MASS SPECTROMETRY-BASED ANALYSIS OF CHONDROITIN SULFATE AND HEPARAN SULFATE GLYCOSAMINOGLYCANS IN LUNG CANCER TISSUE SAMPLES USING OPTIMIZED SAMPLE PREPARATION CONDITIONS

PhD thesis

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1. Introduction

The investigation of glycosaminoglycans (GAGs) of biological origin requires several sample preparation steps. The general sample preparation workflow of tissue GAG samples involve the cleavage of the large polysaccharide chains into oligo- or disaccharides which can be analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). The cleavage process is followed by solid-phase extraction (SPE) purification, evaporation, freezing and thawing. These steps can include further intermediate steps like sample transfer and storage, which can also lead to significant sample loss. For heparan sulfate (HS) disaccharides the solvent evaporation, freezing and storage are the most critical factors affecting stability and recovery. These factors are heavily influenced by solvent type, storage vessel, temperature and pH.

Several parameters influencing the stability and recovery of chondroitin sulfate (CS) disaccharides have been already investigated. Since HS disaccharides can contain more sulfate groups than CS disaccharides, their stability

could be more significantly affected by the impact of storage and sample preparation, however there is lack of literature regarding the stability and recovery of HS disaccharides.

Proteoglycans (PGs), i.e. proteins carrying covalently attached GAG chains play a crucial role in preserving the structure of the extracellular matrix and controlling signal transduction. Therefore, alterations in their glycosylation can greatly impact their function and disrupt multiple signalling pathways within the tumor microenvironment. In the literature the alteration of protein glycosylation is well-documented and is a commonly observed feature of cancer. Changes in the total quantity and sulfation characteristics of GAG chains have been already observed between healthy and cancerous tissues.

Lung cancer (LC) is the most frequently diagnosed cancer type considering both men and women and holds the highest mortality rate globally (2022). In many cases the treatment options are limited, and metastasis occurs frequently to other organs.

Histologically LC can be categorized into two primary groups: small cell lung cancer (SCLC) and non-small cell

lung cancer (NSCLC). NSCLC constitutes approximately 85%, while SCLC for 15% of all LC cases. NSCLC is a heterogeneous group and can be further categorized into adenocarcinoma (AC), squamous cell carcinoma (SqCC) and large cell carcinoma (LCC). Differences in the total quantity and in the sulfation patterns of GAG chains have already been observed between healthy and tumor tissues in case of lung cancer.

Multiple genetic alterations in key oncogenes have been frequently identified in lung AC, including epidermal growth factor receptor (*EGFR)*, Kirsten rat sarcoma viral oncogene homolog (*KRAS)* and anaplastic lymphoma kinase (*ALK)*. These genetic alterations have been identified as oncogenic drivers of AC possessing predictive value for targeted therapies. Mutations or rearrangements in genes such as EGFR, KRAS and ALK can serve as specific molecular targets offering prognostic value.

2. Objectives

The dissertation is based on three interlinked research projects. The work was approved by the Medical Research Council (TUKEB), ethical permit number: IV/2567-4/2020/EKU, 22/04/2020.

The objective of the first project was to perform a comprehensive study comparing common parameters and methods for analyzing HS samples of biological origin, aiming to reduce biases arising from sample loss during sample preparation. For the sample preparation of HS, the common workflow includes HS chain digestion into disaccharides, purification and analysis. This multi-step process involves several intermediate sample handling steps that have a great impact on the results. Therefore, we have systematically examined the type of buffer used for digestion, injection conditions, solvent evaporation and freezing cycles for storage. An additional aim was to evaluate factors influencing sample stability and recovery to ensure more reliable and reproducible HS disaccharide analysis.

The aim of the second project was to investigate the composition of CS/DS and HS GAGs in different lung tumor phenotypes and adjacent normal lung tissue samples through tissue surface enzymatic digestion. Following the digestion and SPE purification of the samples, HPLC-MS measurements were performed using an in-house packed capillary column with HILIC-WAX mixed solid phase resin, detected in negative ionization mode.

During the third project, we performed a comprehensive investigation of CS/dermatan sulfate (DS) disaccharide composition in lung AC tissues with ALK, EGFR and KRAS genetic alterations, and compared these profiles with triple wild-type (WT) lung AC tumors.

3. Methods

In the first project parameters influencing the stability and recovery of HS disaccharides were investigated and all the samples and their control samples were prepared together in one batch. The impact of evaporation was examined using a heated vacuum concentrator at 55°C for ammonium bicarbonate (AMBIC), tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) and water solvents. Investigating the effect of freezing, samples were dissolved in water and subjected to repeated freeze-thaw cycles (5 or 10 cycles) with standard freezer (−20°C) or with liquid nitrogen (−196°C) and thawed in block heater at 37°C. The storage temperature was investigated following 48 h storage of 50 µL water dissolved samples at −18, 4, 20, 37 and 55°C temperatures. The storage pH was examined with 50 µL 1 mM hydrochloric acid (pH 3), water (pH 7) and 1 mM NaOH (pH 11) solutions at 37°C for 0, 6, 12 and 24 h. The effect of the different digestion buffers was examined by dissolving samples in 50 µL of 12.5 mM AMBIC and 20 mM Tris–HCl and incubated for 24 and 48 h at 37°C.

In order to investigate the impact of storage vessels on the recovery of HS disaccharides, samples were dissolved in 20 µL water or in a commonly used HPLC–MS injection solvent (10 mM ammonium formate in 75:25 v/v ACN:water) and stored at 4°C for 24 h. 0.5 mL plastic Eppendorf Safe-Lock Tubes (Eppendorf Corporate) and borosilicate type 70 glass vial with glass insert (LAB-EX Kft.) containers were compared. To investigate the effect of solvents on sample storage, samples were dissolved in 20 µL of the following solvents: 90:10 v/v% ACN:water, 75:25 v/v% ACN:water, 50:50 v/v% ACN:water, 10 mM ammonium formate in 75:25 v/v% ACN:water, 75:25 v/v% MeOH:H2O and 10 mM ammonium formate in 75:25 v/v% MeOH:water. All the samples were stored at 4°C for 0, 6 and 12 h.

In the second project, different LC subtypes and their respective tumor adjacent formalin-fixed, paraffin-embedded (FFPE) human tissue samples were analyzed from the Department of Pathology, Medical School, and Clinical Center, University of Pécs, and the Teaching Hospital Markusovszky, Szombathely, Hungary. The LC

types investigated were the following: SCLC and three NSCLC subtypes, AC, SqCC and LCC.

In the third project, lung AC tissue samples were examined with the following genetic alterations: ALK, EGFR or KRAS and compared to wild-type. For the experiment the FFPE tissue samples were obtained from the National Korányi Institute for Pulmonology.

Both in the second and third projects, three micrometer-thick tissue samples were fixed in 10% buffered formaldehyde and embedded in paraffin. The samples were dewaxed by washing the tissues with xylene, 100%, 90% and 70% ethanol, 10 mM ammonium bicarbonate solution and water. In the following step, antigen retrieval of the samples was performed with 95 mM trisodium citrate and 21 mM citric acid (pH 6) at 85 °C for 30 min. CS/DS chains were digested on the tissue surface using a previously developed methodology. The composition of the digestion solution was the following: in the second project 20 mM Tris–HCl, 2.5 mM ammonium acetate, and 2 mU/μL chondroitinase ABC (pH = 7.6) and 10% glycerol containing solution, while in the third project: 25 mM AMBIC, 2.5 mM ammonium

acetate, 2 mU/μL Chondroitinase ABC and 10% glycerol. The digestion of HS chains was also performed based on a previously developed methodology with 20 mM Tris–HCl, 2.5 mM $Ca(OH)_2$, 0.5 mU/μL of heparin lyase I, 0.1 mU/μL of heparin lyase II, and 0.1 mU/μL of heparin lyase III and 10% glycerol digestion solution. After digestion a two-step SPE purification combined clean-up method was performed.

In all the projects the chromatographic separation was carried out on fritted and self-packed, 250 μm i.d. fused silica capillaries using GlycanPac AXH-1 1.9 μm solid phase (Thermo Fisher Scientific). For the separation the following HPLC eluents were used: 10 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4) (solvent A) and 65 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4) (solvent B). The detection was carried out in negative ionization mode. HS and CS/DS disaccharides were detected in MS1 mode, while CS/DS disaccharide positional isomers were differentiated using MS/MS.

Waters nano-Acquity ultrahigh performance liquid chromatography (UPLC) system attached to Waters quadruple time of flight (Q-TOF) Premier mass

spectrometer was used for the investigation of the stability and recovery of HS disaccharides and for analyzing the CS/DS disaccharides in the second project.

The HPLC–MS investigation of HS disaccharides in the second- and the CS/DS disaccharides in the third project were done by a Waters Acquity I-class UPLC (Milford, MA) coupled to a Waters Select Series Cyclic Ion Mobility (Milford, MA, USA) mass spectrometer.

In the first and third project the statistical analysis and the data visualization were conducted using Microsoft Office Excel and Python (3.11.7) with Spyder (5.4.3), while for the second project R (4.0.5) in RStudio (1.4.1106) was used.

4. Results

4.1. Stability studies of HS disaccharides

We investigated the impact of the solvent used during evaporation. For most HS disaccharides, recovery rates ranged between 80% and 100% using either 12.5 mM AMBIC or water as solvent. However, using 20 mM Tris-HCl the recovery drastically reduced.

Investigating the freezing methods the disaccharide content was lower in the −20°C freezer (5 cycles). For 10-cycle freezing, liquid nitrogen was more favorable.

Investigating the effect of storage temperature indicates that storing samples at or below 4°C for 48 h is a viable option, as disaccharide recovery at −18 and 4°C ranged between 85% and 95%.

Examining the effect of storage pH, the most significant sample loss occurred at basic pH (pH 11), with recovery values ranging between 10% and 60%. At pH 3, recovery ranged from 60% to 100%. Under neutral conditions (pH 7), recovery varied between 75% and 100%. Next, we investigated the effect of the most commonly used digestion buffers during enzymatic cleavage on sample

recovery. During 24 and 48 h storage in AMBIC solution at 37°C the recovery was between 70% and 100%. The recovery of most disaccharides dropped significantly after 24 and 48 h storage in 20 mM Tris–HCl solvent. Examining the effect of storage vessel type, for aqueous solutions higher recovery values were observed in plastic tubes. In glass vial the D0S6 and D2S6 disaccharides showed a significantly higher recovery in water than in the injection solvent. The ACN-based injection solvents provided more reliable results over 12 h storage compared to the MeOH–containing solvents. In the presence of 10 mM ammonium formate the recovery increased for most disaccharides in both ACN and MeOH containing injection solvents.

4.2. Analysis of lung tumor sections with different cancer subtypes

Total CS/DS disaccharide quantity was found to be significantly higher in the tumor tissues compared to adjacent samples. Comparing CS/DS disaccharides between all the tumor and adjacent sample groups, significant differences were observed in the D0a0, D0a4, and D0a6 disaccharide content. In the tumor samples the

D0a0 abundance decreased, while the D0a4 and D0a6 content increased. For CS/DS disaccharides the average degree of sulfation was found to be higher, while the 6-*O*-/4-*O*-sulfation ratio was lower between all tumor and all adjacent samples.

The total HS disaccharide content showed almost identical values both in tumor and tumor-adjacent regions. Comparing all the tumor and all the tumor-adjacent samples, the relative quantity of D2A0 + D0A6 was higher, while it was lower for D2S0 + D0S6 in the tumor samples. A decrease of the mono-, di-, and total *N*/*O*-sulfation ratios were observed in the tumor samples. Between tumor and their respective adjacent samples, the abundance of D0a0 CS disaccharide decreased, however the relative abundance of D0a4 and D0a6 CS disaccharides, and the average degree of sulfation, increased in all the examined lung tumor subtypes. The total CS/DS abundance showed an increased level for AC, SqCC, LCC. The 6-*O*/4-*O*-sulfation ratio decreased in the case of SCLC, SqCC, and LCC groups.

4.3. Analysis of lung adenocarcinoma sections with different genetic alterations

Significantly higher relative abundance was observed for ALK and WT groups compared to EGFR group in the case of D0a4 disaccharide. The 6-*O*-/4-*O*-sulfation ratio was significantly higher in the EGFR group compared to ALK sample group. The different mutation types within the EGFR sample group did not show any separation in terms of CS/DS disaccharide abundance.

5. Conclusions

We identified several parameters, which are favorable to use and others that should be avoided during the sample preparation process of HS disaccharides. Evaporation of HS disaccharide samples from Tris-HCl containing solvent in heated vacuum centrifuge should be avoided. Storage of HS disaccharide samples under alkaline conditions is not recommended. It is advisable to avoid frequent thawing and freezing of HS disaccharide samples, however if it is necessary rapid freezing is preferable with liquid nitrogen (-196 °C). The storage of HS disaccharides is safe under neutral pH conditions for up to 24 h at room temperature and even up to 48 h at or under 4 °C. Ammonium formate salt additive enhances the stability of the multiply sulfated HS disaccharides in both ACN and MeOH-based solvents. HS disaccharide precipitation can be reduced by using aqueous solution and storage in plastic tubes.

We examined the CS/DS and HS disaccharide content and sulfation of various lung tumor phenotypes and corresponding adjacent normal tissues. The total

abundance of CS/DS disaccharides was doubled in tumor samples, while the total abundance of HS disaccharides did not change significantly in line with previous literature. The average degree of CS/DS sulfation significantly increased in all the investigated tumor phenotypes compared to the adjacent normal tissue. The CS 6-*O*-/4-*O*-sulfation ratio was elevated in AC compared to the other lung tumor phenotypes investigated. *O*-sulfated HS components increased in tumor samples.

We identified a few differences in CS/DS disaccharide composition and sulfation patterns between the three key oncogenes including ALK, EGFR and KRAS and WT lung AC tissue. The D0a4 relative abundance for the ALK and EGFR groups was significantly different compared to the WT sample group. In the case of 6-*O*-/4-*O*-sulfation ratio the ALK and EGFR sample groups were found to be significantly different from each other, suggesting different roles in receptor tyrosine kinase binding of the CS chains.

6. Bibliography of the candidate's publications

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