

Microanalysis of stomach cancer glycosaminoglycans

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Abstract Glycosaminoglycans (GAGs) are anionic, linear, polysaccharides involved in cell signaling. The GAG content, composition and structure of human tissue have been suggested to play a role in cancer and might provide useful diagnostic or prognostic markers. The current study examines 17 stomach tissue biopsy samples taken from normal individuals and from patients with gastric cancers. An ultrasensitive liquid chromatography (LC)–mass spectrometry assay was applied to individual biopsy samples as small 250 µg providing GAG content and disaccharide composition. The results of these analyses show a significant

increase in non-sulfated chondroitin/dermatan sulfate concentration in all cancer samples when compared to normal tissues. In addition in advanced gastric cancer, a significant decrease is observed in hyaluronan.

Keywords Stomach cancer · Glycosaminoglycan · Chondroitin sulfate · Hyaluronan · Disaccharide analysis

Abbreviations

AGC	Advanced gastric cancer
AMAC	2-aminoacridone
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
CS	Chondroitin sulfate
DS	Dermatan sulfate
EGC	Early gastric cancer
ESI	Electrospray ionization
GAG	Glycosaminoglycan
HA	Hyaluronan
HS	Heparan sulfate
LOD	Limit of detection
MWCO	Molecular weight cut-off

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Introduction

While mortality rates of gastric cancer have fallen in the United States over the past few decades, gastric cancer is still the fourth leading cause of cancer in the world and the second most common cause of cancer related deaths worldwide [1, 2]. Although the majority of cases occur in developing nations, the incidence of gastric cancer varies widely from nation to nation, with China, Japan and Korea have been found to have the highest rates of occurrence [1, 2]. Gastric cancer is associated with a wide range of risk factors, including obesity, tobacco use, and infection with gram-negative *Helicobacter pylori* [1].

Over the years many characteristics of gastric tumors have been studied, especially those pertaining to carbohydrate expression levels, which are of particular interest here. Among other things, these studies have analyzed glycosaminoglycan (GAG) structural composition, special patterns of carbohydrate expression, hyaluronan (HA) expression levels, and the expression of carbohydrate receptors such as CD44 [3–7]. Since carbohydrates, and GAGs in particular, are important in cellular signaling processes, many of these studies are designed to uncover links between the metastatic ability or the lethality of gastric cancer and particular carbohydrate-based biomarkers. Especially relevant to this work are studies in the sulfation patterns of GAGs and in the HA expression levels.

Cancer has long been associated with changing carbohydrate structures [8]. However, there is often a lack of consensus in the literature over the levels present and the importance of various sulfation patterns in gastric cancer [4], or a lack of comprehensive data on sulfation patterns. For example, a study on sulfation patterns in gastric cancer combined several tissue samples together to amass enough material for analysis, obscuring information on natural variation of GAG sulfation levels. This study found a higher expression of 6-*O*-sulfo (6S) group and non-sulfated (0S) GAG disaccharides in gastric cancer [4], while a complementary study found that expression of a 6-*O*-sulfatase (which removes 6-*O*-sulfo groups GAGs lowering the 6S disaccharide content) suppresses a Wnt signaling pathway, restricting the tumor's ability to proliferate and invade [9]. Other studies have focused on HA levels in gastric cancer. These studies have found increased HA levels in tumors [4] and increased HA levels in individual tumor cells [10, 11], but subsequent work found no statistical correlation to lymphatic or blood vessel invasion [12], and even varying HA levels within different histological subtypes [13, 14]. Many of these studies show over a 100-fold variation in the range of measured HA levels for any particular diagnostic grouping, making it difficult to use this assay (based on a radiolabel) as a diagnostic method [11, 12].

In this paper, a simple, direct and ultra-sensitive method of disaccharide analysis was used to measure the heparan sulfate (HS), chondroitin sulfate (CS) and HA levels (Fig. 1). This analysis relied on the fluorescent labeling of disaccharides followed by their analysis using liquid chromatography (LC)-mass spectrometry (MS). This provided a limit of detection (LOD) of 0.1 ng of GAG allowing for the analysis of as little as 250 μ g of biopsy sample. In this study, 17 stomach cancer samples were analyzed representing four different types of cancer or precancerous tissues of varying histology. These four different types are normal tissue, gastric adenoma, early gastric cancer (EGC) and advanced gastric cancer (AGC). The sensitivity and reproducibility of this method make it a good

candidate for the development of future diagnostic and prognostic methods. Differences in the disaccharide composition between the different tissue types may provide signature disaccharide patterns useful for diagnosis.

Materials and methods

Tissue samples

Stomach tissue samples collected from each of 14 different patients were obtained at Konyang University Hospital Daejeon, South Korea. Biopsy samples were taken from separate patients, except for two patients who gave both normal and advanced gastric cancer (AGC) samples (samples 4 & 16, 5 & 17, respectively). Gastric adenoma tissue samples were obtained from endoscopic submucosal dissection and all other malignant tissue samples were obtained surgically from gastrectomy. Normal tissue samples were obtained as distal as possible from tumor mass at gastrectomy. Samples were stored in liquid nitrogen before use. The clinicopathologic data were collected by reviewing medical charts and pathologic records (Table 1). All patients provided written informed consent before the collection of gastric tissues samples. The collection and use of the samples were approved by the Institutional Review Board of the Konyang University Daejeon Hospital.

Reagents

Amicon Ultra centrifugal filters (10 kDa and 30 kDa) were from Millipore (Billerica, MA). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Heparin was from Celsus Laboratories (Cincinnati, OH). Vivapure Mini Q-H columns were from Sartorius Stedium Biotech (Goettingen, Germany). Hexylamine and hexafluoroisopropanol were from Sigma Chemical Company (St. Louis, MO). CS and DS unsaturated disaccharides standards, including C-0S, Δ UA(1 \rightarrow 3)GalNAc; C-4S, Δ UA(1 \rightarrow 3)GalNAc4S; C-6S, Δ UA(1 \rightarrow 3)GalNAc6S; C-2S, Δ UA2S(1 \rightarrow 3)GalNAc; C-2S4S or C-SB, Δ UA2S(1 \rightarrow 3)GalNAc4S; C-2S6S or C-SD, Δ UA2S(1 \rightarrow 3)GalNAc6S; C-4S6S or C-SE, Δ UA(1 \rightarrow 3)GalNAc4S6S; and C-2S4S6S or C-TriS, Δ UA2S(1 \rightarrow 3)GalNAc4S6S, and chondroitin lyases ABC and ACII were purchased from Associates of Cape Cod, Inc. (East Falmouth, MA). HS unsaturated disaccharides standards, H-0S, Δ UA(1 \rightarrow 4)GlcNAc; H-NS, Δ UA(1 \rightarrow 4)GlcNS; H-6S, Δ UA(1 \rightarrow 4)GlcNAc6S; H-2S, Δ UA2S(1 \rightarrow 4)GlcNAc; H-2SNS, Δ UA2S(1 \rightarrow 4)GlcNS; H-NS6S, Δ UA(1 \rightarrow 4)GlcNS6S; H-2S6S, Δ UA2S(1 \rightarrow 4)GlcNAc6S; and H-TriS, Δ UA2S(1 \rightarrow 4)GlcNS6S, and HA unsaturated disaccharide standard, HA-OS, Δ UA(1 \rightarrow 3)GlcNAc were from Iduron (Manchester, UK). Cloning,

Table 1 Stomach tissue sample descriptions

Case	Tissue type	Tissue dry weight (mg)	Sex	Age	Tumor size	Lymph nodes	Metastasis	Prognosis ^a	Histology ^b
1	Normal	0.694	M	65					
2	Normal	0.693	F	63					
3	Normal	1.53	M	72					
4	Normal	45.0	M	35					
5	Normal	59.0	M	43					
6	Gastric adenoma	1.56	F	72					Tubular adenoma, Low grade
7	Gastric adenoma	1.48	M	59					Tubular adenoma, Low grade
8	Gastric adenoma	0.541	F	68					Tubular adenoma, Low grade
9	EGC	2.97	M	71	T1b	Negative	Negative	Alive	Adenocarcinoma (MD)
10	EGC	17.9	F	57	T1a	Negative	Negative	Alive	Adenocarcinoma (WD)
11	EGC	3.00	F	75	T1a	Negative	Negative	Alive	Adenocarcinoma (MD)
12	AGC	0.263	M	72	T4a	Positive	Negative	Alive	Adenocarcinoma (MD)
13	AGC	26.3	M	75	T4a	Positive	Negative	Dead	Signet ring cell carcinoma
14	AGC	88.3	M	59	T4	Positive	Negative	Alive	Adenocarcinoma (PD)
15	AGC	4.29	F	72	T2	Positive	Negative	Alive	Adenocarcinoma (MD)
16	AGC	44.5	M	35	T3	Positive	Negative	Alive	Adenocarcinoma (PD)
17	AGC	43.4	M	43	T1	Negative	Negative	Alive	Adenocarcinoma (PD)

^a State of patient 2 years or longer after biopsy was taken

^b PD, MD, WD poorly, moderately and well defined tumors

E. 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 *F. heparinum* were

performed in our laboratory as described [15–17]. All other chemicals were of reagent grade.

Carbohydrate isolation

Two years after biopsy, the tissues were freeze-dried and transferred from Konyang Hospital in Korea to Rensselaer for glycan analysis. Lyophilized tissue samples were weighed and individually proteolyzed using Actinase E (55 °C, 800 μ L of 2 mg/mL aqueous solution) for 2 days.

GAGs were next isolated using Vivapure Mini Q-H spin columns. Each spin column was first equilibrated with a urea 3-[(3-drolamidopropyl) dimethylammonio]-1-propane sulfate (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.

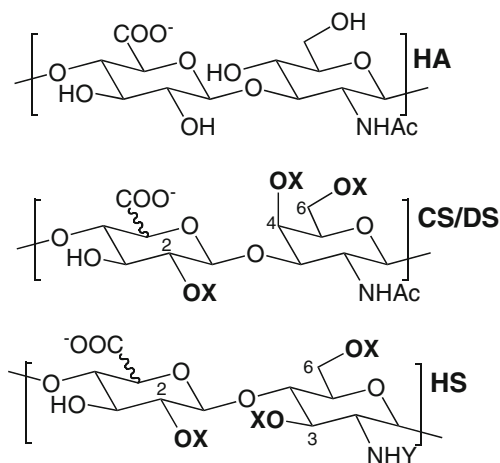
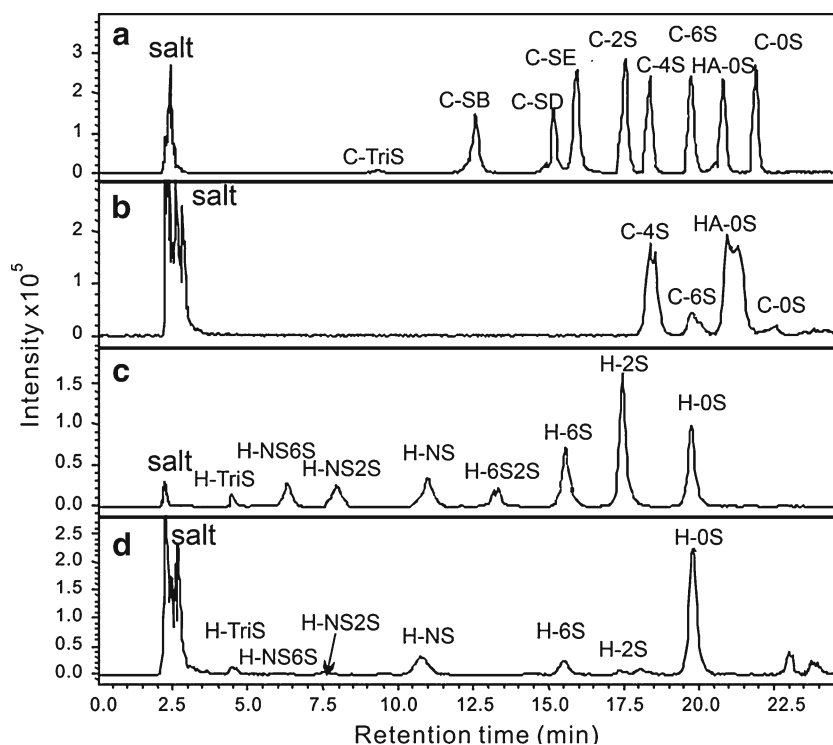


Fig. 1 Structures of the GAGs analyzed in this study. Glycosaminoglycans are long, highly charged, linear polysaccharides comprised of repeating disaccharide units. HA, hyaluronan [\rightarrow 4)GlcA(1 \rightarrow 3)GlcNAc(1 \rightarrow 3)]; CS, chondroitin sulfate [\rightarrow 4)GlcA2S/OH(1 \rightarrow 3)GalNAc4S/OH6S/OH(1 \rightarrow 3)]; DS, dermatan sulfate [\rightarrow 4)IdoA2S/OH(1 \rightarrow 3)GalNAc4S/OH6S/OH(1 \rightarrow 3)]; and HS, heparan sulfate [\rightarrow 4)GlcA/IdoA2S/OH(1 \rightarrow 4)GlcNAc/S/3S/OH6S/OH(1 \rightarrow 3)]. Potential sulfo group locations are highlighted in bold; X=H or SO₃⁻; Y=COCH₃ or SO₃⁻, or H. The numbers on the HS and CS/DS structures indicate carbons where O-sulfo groups can be found

Fig. 2 LCMS separation and quantification of HA, CS and HS disaccharides. Shown are (a) CS/DS and HA disaccharide standards; b CS/DS and HA disaccharides isolated from sample #6, a typical gastric adenoma tissue sample; c HS disaccharide standards; d HS disaccharides isolated from sample #6, a typical gastric adenoma tissue sample



LC-MS disaccharide analysis

The isolated GAGs were subject to enzymatic depolymerization. GAGs (20 μg) were digested with chondroitinase ABC (10 mU) and ACII (5 mU) at 37 $^{\circ}\text{C}$ for 10 h and the resulting disaccharide products were isolated by centrifugal filtration (30 kDa molecular weight cut-off (MWCO)), freeze-dried, and were ready for LC-MS analysis. Centrifugal filters having various MWCO values were tested for

disaccharide recovery without loss of GAG chains. The use of a 30 kDa MWCO membrane resulted in the nearly quantitative recovery of disaccharide products of polysaccharide lyase-treatment of GAGs obtained from cultured cells without the loss of GAG chains of MW $\geq 1,200$ [18, 19]. The remaining undigested GAGs were then incubated with heparinases I, II and III at 37 $^{\circ}\text{C}$ for 10 h. The resulting disaccharide products were similarly isolated (30 kDa filter), freeze-dried, and subjected to LC-MS analysis [20]. Fluorescent labeling of disaccharide was next undertaken to ensure highly sensitive detection and to eliminate potential complications associated with UV absorbing impurities [18] coming from the biopsy tissues being analyzed. The freeze-dried sample containing the GAG-derived disaccharides ($\sim 2 \mu\text{g}$) was added 10 μL a 0.1 M 2-aminoacridone (AMAC) solution in acetic acid dimethyl sulfoxide (3:17, v/v) and mixed by vortexing for 5 min. Next, 10 μL of 1 M NaBH_3CN was added in the reaction mixture and incubated at 45 $^{\circ}\text{C}$ for 4 h [21]. LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with a 6300 ion-trap and a binary pump followed by a UV detector equipped with a high-pressure cell. The column used was a poroshell120 C18 column (2.1 \times 150 mm, 2.6 μm , Agilent) at 45 $^{\circ}\text{C}$. Solution A was 80 mM ammonium acetate solution and Solution B was methanol. Solution A and 15 % solution B was flowed (150 $\mu\text{L}/\text{min}$) through the column for 5 min followed by a linear gradient of 15–30 % solution B from 5 to 30 min.

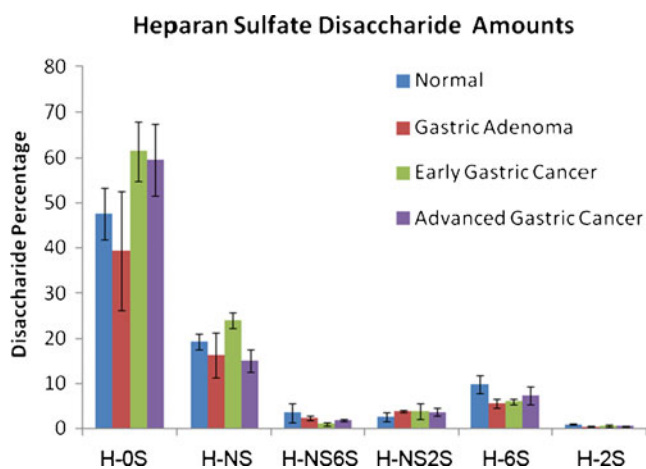


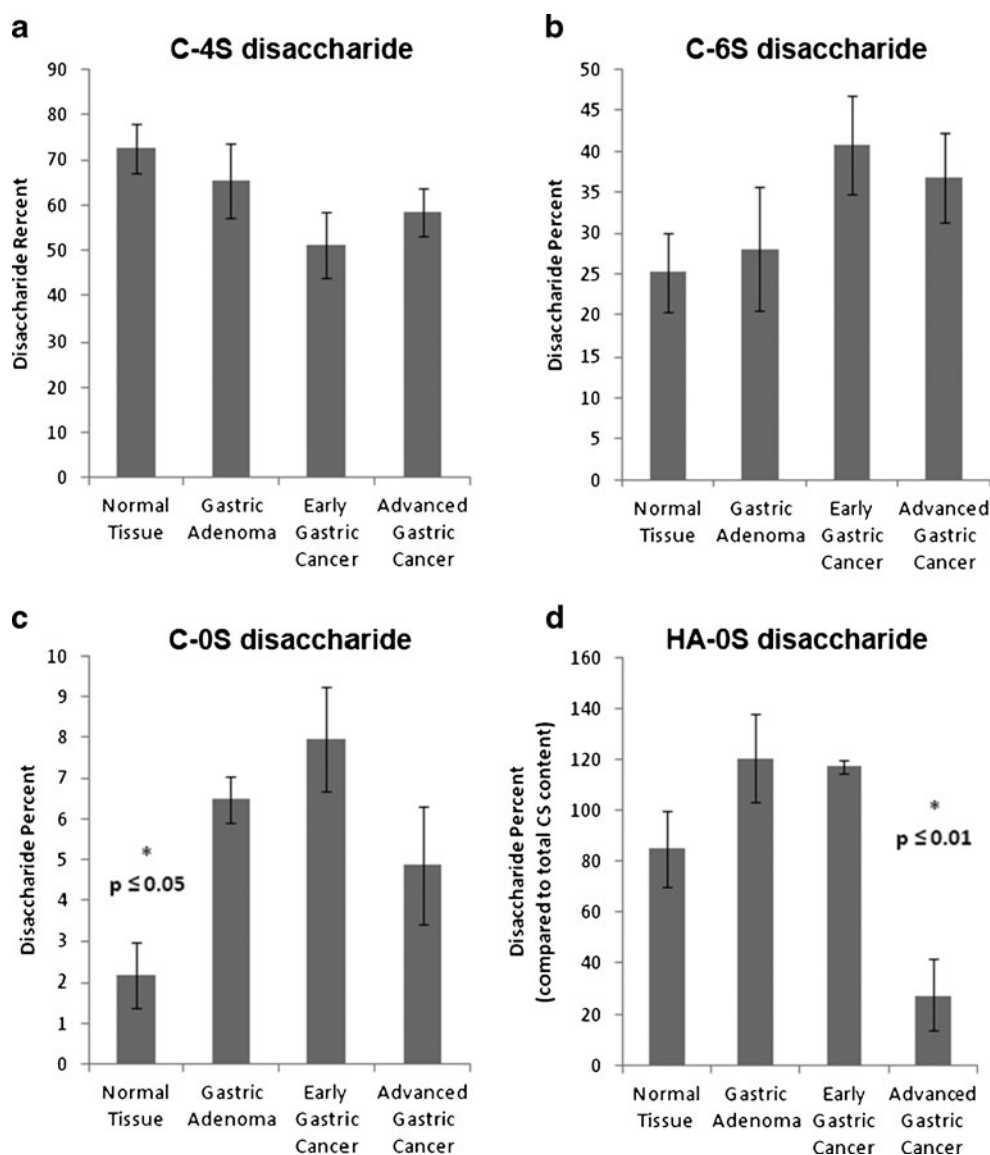
Fig. 3 Measured levels of HS disaccharides in GAGs present in stomach tissues. The HS disaccharide amounts, calculated by comparison to HS disaccharide standards, were normalized to the dry tissue weight of each sample. No significant differences were seen between the different stomach tissue types

The column effluent entered the electrospray ionization (ESI)-MS source for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of -40.0 V, a capillary exit of -40.0 V, and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (150–1200 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas [22].

Statistical analysis of data

All statistical calculations were done using IBM SPSS Statistics 20. Comparisons of disaccharide content amongst the four tissue types (normal, gastric adenoma, early gastric cancer, advanced gastric cancer), were done using a one-way Welch ANOVA (analysis of variance) followed by the post-hoc Dunnett's T3 multiple pairwise test for unequal variance.

Fig. 4 Measured levels of CS disaccharides in GAGs present in stomach tissues. **a** C-4S disaccharide obtained from the different tissue samples; **b** C-6S disaccharide obtained from the different tissue samples; **c** C-0S disaccharide obtained from the different tissue samples; **d** HA-0S disaccharide obtained from the different tissue samples. The CS disaccharide amounts, calculated by comparison to CS disaccharide standards, were also normalized to the dry tissue weight of each sample. No significant differences were observed in different stomach tissue types for content of 4S and 6S disaccharides. Note that the C-4S + C-6S + C-0S = 100 for each tissue sample. The HA-0S disaccharide is presented relative to the total CS found in each sample. Significant differences were observed in both non-sulfated chondroitin (C-0S) and hyaluronan (HA-0S) disaccharides



Results and discussion

The CS, HS, and HA content of the stomach cancer samples were analyzed using LC-MS to quantify the disaccharide composition and content of each type of GAG. The total quantity of each disaccharide type was then calculated based on a standard curve for each disaccharide [22] and the mole percentage of each were compared across the different biopsy samples.

There was no notable difference between the different tissue types in the average content of CS, HS and HA. The average total content of CS, HS and HA was $2\text{-}\mu\text{g mg}^{-1}$ of freeze-dried tissue sample. Next, the HS disaccharide composition was compared and no apparent trends or major differences were observed across the four tissue types (Figs. 2 and 3). In contrast there were several significant differences in the CS disaccharide composition of the four

tissue types (Fig. 4a–c). There were decreases in the average level of 4-sulfo group substitution of the tissue samples, and elevations in the average level 6-sulfo group substitution in the three cancer samples, particularly the two most developed cancer tissue types, EGC and AGC. However, these differences were not significant. There were, however, differences in the levels of HA-OS and C-OS disaccharides found in the tissue samples analyzed (Fig. 4d). Specifically, the HA levels of the most developed cancer tissue type, AGC showed a significantly lower amount of HA than all other biopsy samples. Normal, non-cancerous tissue showed a significantly lower content of non-sulfated disaccharide than the cancerous tissue samples.

The impact of GAG content, disaccharide content sulfation patterns, and GAG sequence on signaling in cancerous tissue is still an open question. The current study on stomach cancer tissue shows that HA content is higher in AGC. HA has been suggested to play an important role in the metastasis of cancerous cells based on its linkage to cell mobility in embryonic tissue [14]. Similarly, in cancer HA may support cell migration signaling and provide the cells with a coating of a GAG with low protein binding affinity, a role played by HA in wound healing [14, 23].

Decreases in HA levels in the most severe type of gastric cancer tissue measured, AGC, must be interpreted with caution. While many studies have found links between HA expression and high metastasis, other studies have shown that HA levels are relatively specific to the tissue of origin, and are spatially dependent (different levels at different places on the tumor) [13, 14]. Specifically, Wang and co-workers found little to no HA expression within the body of the tumor in a set of 10 stomach cancer samples, finding that HA was limited to the outer stromal layer [14]. The differences observed here may in some ways be a reflection of the tumor morphology—in the more developed, cohesive AGC tumors, the overall HA content decreases as a larger percentage of the tissue mass is the HA deficient tumor body.

The significantly decreased levels of undersulfated CS, rich in C-OS disaccharide, observed in the stomach cancer samples in the current study, is entirely consistent with other observations of lowered sulfation levels in cancerous tissues [24–26], including gastric cancers [4]. The shift in cancerous tissues to a lower sulfation pattern may reflect a larger metabolic shift in enzyme expression levels and biosynthetic pathways that have yet to be determined. Of note here is the increase in undersulfated CS tissue in all pre-cancerous and early cancerous tissue, indicating that these tissues already share significant metabolic patterns with advanced tumors.

Although the results of this study do not provide disaccharide composition patterns that could provide significant diagnostic or prognostic matrices, they do shed light on the changing metabolism and GAG expression of cancerous and

pre-cancerous tissue. In addition the current study provides a sensitive analytical method with an LOD of 0.1 ng GAG capable of providing GAG composition on very small (250 µg) biopsy samples.

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