. Although these findings come from a limited sample size, they indicate that structural changes in GAGs exist between cancerous and noncancerous tissues and between tissues from patients with highly metastatic cancer and cancer that was successfully treated by chemotherapy. Based on these findings, we hypothesize that (1) there are putative changes in the body's construction of GAGs as tissue becomes cancerous; (2) there may be innate structural person-to-person variations in GAG composition that facilitate the metastasis of tumors in some patients when they develop cancer.

## Introduction

Cancer is one of the leading noncommunicable diseases that vastly impact both developed and developing countries (The Lancet Oncology, 2011). Novel diagnostics that inform disease susceptibility, prognosis, and response to treatment (theragnostics: the fusion of therapeutics with diagnostic medicine) are seriously needed for global public health. Glycosaminoglycans (GAGs), highly sulfated, negatively charged polysaccharide chains are found throughout the tissues of the body. GAGs mediate a wide variety of biological functions and are often covalently attached to proteins, as proteoglycans (PGs). The properties of GAGs are governed by their structures, and GAGs are classified into families based on the sugars, which make up their composite disaccharide units. These are hyaluronan (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, and keratan sulfate. GAGs are used to transduce and propagate signals in development, coagulation, cell adhesion, immunity, cell replication, obesity, diabetes, and many other pathways (Bishop et al., 2007; Bulow and Hobert, 2006; Linhardt and Toida, 2004). Because the major signaling pathways that govern the appearance and spread of cancer—namely differentiation, replication, and migration—are all controlled

[1]Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York.
[2]Department of Surgery, College of Medicine, Konyang University, Konyang Hospital, Daejeon, Korea.
[3]Department of Biochemistry, College of Medicine, Konyang University, Daejeon, Korea.
[4]Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York.
[5]Department of Biology and Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York.

on some level by GAGs, the investigation of the role that PGs and GAGs play in cancer has been the focus of much recent study (Blackhall et al., 2001; Fjeldstad & Kolset, 2005; Gotte & Yip, 2006; Itano & Kimata, 2008; Muramatsu & Muramatsu, 2008; Sasisekharan et al., 2002; Yip et al., 2006).

In cancer, the study of GAGs has been primarily twofold: first, in aiding diagnosis, particularly in developing methods to differentiate the severity or aggressiveness of the disease, and second, in developing alternative treatments. GAG-focused cancer treatments that have been explored are generally concentrated on inhibiting the synthetic pathways or signaling pathways related to the GAGs and PGs in cancerous tissue. Specifically, targets have ranged from disruption of the CD44-HA interaction, a major receptor of HA regulating growth of cancer tissue (Platt and Szoka, 2008), inhibition of the HA biosynthetic pathway (Simpson et al., 2002), to the use of modified GAGs and PGs to disrupt angiogenesis and growth (Yip et al., 2006).

Diagnostic methods based on GAGs have typically centered around the analysis of GAG structure and concentration. Elevated levels of HA, a major component of the extracellular matrix (ECM), were found to correlate with several types of cancers (Itano and Kimata, 2008), including gastric (Vizoso et al., 2004), prostate (Lokeshwar et al., 2001), endometrial (Paiva et al., 2005), and bladder (Kramer et al., 2010; Lokeshwar et al., 1997, 1999; Pham et al., 1997). In several studies, the level of HA was found to be associated with the degree of severity and spread of the disease through the body (Kramer et al., 2010; Paiva et al., 2005; Pham et al., 1997; Vizoso et al., 2004). Other studies found a link between poor prognosis in prostate cancer and undersulfation in CS (Teng et al., 2008) or elevated levels of CS (Ricciardelli et al., 1997, 1999). Undersulfation in CS has also been linked to higher levels of prostate cancer relapse (Sakko et al., 2008). A further study in hepatic carcinoma revealed both an increase in overall levels of CS and an undersulfation of that CS was associated with more aggressive forms of the cancer (Lv et al., 2007).

Several studies have also investigated the link between changes in GAG structure or expression levels and breast cancer. Investigators have examined the influence various GAGs on breast cancer cell lines, including the regulation of FGF response to HS chains (Delehedde et al., 1996; Nurcombe et al., 2000), the ability of heparin and low molecular weight heparins to inhibit metastisis (Mellor et al., 2007), increases in motility on HA exposure (Herrera-Gayol and Jothy, 2001), and the inhibitory effect of lowering HS sulfation (Delehedde et al., 1996; Guo et al., 2007). Breast cancer cell lines were also found to secrete factors, which unregulated HA production in other cell lines (Corte et al., 2006).

However, there been relatively few studies that investigate GAG changes *in vivo* in cancerous breast tissue. Among the findings were the association of high levels of HS with phyllodes tumors (Koo et al., 2006), elevated levels of HA in the invasive components of ductal carcinoma (Corte et al., 2010), high levels of an unusual unsubstituted GlcA-GlcNH$_3^+$ in invasive mammary carcinomas (Fujii et al., 2010), and a twofold increase in proteoglycan content and CS GAG content in mammary tumors (Alini and Losa, 1991). Structural changes in cancerous tissues are typically established against a baseline of "normal" breast tissue; few, if any, studies include benign or nonlethal tumor breast tissue (Alini and Losa, 1991).

In the present study, we have examined the content and structure of GAGs isolated from neoplastic ("cancerous") tissue and "normal" tissue from two sets of patients with breast cancer. Cancer that metastasized to another major organ system in which the patients succumbed to the disease was termed "lethal" cancer and cancer that did not metastasize and showed no further signs of reoccurrence 5 years after the original diagnosis was termed "nonlethal" cancer. Our analysis, to our knowledge the first of its kind, aimed to uncover structural and compositional differences in the glycome to inform our understanding of how carbohydrates may influence the progression and spread of breast cancer in the body.

## Materials and Methods

### *Biological samples and materials*

Two breast tissue samples, one neoplastic (hereafter "cancerous") and one normal, were collected from six separate patients at Konyang Universtiy Hospital in Daejon, South Korea. Samples were obtained surgically by mastectomy. Cancerous mammary gland tumor samples and normal tissue samples (as distal as possible from the tumor mass) were isolated and stored in liquid nitrogen until use. All patients provided written informed consent before the collection of breast tissue samples. The collection and use of the samples was approved by the institutional review board of the Konyang University Hospital.

Amicon ultracentrifugal filters (10 and 30 K) were from Millipore (Billerica, MA). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Heparin was from Celsus Laboratories (Cincinnati, OH). Vivapure Q Mini H colums were from Sartorius Stedium Biotech (Goettingen, Germany). Alcian blue dye, CHAPS, hexylamine, hexafluoroisopropanol, polyacrylamide, and urea were from Sigma Chemical Company (St. Louis, MO). Unsaturated disaccharides standards of CS/DS (0S, ΔUA-GalNAc; 4S, ΔUA-GalNAc4S; 6S, ΔUAGalNAc6S; 2S, ΔUA2S-GalNAc; 2S4S or SB, ΔUA2S-GalNAc4S; 2S6S or SD, ΔUA2S-GalNAc6S; 4S6S or SE, ΔUA-GalNAc4S6S; and TriS, ΔUA2SGalNAc4S6S), and Chondroitin lyases ABC and ACII were purchased from Associates of Cape Cod, Inc. (East Falmouth, MA). Unsaturated disaccharides standards of heparin/HS (0S, ΔUA-GlcNAc; NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; and TriS, ΔUA2S-GlcNS6S) were purchased from Seikagaku Corporation (Japan). Cloning, *Escherichia coli* expression, and purification of the recombinant heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC assigned), and heparin lyase III (EC 4.2.2.8) from *Flavobacterium heparinum* were performed in our laboratory as described (Godavarti et al., 1996; Shaya et al., 2006; Yoshida et al., 2002). All other chemicals were of reagent grade.

### *Isolation, purification of GAGs*

Lyophilized tissue samples were weighed and individually proteolyzed using Actinase E (55°C, 800 $\mu$L of 2 mg/mL aqueous solution) for 2 days. Protease digested samples were then homogenized using a Labgen 7 tissue homogenizer (Cole-Parmer, Vernon Hills, IL).

GAGs were next isolated from the homogenized tissue sample solutions using Vivapure Q Mini H spin columns.

Each spin column was first equilibrated with a urea/CHAPS solution (2 wt.% CHAPS, 8 M urea). CHAPS and urea were added to each tissue isolate solution to afford a final concentration of 2 wt.% CHAPS and 8 M urea. Undigested particulates were removed from these solutions by centrifugation ($5000\times g$, 30 min), and the resulting tissue solutions were then loaded on the Vivapure spin columns ($500\times g$). Spin columns were washed with three column volumes of 200 mM NaCl to remove impurities. GAGs were released from the spin columns using two washes of 16% NaCl (0.5 column volumes). Methanol (80% total volume) was added to the released GAG solutions, precipitating the GAGs overnight at 4°C. Precipitated GAGs were recovered by centrifugation ($5000\times g$, 30 min) and the isolated GAG precipitate was then dissolved in doubly distilled water and analyzed. The amount of GAGs isolated from each tissue was quantified using a carbazole assay (Bitter and Muir, 1962) using heparin as a standard.

### *Analysis of isolated GAGs using PAGE*

Each GAG isolate was analyzed using polyacrylamide gel electrophoresis (PAGE). To each lane $\sim 1\,\mu$g of isolated GAG was subjected to electrophoresis against a heparin oligosaccharide standard prepared from bovine lung heparin enzymatically. GAGs were visualized on the gels using alcian blue stain and the resulting gel was scanned and digitized using UN-Scan-it software (Silk Scientific, Orem, UT). The number average molecular weight ($M_N$, the arithmetic mean of the chain distribution) and weight average molecular weight ($M_W$, a weighted average of the chain distribution), as well as polydispersity (a ratio of $M_N$ to $M_w$, which describes the degree of asymmetry in the chain distribution curve) of each GAG sample was then calculated. These weights were calculated based on the molecular weight of the bovine heparin lung standard using previously established methods (Edens et al., 1992); briefly, each lane of the gel was converted to a digital image, with the distribution of pixel intensity converted to a 2D lane distribution curve. For each point $i$, $y_i$ was then directly correlated to an absorbance ($A_i$) and each $x_i$ to a chain length ($M_i$) such that:

$$M_N = \Sigma \mathrm{A_i}/\Sigma(\mathrm{A_i}/\mathrm{M}i) \tag{1}$$

$$M_W = \Sigma(\mathrm{A_i M_i})/\Sigma \mathrm{A_i} \tag{2}$$

$$P = M_W/M_N \tag{3}$$

### *Disaccharide compositional analysis using liquid chromatography-mass spectrometry (LC-MS)*

The isolated GAGs were subject to enzymatic depolymerization. GAGs ($20\,\mu$g) were digested with chondrontinase ABC (10 mU) and ACII (5 mU) at 37°C for 10 h and the resulting dissacharide products were isolated by centrifugal filtration (AmiconUltra centrifugal filters, 10 k and 30 k MWCO, Millipore, Bedford, MA), freeze dried, and were ready for LC-MS analysis. The remaining undigested GAGs were then incubated with heparinses I, II, and III at 37°C for 10 h. The resulting disaccharide products were similarly isolated (10-k filter), freeze dried, and subject to LC-MS analysis.

LC-MS analyses were performed on an Agilent 1100 LC/MSD instrument (Agilent Technologies, Inc., Wilmington, DE) equipped with an ion trap, binary pump followed by microflow, and a UV detector. The column used was a 1.7 $\mu$m Acquity UPLC BEH C18 column (2.1 × 150 mm, Waters, Milford, MA). For CS/DS disaccharides analysis, solutions A and B for UPLC were 0 and 75% acetonitrile, respectively, containing the same concentration of 15 mM hexylamine as an ion-pairing reagent and 100 mM hexafluoroisopropanol as an organic modifier. The column temperature was maintained at 45°C. Solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0 to 50% solution B at the flow rate of 100 $\mu$L/min was used for disaccharides analysis. The electrospray interface was set in positive ionization mode with the skimmer potential 40.0 V, capillary exit 40.0 V, and a source of temperature of 350°C to obtain maximum abundance of the ions in a full-scan spectra (350–1500 Da, 10 full scans/s) (Solakyildirim et al., 2010). Nitrogen was used as a drying gas (8 L/min) and a nebulizing gas (40 psi). For HS disaccharides analysis, eluent A was water/acetonitrile (85:15) v/v, and eluent B was water/acetonitrile (35:65) v/v. Both eluents contained 12 mM tributylamine (TrBA) and 38 mM $NH_4OAc$ with pH adjusted to 6.5 with HOAc. The column temperature was maintained at 45°C. Solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0 to 50% solution B at the flow rate of 100 $\mu$L/min was used for disaccharides analysis. The electrospray interface was set in negative ionization mode with the skimmer potential −40.0 V, capillary exit −40.0 V, and a source of temperature of 350°C to obtain maximum abundance of the ions in a full-scan spectra (350–1500 Da, 10 full scans/s). Nitrogen was used as a drying gas (8 L/min) and a nebulizing gas (40 psi) (Yang et al., 2011).

### *Statistical analysis of data*

All statistical calculations were carried out using IBM SPSS Statistics 20 and StatCrunch (www.statcrunch.com). Comparisons of GAG content amongst the four tissue groups (lethal cancerous, lethal normal, nonlethal cancerous, and nonlethal normal), were done using a one-way Welch ANOVA followed by the post hoc Dunnett's T3 multiple pairwise test for unequal variance. Comparisons of GAG structure between cancerous and normal tissue (including samples from patients with lethal and nonlethal forms) were done using a paired-samples $t$-test. Comparisons of GAG structure between tissues coming from patients with lethal and nonlethal forms of cancer were done using a two-way ANOVA examining both the effect of cancer type (lethality) and tissue type (cancerousness); to ensure that including more than one sample from a patient did not create clustering in the resulting data, a one-way ANOVA was performed to examine the effects of patient number on the GAG structure ratios. No significant influence was found ($p > 0.10$). Significance was taken to be $p < 0.001$ for one-way ANOVA and $p < 0.05$ for all other tests.

## Results

### *GAG isolation and purification*

Tissue samples from six separate individuals with breast cancer were collected during surgical mastectomies at Konyang University Hospital in Daejeon, South Korea. Patient

Table 1. Tissue Samples and Related Patient Outcomes

| *Patient* | *Sample* | *Tissue type* | *Tumor size* | *Spread to lymph node* | *Metastasis* | *Patient outcome* |
|---|---|---|---|---|---|---|
| P1 | 1 | Cancer | T2 (3.5 cm) | negative | lung | lethal |
| | 2 | Normal | | | | |
| P2 | 3 | Cancer | T1 (1.5 cm) | positive | lung | lethal |
| | 4 | Normal | | | | |
| P3 | 5 | Normal | | | | |
| | 6 | Cancer | T2 (3.0 cm) | positive | bone | lethal |
| P4 | 7 | Normal | | | | |
| | 8 | Cancer | T2 (2.5 cm) | positive | negative | cured |
| P5 | 9 | Normal | | | | |
| | 10 | Cancer | T2 (2.0 cm) | positive | negative | cured |
| P6 | 11 | Normal | | | | |
| | 12 | Cancer | T1 (1.5 cm) | negative | negative | cured |

outcomes and cancer staging details were recorded. Tissue samples were selected for study to include patients with both lethal and nonlethal forms of the disease (Table 1). Lethal cancer was characterized by both a metastasis to a separate organ system and a lethal outcome for the patient. Nonlethal cancer was characterized by a lack of metastasis to other organ systems and by a lack of discernable cancer after treatment (no observed reoccurrence of cancer in 5 years' time). Normal tissue samples were taken as distal as possible from the tumor site on the masected tissue.

The dry weight of the tissues was recorded and the samples were subjected to a three-step procedure previously developed to quantitatively isolate heparin from human plasma (Zhang et al., 2006). Namely, tissues were proteolyzed, isolated by strong-anion exchange chromatography, and purified via methanol precipitation. GAG content was quantified using the carbazole assay for uronic acid (Table 2) and normalized against tissue weight. The results for each sample were analyzed using a one-way Welch ANOVA according to tissue type (lethal/nonlethal, cancerous/normal). The pooled averages are shown in Figure 1. Lethal cancerous tissue was found to contain significantly more GAG content per tissue mass (4.94 $\mu$g/mg) than any other type of tissue analyzed. No significant difference was found between any other type of tissue.

### *PAGE analysis of isolated GAGs*

GAGs were isolated by PAGE and imaged with alcian blue staining (Fig. 2). Gels were scanned, and the resulting image digitized and analyzed using UnScan it. The number average molecular weight ($M_N$) and weight average molecular weight ($M_W$), as well as polydispersity of each GAG was then calculated (Table 3). GAGs isolated from cancerous tissues had higher $M_W$ (22.0 kDA vs. 18.9 kDA) than the normal tissue isolated from the same patients. The longer chains isolated from the cancerous tissue were an average of 5 dissacharide units longer.

### *LC-MS analysis of disaccharide composition*

The isolated GAGs were subject to digestion using chondrotinase ABC and ACII, and then by heparinases I, II, and III. The isolated CS and HS disaccharides were both analyzed using LC-MS, each along with its own set of known disaccharide standards (see Materials and Methods) for ease of identification and quantification (Fig. 3). For each sample, the relative amount of each disaccharide was calculated, converted to a percentage of the total disaccharide content, and used to compare relative tissue expression levels between samples. The resulting amounts are shown in Tables 4 and 5.

Table 2. Carbazole Assay of Glycosaminoglycan Content in Different Cancerous and Normal Breast

| *Sample* | *Tissue type* | *Tissue dry weight (mg)* | *Isolated GAG ($\mu$g)* | *GAG/tissue weight ($\mu$g/mg)* | *Average* |
|---|---|---|---|---|---|
| Lethal outcome cancer | | | | | |
| 1 | Cancer | 10.5 | 74.3 ± 2.8 | 7.08 ± 0.3 | |
| 3 | Cancer | 66.5 | 275.7 ± 11 | 4.15 ± 0.2 | 4.94 ± 0.5 |
| 6 | Cancer | 44.1 | 158.5 ± 10 | 3.59 ± 0.2 | |
| 2 | Normal tissue | 10.3 | 36.7 ± 0.9 | 3.56 ± 0.1 | |
| 4 | Normal tissue | 41.8 | 40.2 ± 7.7 | 0.96 ± 0.2 | 2.27 ± 0.3 |
| 5 | Normal tissue | 28.3 | 64.8 ± 4.0 | 2.29 ± 0.1 | |
| Nonlethal outcome cancer | | | | | |
| 8 | Cancer | 34.6 | 74.6 ± 4.7 | 2.16 ± 0.1 | |
| 10 | Cancer | 64.7 | 106.5 ± 4.4 | 1.65 ± 0.1 | 2.34 ± 0.2 |
| 12 | Cancer | 59.1 | 190.9 ± 8.6 | 3.23 ± 0.1 | |
| 7 | Normal tissue | 20.6 | 25.5 ± 2.6 | 1.24 ± 0.1 | |
| 9 | Normal tissue | 83.8 | 145.3 ± 3.0 | 1.73 ± 0.04 | 1.71 ± 0.1 |
| 11 | Normal tissue | 79.9 | 173.3 ± 5.5 | 2.17 ± 0.1 | |

[a]Average value ± standard error in triplicate to sextuplicate experiments.

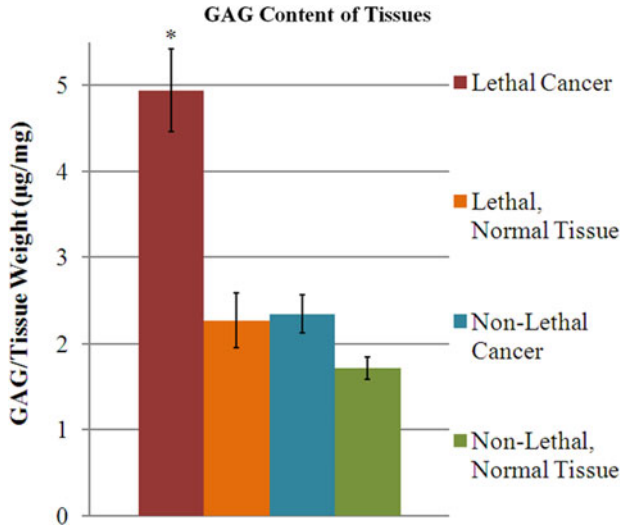


**FIG. 1.** GAG content (as determined via Carbazole Assay) per tissue type; total GAG amount is normalized by dry tissue weight (y-axis). From left to right, data is as follows: lethal cancerous tissue, lethal normal tissue, nonlethal cancerous tissue, nonlethal normal tissue. Lethal cancerous tissue was found to be significantly different from all other tissues ($p < 0.001$).

CS disaccharides were analyzed using to different isolation methods. The disaccharides were first purified through 10-kDa molecular weight cutoff membranes, which preferentially allowed only the smaller, singly sulfated disaccharides to pass through the filter. The samples were then digested with chondroitinases a second time, and the disaccharides were isolated through larger, 30-kDa filters to allow the larger

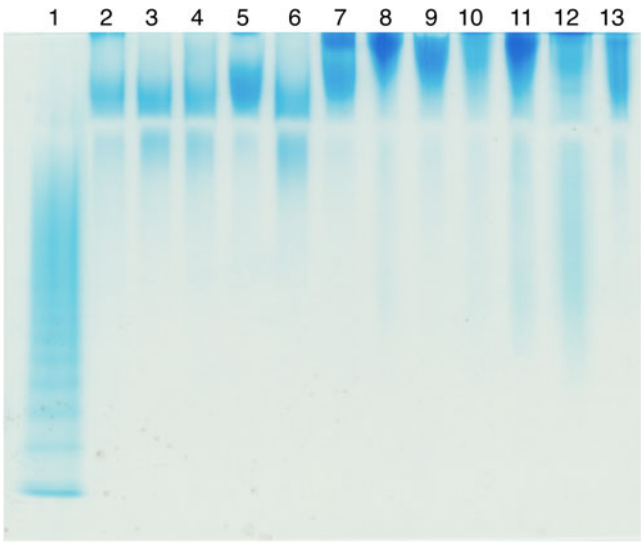


**FIG. 2.** Alcian blue stained PAGE of isolated GAGs. From left to right, lanes are: (1) bovine heparin lung (digested); (2–4) normal tissue from patients with lethal cancer, samples 2, 4, 5; (5–7) normal tissue from patients with nonlethal cancer, samples 9, 7, 11; (8–10) lethal cancerous tissue, samples 1, 3, 6; (11–13) nonlethal cancerous tissue, samples 8, 10, 12.

Table 3. Calculated GAG Molecular Weights From PAGE Analysis

| *Sample* | *Tissue type* | *Number average M.W. (kDa)* | *Weight average M.W. (kDa)* | *Polydispersity* |
|---|---|---|---|---|
| 1 | Cancer | 16,400 | 23,000 | 1.40 |
| 3 | Cancer | 16,900 | 22,700 | 1.34 |
| 6 | Cancer | 15,700 | 22,700 | 1.47 |
| | *Average* | **16,400** | **22,800** | **1.40** |
| 2 | Normal tissue | 12,700 | 18,600 | 1.47 |
| 4 | Normal tissue | 13,300 | 17,600 | 1.33 |
| 5 | Normal tissue | 13,400 | 17,800 | 1.33 |
| | *Average* | **13,100** | **18,000** | **1.38** |
| 8 | Cancer | 14,900 | 21,400 | 1.43 |
| 10 | Cancer | 12,500 | 18,700 | 1.50 |
| 12 | Cancer | 18,800 | 23,300 | 1.24 |
| | *Average* | **15,400** | **21,200** | **1.39** |
| 7 | Normal tissue | 13,700 | 17,000 | 1.25 |
| 9 | Normal tissue | 15,000 | 19,600 | 1.31 |
| 11 | Normal tissue | 17,000 | 22,600 | 1.33 |
| | *Average* | **15,200** | **19,800** | **1.30** |

disulfated disaccharides to be better analyzed. In the second set of samples, using 30-kDa filters, the presence of salt obscured the 0S peak preventing its accurate analysis.

In the CS disaccharide analysis only one significant difference was found between the cancerous and normal tissues (Fig. 4). In the first set of CS disaccharides, the levels of 6S disaccharide in cancerous (lethal and nonlethal) tissue was found to be significantly higher than that found in normal tissues.

In the HS disaccharides significant differences found were between the lethal (both cancerous and normal tissues) and nonlethal tissues (Fig. 5). Notably, there is a much lower amount of 6S disaccharide and higher level of 0S disaccharide in both the cancerous and normal tissues from patients with lethal forms of the disease. Levels of TriS were also found to be significantly lower in patients with lethal forms of cancer; however, this difference may be influenced by clustering effects in the data resulting from comparing more than one tissue sample from the same patient ($0.10 > p > 0.05$). One significant difference was also found between the cancerous and normal tissues; levels of NS2S were higher in cancerous tissues than in normal tissues (Fig. 6).

Recently, a large-scale study done by Fujii et al. (2010) on breast cancer in Japan found a high expression level of $GlcA - GlcNH_3^+$ in carcinogenic breast tissue using an epitope specific antibody. Although Fujii et al. (2010) clearly could only measure the presence of certain epitopes (which would correspond to $\Delta UA\text{-}GlcNH_2$ or $\Delta UA\text{-}GlcNH_26S$), presumably the biological process (either biosynthesis or catabolism) that creates N-unsubstituted residues would result in the presence of $\Delta UA2S\text{-}GlcNH_2$ or $\Delta UA2S\text{-}GlcNH_26S$ disaccharides as well (Westling and Lindahl, 2002). In our disaccharide analysis three of these structures were either not observed or indistinguishably combined with a more prevalent structure ($\Delta UA\text{-}GlcNH_2$ might coelute with the 0S disaccharide and $\Delta UA2S\text{-}GlcNH_2$ and $\Delta UA\text{-}GlcNH_26S$ might coelute with the 2S, 4S, or 6S disaccharide). The fourth, $\Delta UA2S\text{-}GlcNH_26S$,

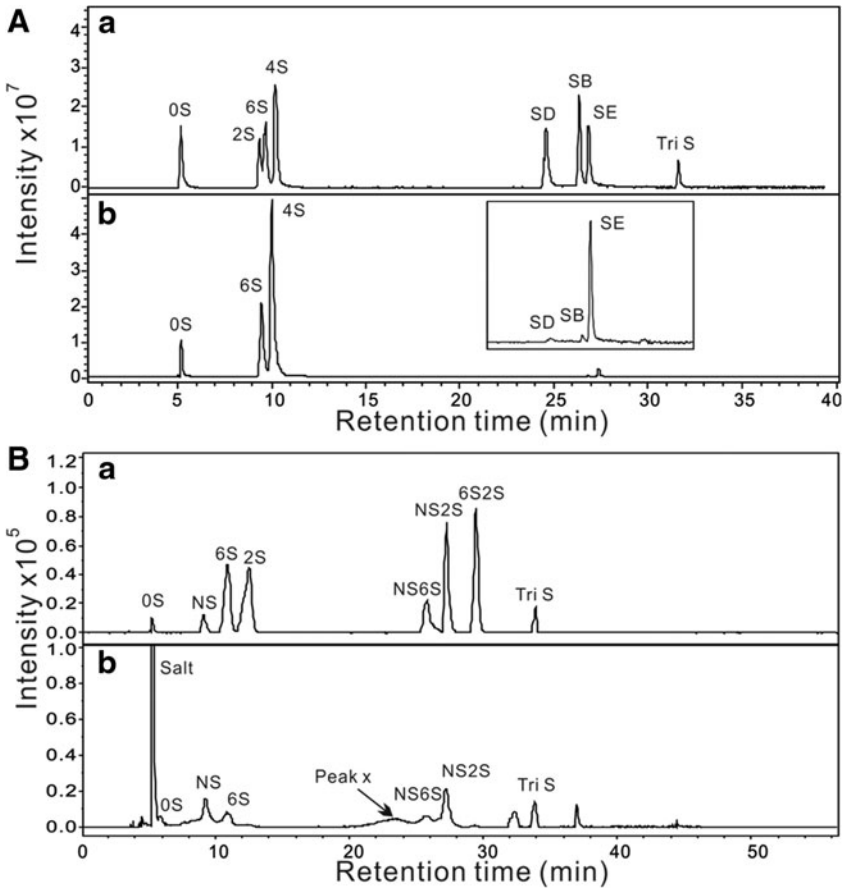


**FIG. 3.** Disaccharides composition analysis of GAGs from breast cancer sample by LC/MS. Shown are (**A**) CS/DS disaccharide analysis; (a) Extracted ion chromatography (EIC) of CS/DS disaccharide standard and (b) lethal breast cancer sample #3; (**B**) HS/HP disaccharide analysis; (a) EIC of HS/HP disaccharide standard and (b) lethal breast cancer sample #3.

Table 4. Results of LCMS Analysis of CS Disaccharide Content

| | | *CS Dissacharide Content (%)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Disaccharides isolated through 10-k filter* | | | *Disaccharides isolated through 30-k filter* | | | |
| *Sample* | *Tissue type* | *0S* | *6S* | *4S* | *6S* | *4S* | *SB* | *SE* |
| Lethal | | | | | | | | |
| 1 | Cancer | 22.3 | 22.8 | 55.0 | 27.4 | 64.7 | 2.3 | 5.7 |
| 3 | Cancer | 10.9 | 34.2 | 54.8 | 48.7 | 46.1 | 1.1 | 4.1 |
| 6 | Cancer | 21.7 | 39.0 | 39.3 | 67.0 | 32.0 | 0.3 | 0.7 |
| | *Average* | **18.3** | **32.0** | **49.7** | **47.7** | **47.6** | **1.2** | **3.5** |
| 2 | Normal tissue | — | — | — | 62.8 | 27.7 | 0.6 | 8.9 |
| 4 | Normal tissue | 22.3 | 9.5 | 68.3 | 3.2 | 83.9 | 0.0 | 12.9 |
| 5 | Normal Tissue | 56.6 | 11.6 | 31.8 | 18.1 | 62.6 | 0.0 | 19.3 |
| | *Average* | **39.4** | **10.5** | **50.0** | **28.0** | **58.1** | **0.2** | **13.7** |
| Nonlethal | | | | | | | | |
| 8 | Cancer | 34.9 | 25.2 | 40.0 | 43.7 | 53.0 | 1.7 | 1.7 |
| 10 | Cancer | 30.8 | 52.9 | 16.3 | 46.9 | 45.8 | 0.0 | 7.3 |
| 12 | Cancer | 30.1 | 32.2 | 37.8 | 62.0 | 35.3 | 0.0 | 2.6 |
| | *Average* | **31.9** | **36.8** | **31.3** | **50.9** | **44.7** | **0.6** | **3.9** |
| 7 | Normal tissue | 42.0 | 8.0 | 50.0 | 3.6 | 77.1 | 0.0 | 19.4 |
| 9 | Normal tissue | 19.1 | 8.8 | 72.1 | 48.8 | 50.4 | 0.0 | 0.9 |
| 11 | Normal tissue | 18.8 | 33.9 | 47.3 | 52.8 | 43.7 | 0.4 | 3.2 |
| | *Average* | **26.6** | **16.9** | **56.5** | **35.0** | **57.0** | **0.1** | **7.8** |

Table 5. Results of LCMS Analysis of HS Disaccharide Content

| Sample | Tissue type | HS dissacharide dontent (%) 0S | NS | 6S | NS6S | NS2S | TriS |
|---|---|---|---|---|---|---|---|
| Lethal | | | | | | | |
| 1 | Cancer | 95.0 | 3.1 | 0.4 | 0.3 | 1.0 | 0.3 |
| 3 | Cancer | 89.5 | 4.4 | 0.6 | 0.8 | 1.6 | 3.1 |
| 6 | Cancer | 93.8 | 1.4 | 0.5 | 0.7 | 1.0 | 2.7 |
| | *Average* | **92.8** | **3.0** | **0.5** | **0.6** | **1.2** | **2.0** |
| 2 | Normal tissue | 95.6 | 3.4 | 0.6 | 0.0 | 0.5 | 0.0 |
| 4 | Normal tissue | 96.0 | 2.3 | 0.5 | 0.5 | 0.8 | 0.0 |
| 5 | Normal tissue | 92.4 | 3.5 | 0.5 | 0.5 | 0.8 | 2.2 |
| | *Average* | **94.7** | **3.1** | **0.5** | **0.3** | **0.7** | **0.7** |
| Nonlethal | | | | | | | |
| 8 | Cancer | 89.0 | 3.1 | 1.1 | 0.8 | 2.0 | 4.1 |
| 10 | Cancer | 91.1 | 4.2 | 0.6 | 0.8 | 1.3 | 1.9 |
| 12 | Cancer | 82.3 | 6.3 | 1.4 | 1.3 | 1.1 | 7.7 |
| | *Average* | **87.5** | **4.5** | **1.0** | **1.0** | **1.5** | **4.6** |
| 7 | Normal tissue | 92.5 | 2.3 | 0.6 | 0.3 | 0.5 | 3.9 |
| 9 | Normal tissue | 83.0 | 5.7 | 1.4 | 1.0 | 1.2 | 7.8 |
| 11 | Normal tissue | 86.7 | 3.1 | 1.3 | 0.8 | 0.8 | 7.4 |
| | *Average* | **87.4** | **3.7** | **1.1** | **0.7** | **0.9** | **6.4** |

with an exact mass of 497.0, matches the mass of an unknown peak ("peak x;" Fig. 3) observed in our disaccharide analysis, with an elution time that did not correspond with any known standard. We suggest that the identity of this peak x observed by LC-MS is in fact the ΔUA2S-GlcNH$_2$6S dissacharide peak. As shown in Table 6, the normalized peak area was almost always observed to be greater in cancerous tissue than in normal tissue. Although this value was not found to be statistically significant, this is possibly indicative of a trend that may become clearer with the study of more tissue samples.

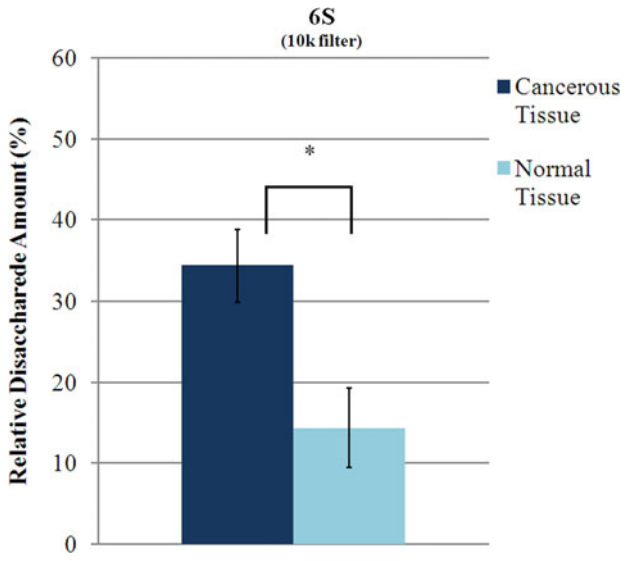


**FIG. 4.** Comparison of representative CS disaccharides in cancerous and normal tissues. Relative amounts (as a percent of all analyzed disaccharides) per tissue were determined by integrating the corresponding LC peaks. The CS 6S disaccharide (isolated through a 10-k filter) was found to be significantly higher in cancerous tissue than in normal tissue ($p < 0.05$).

## Discussion

In this study, the GAG content and structure of four different breast tissue types (lethal cancerous and normal, nonlethal cancerous and normal) were examined. Lethal cancerous tissue was found to contain higher levels of GAG, longer weight average chain lengths, lower HS sulfation levels, and shows indications of having higher levels of a rare disaccharide epitope (ΔUA2S – GlcNH$_3^+$ 6S).

Lethal cancerous tissue was found to express nearly twice as much GAG content as any other tissue. As cancerous tissue is by nature highly prolific tissue, it stands to reason that the proteoglycans and glycosaminoglycans, which direct key metaboltic functions, would be overexpressed in cancerous, highly metastatic cancer. Cancerous tissue has in fact long been linked to higher levels of GAGs, especially higher CS levels (Lv et al., 2007; Ricciardelli et al., 1997, 1999) and in breast cancer, higher HS content (Koo et al., 2006) and higher levels of both HS and CS (Alini and Losa, 1991). In melanoma, a CS proteoglycan has been specifically linked to the ability of tumor cells to metastasize (Knutson et al., 1996) and chemotherapy drugs are being developed to specifically bind to metastatic tumor cells which overexpress CS (Lee et al., 2002). Although GAG content and $M_w$ chain length both increase in the lethal cancerous tissue, the longer GAG chain lengths can only be partially responsible for the increased GAG content. The elongation of GAG chains is probably the result of enhanced GAG chain polymerization (Esko and Selleck, 2002) rather than decreased GAG chain catabolism (Levy-Adam et al., 2010). It is unclear how GAG chain length might impact carcinogenesis.

There were distinct sulfation patterns indicative of the separate tissue types. Cancerous tissue, regardless of type, was found to have higher 6S sulfation. Higher CS-6S sulfation has been associated with cell proliferation (Schonherr et al., 1993). Cancerous tissue was also found to contain higher levels of the HS disaccharide NS2S.

Tissue from patients with lethal cancer, regardless if the tissue was cancerous or normal, was found to have lower HS sulfation, represented by a lower level of 6S sulfation and a higher level of unsulfated (0S) dissacharide units. Also, the lower levels of trisulfated dissacharides (TriS), although not significant, supports this overall trend in lower sulfation levels and may again point to a trend in lethal tissues that might prove to be important as more tissue samples are analyzed.

Interestingly, two *in vitro* studies on breast cancer cell lines show that lower HS sulfation patterns decrease cell migration (Guo et al., 2007) and inhibit fibroblast growth factor (FGF)-2 interactions (Delehedde et al., 1996), which inhibited growth. However, in studying the FGF-2/HS-proteogycan interactions, Delehedde and coworkers (1996) also found that decreasing the HS sulfation was not exclusively inhibitory and was, in fact, a growth promoter, within certain limitations, for one of the two breast cancer cell lines studied. Changing HS sulfation patterns have also been linked to changing inhibition of heparanase, an enzyme that is a potent metastatic agent. High heparanase activity and expression levels have been found to correlate with greater abilities to degrade the extracellular matrix and higher metastatic potentials (Nakajima et al., 1983; Vlodavsky et al., 1999). Irimura et al. (1986) found that decreasing the sulfation levels of heparin *decreased* the ability of the chains to inhibit heparanase. The increased

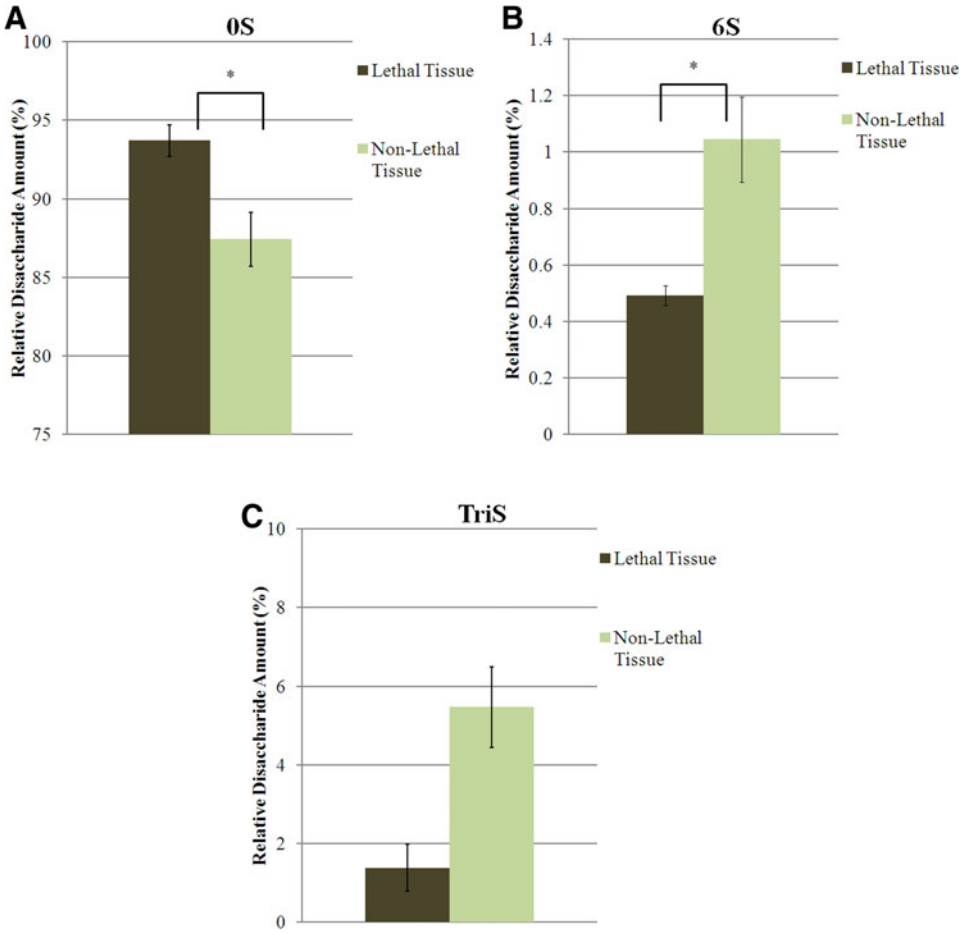


**FIG. 5.** Comparison of representative HS dissacharides in lethal and nonlethal tissues. Relative amounts (as a percent of all analyzed disaccharides) per tissue were determined by integrating the corresponding LC peaks. Shown are: (**A**) 0S (unsulfated) disaccharide levels; (**B**) 6S disaccharide levels; (**C**) TriS (trisulfated) disaccharide levels; 0S and 6S levels were found to be significantly different in the two tissue types ($p < 0.05$).

lethality observed in tissues with lower HS sulfation levels may be a result of decreased inhibition and thus increased activity in heparanase.

A recent study by Fujii and coworkers (2010) found a high expression level of GlcA – $GlcNH_3^+$ in malignant mammary carcinomas. Here we found what we propose to be levels of a related structure, (ΔUA2S – $GlcNH_3^+$ 6S), at roughly threefold higher levels in cancerous tissue than in normal tissue. Again, although these differences were not significant, they should be examined in future breast cancer tissue analysis and may prove to be important in metabolic signaling. We had also previously suggested (Toida et al., 1997) that glucosamine residues without N-substitution ($GlcNH_3^+$) are preferentially localized within the HS chain between the high sulfation and low sulfation domains whose spacing and distribution is crucial for FGF signaling. Thus, the presence of positive charges in the HS chains may impact binding and signaling characteristics, changing the metabolism and signaling of the cancerous tissue; the biological impact of this structural change has yet to be unraveled.

## Conclusions

The impact of differing sulfation patterns in either CS or HS chains on metabolitic forces is not well understood. Some natural variation in sulfation levels is bound to exist, even in the limited samples analyzed here. Our current analysis leads us to conclude that there may be some natural HS variations that exist, which in fact, drive metastasis and promote the spread of the disease through the body. However, these findings must be understood to be limited in strength by the size of the study.

Although the direct effects of changing sulfation patters are not known, sulfation patterns are known to be tissue and temporally specific (Dietrich et al., 1976; Sampaio et al., 1977). In their study on hepatic carcinoma (Lv et al., 2007), Lv and coworkers discuss the link between GAG structure and metabolic changes. Lv et al. (2007) cite research that found similarities in the structural and temporal expression of CS in tumors and developing fetal liver tissue (Dietrich et al., 1977; Gressner, 1983; Sampaio et al., 1977) and further speculate that the observed changes in CS composition may, in fact, be a reflection of a larger metabolic shift in the tumor, reverting the tissue to fetal pathways, presumably with correspondingly high growth rates. In this way, malignancy and high metastatic drive may then be interpreted as nothing more than the upregulation of rapid growth metabolism at inappropriate times.

Perhaps equally as interesting as the innate variation in sulfation patterns across patients is the observed structural

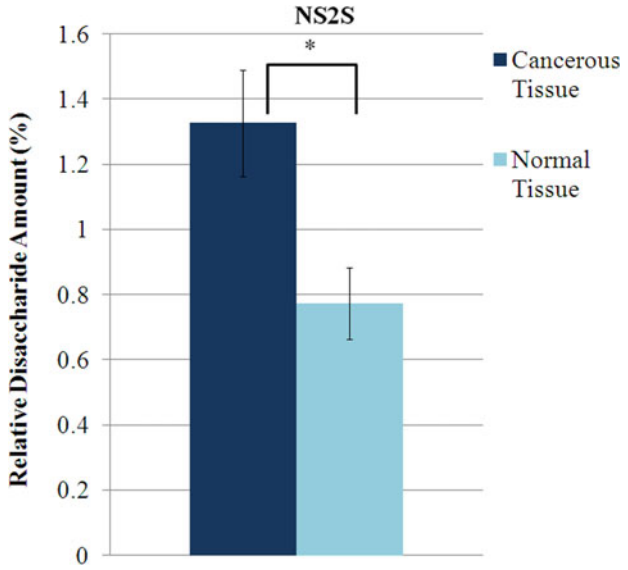


**FIG. 6.** Comparison of representative HS dissacharides in cancerous and normal tissues. Relative amounts (as a percent of all analyzed disaccharides) per tissue were determined by integrating the corresponding LC peaks. The NS2S peak was found to be significantly higher in cancerous tissue than in normal tissue ($p < 0.05$).

changes in cancerous tissue when compared with normal tissue. The increase in $M_w$ chain length suggests that the breast tissue undergoes a change in pathways that regulate or govern chain elongation as the tissue becomes cancerous. Further observed differences in GAG chain sulfation patterns suggest changes may also be happening in the pathways that regulate the production and modification of GAGs as cells become cancerous.

This study, to our knowledge the first of its kind, found significant changes in amounts and structural composition of CS and HS GAG chains isolated from lethal and nonlethal cancers. Although the full impact of changing sulfation patterns on the metabolism and signaling pathways of tissue are not fully known, these changes suggest that higher amounts of GAG and lowered HS GAG sulfation content are an important factor in the survival and spread of malignant tissue. However, these findings must be seen as preliminary, as they are based on a very small set of samples. Further studies, on larger sample sets, should be performed to verify these findings and check the significance of the trends seen here. These preliminary findings, however, point the way to a new and crucial understanding of cancer, where the metastatic potential of each patient's cancerous tissue may be independently assessed, with treatment and monitoring options tailored to match. Ultimately, these observations reported in this article may inform the discovery and development of a highly innovative class of theragnostics in oncology.

Table 6. Results of LCMS Analysis of Peak X[a]

| *Sample* | *Tissue type* | *Raw signal peak area* | *Normalized peak area*[b] |
|---|---|---|---|
| Lethal | | | |
| 1 | Cancer | 135,261 | 12,882 |
| 3 | Cancer | 304,497 | 4,579 |
| 6 | Cancer | 259,112 | 5,876 |
| Lethal | | | |
| 2 | Normal | 17,234 | 1,673 |
| 4 | Normal | 47,843 | 1,145 |
| 5 | Normal | 127,322 | 4,499 |
| Nonlethal | | | |
| 8 | Cancer | 29,717 | 859 |
| 10 | Cancer | 464,774 | 7,184 |
| Nonlethal | | | |
| 7 | Normal | 35,062 | 1,702 |
| 9 | Normal | 164,131 | 1,959 |

[a]Exact mass = 497.0.
[b]Peak area normalized to sample tissue weight.

## Acknowledgments

The authors thank the NIH for generously supporting this research in the form of Grants 2T32GM067545 and GM38060. The authors also thank Dr. Brad Lister of RPI and Dr. Michael Racz of ACP for their helpful suggestions on the statistical analysis of our data.

## Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

## References

Alini, M., and Losa, G.A. (1991). Partial characterization of proteoglycans isolated from neoplastic and nonneoplastic human breask tissues. Cancer Res. 51, 1443–1447.

Bishop, J.R., Schuksz, M., and Esko, J.D. (2007). Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature 446, 1030–1037.

Bitter, T., and Muir, H.M. (1962). A modified uronic acid carbazole reaction. Anal. Biochem. 4,330.

Blackhall, F.H., Merry, C.L.R., Davies, E.J., and Jayson, G.C. (2001). Heparan sulfate proteoglycans and cancer. Br. J. Cancer 85, 1094–1098.

Bulow, H.E., and Hobert, O. (2006). The molecular diversity of glycosaminoglycans shapes animal development. Annu. Rev. Cell Dev. Biol. 22, 375–407.

Corte, M.D., Gonzalez, L.O., Lamelas, M.L., Alvarez, A., Junquera, S., Allende, M.T., et al. (2006). Expression and clinical signification of cytosolic hyaluronan levels in invasive breast cancer. Breast Cancer Res. Treat. 97, 329–337.

Corte, M.D., Gonzalez, L.O., Junquera, S., Bongera, M., Allende, M.T., and Vizoso, F.J. (2010). Analysis of the expression of hyaluronan in intraductal and invasive carcinomas of the breast. J. Cancer Res. Clin. Oncol. 136, 745–750.

Delehedde, M., Deudon, E., Boilly, B., and Hondermarck, H. (1996). Heparan sulfate proteoglycans play a dual role in regulating fibroblast growth factor-2 mitogenic activity in human breast cancer cells. Exp. Cell Res. 229, 398–406.

Dietrich, C.P., Sampaio, L.O., and Toledo, O.M.S. (1976). Characteristic distribution of sulfated mucopolysaccharides in different tissues and in their respective mitochondria. Biochem. Biophys. Res. Commun. 71, 1–10.

Dietrich, C.P., Sampaio, L.O., Toledo, O.M.S., and Cassaro, C.M.F. (1977). Cell recognition and adhesiveness—possible biological role for sulfated mucopolysaccharides. Biochem. Biophys. Res. Commun. 75, 329–336.

Edens, R.E., Alhakim, A., Weiler, J.M., Rethwisch, D.G., Fareed, J., and Linhardt, R.J. (1992). Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low molecular weight heparin derivatives. J. Pharm. Sci. 81, 823–827.

Esko, J.D., and Selleck, S.B. (2002). Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. 71, 435–471.

Fjeldstad, K., and Kolset, S.O. (2005). Decreasing the metastatic potential in cancers—targeting the heparan sulfate proteoglycans. Curr. Drug Targets 6, 665–680.

Fujii, M., Yusa, A., Yokoyama, Y., Kokuryo, T., Tsunoda, N., Oda, K., et al. (2010). Cytoplasmic expression of the JM403 antigen GlcA-GlcNH(3)(+) on heparan sulfate glycosaminoglycan in mammary carcinomas—a novel proliferative biomarker for breast cancers with high malignancy. Glycoconj. J. 27, 661–672.

Godavarti, R., Davis, M., Venkataraman, G., Cooney, C., Langer, R., and Sasisekharan, R. (1996). Heparinase III from *Flavobacterium heparinum*: cloning and recombinant expression in *Escherichia coli*. Biochem. Biophys. Res. Commun. 225, 751–758.

Gotte, M., and Yip, G.W. (2006). Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. Cancer Res. 66, 10233–10237.

Gressner, A.M. (1983). Hepatic proteoglycans—a brief survey of their patho-biochemical implications. Hepato-Gastroenterology 30, 225–229.

Guo, C.H., Koo, C.Y., Bay, B.H., Tan, P.H., and Yip, G.W. (2007). Comparison of the effects of differentially sulphated bovine kidney- and porcine intestine-derived heparan sulphate on breast carcinoma cellular behaviour. Int. J. Oncol. 31, 1415–1423.

Herrera-Gayol, A., and Jothy, S. (2001). Effects of hyaluronan on the invasive properties of human breast cancer cells in vitro. Int. J. Exp. Pathol. 82, 193–200.

Irimura, T., Nakajima, M., and Nicolson, G.L. (1986). Chemically modified heparins as inhibitors of heparan-sulfate specific endo-beta-glucurongdase (heparanase) of metastatic melanoma-cells. Biochemistry 25, 5322–5328.

Itano, N., and Kimata, K. (2008). Altered hyaluronan biosynthesis in cancer progression. Semin. Cancer Biol. 18, 268–274.

Knutson, J. R., Iida, J., Fields, G.B., and McCarthy, J.B. (1996). CD44/chondroitin sulfate proteoglycan and alpha 2 beta 1 integrin mediate human melanoma cell migration on type IV collagen and invasion of basement membranes. Mol. Biol. Cell 7, 383–396.

Koo, C.Y., Bay, B.H., Lui, P.C.W., Tse, G.M.K., Tan, P.H., and Yip, G.W.C. (2006). Immunohistochemical expression of heparan sulfate correlates with stromal cell proliferation in breast phyllodes tumors. Modern Pathol. 19, 1344–1350.

Kramer, M.W., Golshani, R., Merseburger, A.S., Knapp, J., Garcia, A., Hennenlotter, J., et al. (2010). HYAL-1 hyaluronidase: a potential prognostic indicator for progression to muscle invasion and recurrence in bladder cancer. Eur. Urol. 57, 86–93.

Lee, C.M., Tanaka, T., Murai, T., Kondo, M., Kimura, J., Su, W., et al. (2002). Novel chondroitin sulfate-binding cationic liposomes loaded with cisplatin efficiently suppress the local growth and liver metastasis of tumor cells in vivo. Cancer Res. 62, 4282–4288.

Levy-Adam, F., Ilan, N., and Vlodavsky, I. (2010). Tumorigenic and adhesive properties of heparanase. Semin. Cancer Biol. 20, 153–160.

Linhardt, R.J., and Toida, T. (2004). Role of glycosaminoglycans in cellular communication. Acc. Chem. Res. 37, 431–438.

Lokeshwar, V.B., Obek, C., Soloway, M.S., and Block, N.L. (1997). Tumor-associated hyaluronic acid: a new sensitive and specific urine marker for bladder cancer. Cancer Res. 57, 773–777.

Lokeshwar, V.B., Young, M.J., Goudarzi, G., Iida, N., Yudin, A.I., Cherr, G.N., et al. (1999). Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1. Cancer Res. 59, 4464–4470.

Lokeshwar, V.B., Rubinowicz, D., Schroeder, G.L., Forgacs, E., Minna, J.D., Block, N.L., et al. (2001). Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. J. Biol. Chem. 276, 11922–11932.

Lv, H.Z., Yu, G.L., Sun, L.L., Zhang, Z., Zhao, X., and Chai, W.G. (2007). Elevate level of glycosaminoglycans and altered sulfation pattern of chondroitin sulfate are associated with differentiation status and histological type of human primary hepatic carcinoma. Oncology 72, 347–356.

Mellor, P., Harvey, J.R., Murphy, K.J., Pye, D., O'Boyle, G., Lennard, T.W.J., et al. (2007). Modulatory effects of heparin and short-length oligosaccharides of heparin on the metastasis and growth of LMD MDA-MB 231 breast cancer cells in vivo. Br. J. Cancer 97, 761–768.

Muramatsu, T., and Muramatsu, H. (2008). Glycosaminoglycan-binding cytokines as tumor markers. Proteomics 8, 3350–3359.

Nakajima, M., Irimura, T., Diferrante, D., Diferrante, N., and Nicolson, G.L. (1983). Heparan-sulfate degradation—relation to tumor invasive and metastatic properties of mouse B-16 melanoma sublines. Science 220, 611–613.

Nurcombe, V., Smart, C.E., Chipperfield, H., Cool, S.M., Boilly, B., and Hondermarck, H. (2000). The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates J. Biol. Chem. 275, 30009–30018.

Paiva, P., Van Damme, M.P., Tellbach, M., Jones, R.L., Jobling, T., and Salamonsen, L.A. (2005). Expression patterns of hyaluronan, hyaluronan synthases and hyaluronidases indicate a role for hyaluronan in the progression of endometrial cancer. Gynecol. Oncol. 98, 193–202.

Pham, H.T., Block, N.L., and Lokeshwar, V.B. (1997). Tumor-derived hyaluronidase: a diagnostic urine marker for high grade bladder cancer. Cancer Res. 57, 778–783.

Platt, V.M., and Szoka, F.C. (2008). Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor. Mol. Pharmaceut. 5, 474–486.

Ricciardelli, C., Mayne, K., Sykes, P.J., Raymond, W.A., McCaul, K., Marshall, V.R., et al. (1997). Elevated stromal chondroitin sulfate glycosaminoglycan predicts progression in early-stage prostate cancer. Clin. Cancer Res. 3, 983–992.

Ricciardelli, C., Quinn, D.I., Raymond, W.A., McCaul, K., Sutherland, P.D., Stricker, P.D., et al. (1999). Elevated levels of peritumoral chondroitin sulfate are predictive of poor prognosis in patients treated by radical prostatectomy for early-stage prostate cancer. Cancer Res. 59, 2324–2328.

Sakko, A.J., Butler, M.S., Byers, S., Reinboth, B.J., Stahl, J., Kench, J.G., et al. (2008). Immunohistochemical level of unsulfated chondroitin disaccharides in the cancer stroma is an independent predictor of prostate cancer relapse. Cancer Epidemiol. Biomarkers Prevent. 17, 2488–2497.

Sampaio, L.O., Dietrich, C.P., and Filho, O.G. (1977). Changes in sulfated mucopolysaccharide composision of mammalian tissues during growth and in cancer tissues. Biochim. Biophys. Acta 498, 123–131.

Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanasami, U. (2002). Roles of heparan-sulphate glycosaminoglycans in cancer. Nat. Rev. Cancer 2, 521–528.

Schonherr, E., Jarvelainen, H.T., Kinsella, M.G., Sandell, L.J., and Wight, T.N. (1993). Platelet-derived growth-factor and transforming growth-factor-beta-1 differentially affect the syntesis of biglycan and decorin by monkey arterial smooth-muscle cells. Arterioscler. Thromb. 13, 1026–1036.

Shaya, D., Tocilj, A., Li, Y.G., Myette, J., Venkataraman, G., Sasisekharan, R., et al. (2006). Crystal structure of heparinase II from Pedobacter heparinus and its complex with a disaccharide product. J. Biol. Chem. 281, 15525–15535.

Simpson, M.A., Wilson, C.M., and McCarthy, J.B. (2002). Inhibition of prostate tumor cell hyaluronan synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. Am. J. Pathol. 161, 849–857.

Solakyildirim, K., Zhang, Z.Q., and Linhardt, R.J. (2010). Ultraperformance liquid chromatography with electrospray ionization ion trap mass spectrometry for chondroitin disaccharide analysis. Anal. Biochem. 397, 24–28.

Teng, Y.H.F., Tan, P.H., Chia, S.J., Zam, N., Lau, W.K.O., Cheng, C.W.S., et al. (2008). Increased expression of non-sulfated chondroitin correlates with adverse clinicopathological parameters in prostate cancer. Mod. Pathol. 21, 893–901.

The Lancet Oncology (Editorial). (2011). Two days in New York: reflections on the UN NCD Summit. Lancet Oncol. 12, 981.

Toida, T., Yoshida, H., Toyoda, H., Koshishi, I., Imanari, T., Hileman, R.E., et al. (1997). Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissues and species. Biochem. J. 322, 499–506.

Vizoso, F.J., del Casar, J.M., Corte, M.D., Garcia, I., Corte, M.G., Alvarez, A., et al. (2004). Significance of cytosolic hyaluronan levels in gastric cancer. Eur. J. Surg. Oncol. 30, 318–324.

Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., et al. (1999). Mammalian heparanase: Gene cloning, expression and function in tumor progression and metastasis. Nat. Med. 5, 793–802.

Westling, C., and Lindahl, U. (2002). Location of N-unsubstituted glucosamine residues in heparan sulfate. J. Biol. Chem. 277, 49247–49255.

Yang, B., Weyers, A., Baik, J.Y., Sterner, E., Sharfstein, S., Mousa, S.A., et al. (2011). Ultra-performing ion-pairing liquid chromatography with on-line electrospray ion trap mass spectrometry for heparin disaccharide analysis. Anal. Biochem. 415, 59–66.

Yip, G.W., Smollich, M., and Gotte, M. (2006). Therapeutic value of glycosaminoglycans in cancer. Mol. Cancer Res. 5, 2139–2148.

Yoshida, E., Arakawa, S., Matsunaga, T., Toriumi, S., Tokuyama, S., Morikawa, K., et al. (2002). Cloning, sequencing, and expression of the gene from *Bacillus circulans* that codes for a heparinase that degrades both heparin and heparan sulfate. Biosci. Biotechnol. Biochem. 66, 1873–1879.

Zhang, F.M., Sun, P.L., Munoz, E., Chi, L.L., Sakai, S., Toida, T., et al. (2006). Microscale isolation and analysis of heparin from plasma using an anion-exchange spin column. Anal. Biochem. 353, 284–286.

Address correspondence to:
*Professor Robert J. Linhardt*
*Department of Chemistry and Chemical Biology*
*Center for Biotechnology and Interdisciplinary Studies*
*Rensselaer Polytechnic Institute*
*110 8th Street*
*Troy, NY 12180*

*E-mail:* linhar@rpi.edu

OR

*Professor Kyung Bok Lee*
*Department of Biochemistry*
*College of Medicine*
*Konyang University*
*Daejeon, Korea 302-718*

*E-mail:* kyunglee@konyang.ac.kr