SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Analysis of formalin-fixed and paraffin-embedded tissue samples by capillary electrophoresis

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Doctoral School of Molecular Medicine

Debrecen, 2018

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The Examination takes place at Department of Physiology, Faculty of Medicine, University of Debrecen, March 19, 2018 at 11:00 AM

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, March 19, 2018, 3:00 PM

1. Introduction

Our ideas about carbohydrates have been significantly revalued over the past few decades: they have long been considered as only spare materials (cellulose and chitin), energy sources (starch and glycogen) or constituents of nucleic acids. In the last decades, it has been discovered that carbohydrate-carbohydrate, carbohydrate-protein and carbohydrate-nucleic acid interactions have important physiological significance. Different glycoforms play an important role in biological processes such as viral infection, signal transduction, inflammation, cell-cell interactions, bacterial-host cell interactions, fertility and -/post embrional development. Macro- and microheterogeneity of complex sugar molecules, however, poses a much greater challenge to researchers during isolation than proteins and nucleic acids. As a consequence of frequent branching and link diversity of sugar structures, carbohydrates have the greatest structural complexity among biopolymers.

Ferdinand Blum, a German physician introduced formalin as a tissue fixative in 1893. It protects samples from shrinkage and degradation and stabilizes the structure of cells and tissues. Furthermore, formalin is compatible with the conventional and widely used staining agents such as hematoxylin and eosin. For these reasons, the technique has become the most exclusive fixative in pathology.

Formalin-fixed paraffin-embedded (FFPE) samples are routinely made for histopathological studies in hospitals worldwide. FFPE samples are successfully used for the analysis of biomolecules in genomics, proteomics, metabolomics and lipidomics. glycomics is a relatively new, but developing field of omics. The importance of this area is underlined by the fact that carbohydrates carry vital functions in biological processes. Cancer diseases are usually associated with changes in cell surface carbohydrate structures, therefore, tracking specific glycan structural changes may lead to the discovery of new cancer biomarkers.

2. Objectives

The growing demand for the use of archived samples induced the development of analysis methods. So far LC / MS and MALDI techniques have been mostly utilized as analytical tools. To the best of my knowledge, our team was the first to use CE-LIF (capillary electrophoresis laser-induced fluorescence) to analyze the glycan profile of FFPE samples. During my PhD studies, I wanted to test whether fixation causes structural changes in carbohydrates. For this purpose, formalin-fixed paraffin-embedded glycoproteins, human serum and murine tumor tissue samples were used. The samples were digested by PNGase F endoglycosidase enzyme, and the released sugars were labeled fluorescently (aminopyrenesulphonate-APTS) and then measured by CE-LIF.

The aim of my thesis is to demonstrate that 1) N-linked oligosaccharides do not alter with formalin fixation and paraffin embedding and 2) FFPE tissue samples can be used to determine the total N-glycan profile or provide the possibility to identify sugar structures from archived tissues.

3. Results and discussion

3.1 Investigation of the effect of formalin fixation and paraffin embedment on the glycan profile

3.1.1 Standard glycoproteins

Analysis of standard glycoproteins means the basis of my research, as their N-glycan structures were known and could be measured by CE-LIF. Along these lines, three glycoproteins (Immunoglobulin G, Fetuin and Ribonuclease B) were analyzed in lacking fixation (control), formalin-fixed and formalin-fixed paraffin-embedded forms. These glycoproteins represent important subgroups of N-glycosylation: they contain mainly neutral, sialylated or high mannose sugar structures. The experiments were performed with 3 parallel samples and with 3 repetitions. During the separation, each sample was injected twice. I found that no significant differences were observable between the fixed, embedded and untreated samples.

3.1.2 Human serum

Glycosylation changes that may be caused by formalin fixation were further tested on human serum due to the fact, that its complexity is closer to clinical specimens than glycoproteins. The measurements were similar to the glycoproteins discussed above: I compared non-fixed, formalin-fixed and formalin-fixed paraffin-embedded samples.

In this case, I found some discrepancies between the N-glycan profiles of the three differently prepared samples. However, this deviation (12.68 RSD% relative standard deviation), which is within the 15% ICH guideline (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, http://www.ich.org/home.html) so it has no biological significance.

3.1.3 Mouse tumor tissue sample

Based on the positive results described above, the subject of my next study was to analyze the complete N-glycosylation profile of mouse tumor tissue samples. SCID (severe combined immune deficiency) male mice were used for the experiments from the National Institute of Oncology. These mice do not develop immune response due to missing or atypical T and B lymphocytes. HT-1975 lung cancer cells were injected (each with 2 million cells) to the mice laterally as the generated tumor had to be retrieved and there the skin anatomically was less

vulnerable. The biopsy was made when the tumor diameter reached 0.5 to 0.8 cm. The sample was taken from deceased animals.

For precise structural determination of the oligosaccharides, carbohydrate sequencing was carried out, which is based on the cleavage of the non-reducing chain ends via enzymatic digestion. For this sequencing technique, exoglycosidase enzymes are required with specificity for the monosaccharides or the binding types. The enzymes were: Arthrobacter ureafaciens sialidase (ABS) for the removal of α(2-3,6,8,9) bound sialic acids; bovine kidney fucosidase (BKF) to release α (1-2,3,4,6) fucoses; Jack bean (Canavalia ensiformis) galactosidase (JBG) to remove β(1-4,6) bound galactose and Jack bean hexosaminidase (JBH) for the cleavage of N-acetylglucosamine with β(1-2,3,4,6) linkage (each form Prozyme, 0.5 U). Sequencing was performed by the combined matrix type loading of the enzymes. To this, I divided the sample into equal parts, to the first I was adding only the sialidase enzyme, to the second the sialidase and the fucosidase enzymes, to the third the sialidase, the fucosidase, and the β-galactosidase and finally the sialidase, fucosidase, β-galactosidase, and hexosaminidase. As a result of the sequencing, 22 different sugar structures were identified from the N-glycome of the mouse tumor tissue samples.

The intact, formalin-fixed and FFPE samples were different only in the intensity of three specific structures: each of these peaks had higher abundance (3x) in formalin fixed state than in the control sample. The relative standard deviation of the parallel samples was 12.91-16.22 RSD% for these three peaks. The structures are monosialylated oligosaccharides in which the sialic acid units are linked by α(2,6) bonds and come from three disialylated structure. These structures lost their unstable, α(2,3) linked sialic acid units during fixation.

3.2 Analysis of N-glycans from FFPE mouse tissue samples

Since the results of my previous studies suggested that formalin fixation and paraffin embedding did not have a significant effect on the carbohydrate of the glycoproteins, I extended my research to the complete N-glycan profile determination of various FFPE tissue samples from mouse organs and accurately identified the structures found there. For these experiments, I used the following formalin-fixed paraffin-embedded mouse organs: lung, brain, heart, spleen, liver, kidney and intestine. Different types of tissue samples exhibit different N-glycan profiles, which were specific to the particular tissue. The relative standard deviation of the glycan

profiles was 8.72 RSD% for samples measured on the same day. Additionally, tissues of individual organs contained different ratios of high and low sialylated or neutral structures.

To identify the structures of the N-glycan profiles from each tissue, oligosaccharide sequencing was necessary. To determine the sugar constituents of any oligosaccharide and their type of binding, exoglycosidase matrix digestion was performed as described above. First, I examined N-glycans from lungs and deciphered 16 different N-linked oligosaccharides. I also performed the identification of certain oligosaccharides of purified and APTS labeled N-glycans from other FFPE mouse tissue samples by exoglycosidase enzymatic sequencing in conjunction with an online database.

3.2 Changes in the N-glycan profile postmortem, from death to fixation

In the third part of my work, I was looking for a relationship between the ratio of sugar structures in total N-glycan profile and the time elapsed between sampling and fixation. Mouse brain and lung tissue samples were used. It was clearly discernible that sialic acid structures respond sensitively to long standing at room temperature. In all cases, the sialic acid units are located farthest from the oligosaccharide linkage to the protein (or the fluorophore group) and can often be cleaved without enzymatic digestion when the temperature is increased. This is due to the fact that the tissue samples directly processed at the time of death preserved these structures to the greatest extent.

Interestingly, we cannot clearly demonstrate this regularity on the lung tissue sample. It can be explained by the fact that the N-glycan profile of the two tissues contained different oligosaccharide structures. However, I can state that FFPE samples stored in hospitals and research institutes can be an important source of information for glycomic discoveries.

4. Conclusion

The surface of the living cells is richly decorated with various carbohydrate chains that play important roles in the body and cell life as part of the glycoconjugates. Glycan structures of glycoproteins influence the conformation, determine lifetime of the associated protein, participate in cell-cell interactions, and could be receptors for different lectins or antibodies. The growing field of glycomics explores the structure to function relationship of carbohydrates. As oligosaccharides can change due to diseases, they can be used as biomarkers. FFPE samples represent a good alternative to fresh/frozen samples because of their easier storage and longer shelf-life. So far, FFPE samples have been used to isolate nucleic acids for gene expression analysis in genomics, for reverse phase array Western blotting and mass spectrometry experiments in the proteomics field. In the glycomics field, MSI (mass spectrometry imaging) techniques have only been used to investigate the glycosylation of proteins from FFPE samples. However, despite the fact that it is capable of detecting and determining a large number of different materials at the same time, this technique cannot distinguish between the individual binding and positioning isomers that have an important physiological role and, consequently, biomarker significance. In addition, it requires large amount of sample, as it has relatively low sensitivity. Based on my studies, I have found that carbohydrates are not effected by formalin fixation, so they can be used in glycomic research. Capillary electrophoresis is a high-resolution technique, which enables the separation of isomers and anomers. Using laser-induced fluorescence, high sensitivity can be achieved.

In my thesis, I wanted to answer the following questions: 1) whether the oligosaccharide chain of glycoproteins was altered by formalin fixation and paraffin embedding, and 2) would it be possible to determine the complete N-glycan profile of FFPE tissue samples and identify each oligosaccharide structure. During my research, using standard glycoproteins, human sera and mouse tumor tissue samples I confirmed that formalin fixation and paraffin embedding did not change the number of N-glycan peaks detected and only slightly changed their distribution. In the glycomic analysis of FFPE mouse tissue samples, N-linked glycans extracted from different tissues produced distinct glycosylation profile of the tissue types. The exact structure of the oligosaccharides from the individual tissue samples was released by exoglycosidase matrix digestion. In summary, I have determined that FFPE samples are suitable for comprehensive N-glycan analysis. In the future, it would be possible to identify the sugar structures from

smaller amounts of materials with the development of sample preparation methods, and to carry out an analytical validation of the method with larger sample number.

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Registry number: DEENK/398/2017.PL
Subject: PhD Publikációs Lista

Candidate: Boglárka Döncző
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Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10037975

List of publications related to the dissertation

1. **Döncző, B.**, Szarka, M., Tóvári, J., Ostoros, G., Csánky, E., Guttman, A.: Molecular glycopathology by capillary electrophoresis: analysis of the N-glycome of formalin-fixed paraffin-embedded mouse tissue samples.
Electrophoresis. 38 (12), 1602-1608, 2017.
DOI: http://dx.doi.org/10.1002/elps.201600558
IF: 2.744 (2016)

2. **Döncző, B.**, Szigeti, M., Ostoros, G., Gács, A., Tóvári, J., Guttman, A.: N-Glycosylation analysis of formalin fixed paraffin embedded samples by capillary electrophoresis.
Electrophoresis. 37 (17-18), 2292-2296, 2016.
DOI: http://dx.doi.org/10.1002/elps.201500446
IF: 2.744

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List of other publications

3. **Döncző, B.**, Kerékgyártó, J., Szurmai, Z., Guttman, A.: Glycan microarrays: new angles and new strategies.
   Analyst. 139 (11), 2650-2657, 2014.
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4. Ágoston, K., Gyémánt, G., Kalmár, L., Kerékgyártó, J., Szurmai, Z., **Döncző, B.**, Guttman, A.: Synthesis and MALDI-TOF MS Analysis of Protected Oligosaccharide Components of N-Glycoproteins.
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   J. Carbohydr. Chem. 31 (3), 203-219, 2012.
   DOI: http://dx.doi.org/10.1080/07328303.2011.642433
   IF: 0.847

Total IF of journals (all publications): 11,859
Total IF of journals (publications related to the dissertation): 5,488

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

16 November, 2017