

Glycosylation of proteins in healthy and pathological human renal tissues

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Abstract: Cancer development is associated with the improper glycosylation of proteins. There are alterations in the synthesis and expression of sugar structures. These changes can be important not only in the early stages of tumour development, but also in the next stages connected with cancer invasiveness and its ability to form metastases. Oligosaccharide structures of glycans in tumours deviate from normal cells. Relatively increased degrees of branching and sialylation of N-glycans, enhanced presentation of short-chain mucin-type O-glycans with sialylation, and alterations in the expression of blood group ABO and Lewis epitopes can be observed. The main aim of our study was to assess changes in the glycosylation of proteins in clear cell renal cell carcinoma. This study was performed on tissues taken from 15 patients. The relative amounts of sugar structures of proteins with molecular mass above 30 kDa in tumour (cancer tissue), intermediate zone i.e. tumour-adjacent tissue, and normal tissue uninvolved by tumour, were determined by ELISA-like test with biotinylated lectins highly specific to examined sugar antigens. A higher expression of all examined structures was revealed in cancer tissue. Increased levels of sialic acid, fucose, T and Tn antigens, compared to healthy renal tissue, were characteristic for clear cell renal cell carcinoma. (*Folia Histochemica et Cytobiologica* 2012, Vol. 50, No. 4, 599–604)

Key words: biotinylated lectins, ELISA, glycosylation, clear cell renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) represents the most prevalent malignancy of the kidney and accounts for about 85% of all kidney cancers worldwide [1]. In Poland, renal cancer comprises 3–4% of all malignancies. Clear cell renal cell carcinoma (ccRCC, *carcinoma clarcellulare*) is the most frequent among renal cancers in adults. Despite the application, in recent years, of new diagnostic procedures, RCC is most often recognised at an advanced stage, when distant metastases are present.

Well-documented altered glycosylation in malignancy should be rigorously examined in diverse classes

of tumours by glycomic profiling to precisely define changes in oligosaccharide structures of glycoconjugates or by glycoproteomics to define target proteins.

Glycoproteins coat all eukaryotic cells and play pivotal roles in many aspects of tumour progression. Cancer transformation, which causes alterations in the synthesis and expression of specific sugar structures, is connected with the incorrect glycosylation of proteins. These changes are important not only in the early stages of tumour development, but also in later stages connected with cancer invasiveness and its ability to form metastases which is due to the alterations of adhesion of cells to tumour, endothelium, blood vessels and extracellular matrix. A huge number of bonds between monosaccharides can be formed. Oligosaccharide chains can comprise a significant amount of biological information, which involves sugar composition, its sequence and spacious configuration. The sugar code could represent a third, together with nucleic acids and proteins, biological system of

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Table 1. Tumour size, Fuhrman nuclear grade and TNM tumour stage

Lesion size* and mode of presentation	Fuhrman nuclear grade				TNM tumour stage			
	G1	G2	G3	G4	T1	T2	T3	T4
No.15	2	11	2	0	8	5	2	0

*All tumours were ≤ 3 cm

holding and transferring information between body cells [2–6].

The majority of elements of intercellular connections are components of glycocalyx, surface structures containing oligosaccharide chains. Alterations in the behaviour of cancer cells compared to healthy ones depend, among other things, on mutual interplay of proteins of glycocalyx. It has been suggested that interactions of cell surfaces in high degree depend on reciprocal adjustment of sugar levels, which result in various alterations of cell function [7]. The structure of cell surface oligosaccharides depends on the course of their biosynthesis in Golgi and can depend on activity and substrate specificity of glycosidases. In a case of improper glycosylation, some secreted glycoproteins aggregate or become degraded, which results in the inhibition of their secretion. Other improperly glycosylated proteins become secreted but lose biological activity [8]. Oligosaccharide chains protect polypeptides against proteolytic enzymes, alter their immunological properties, influence the transmission of signals by cell receptors, and modulate the activity of enzymes and glycoprotein hormones [9].

The aim of our work was to assess the differences in the glycosylation of proteins in clear cell renal cell carcinoma in tumour, tumour-adjacent intermediate tissue, and normal renal cortex/medulla uninvolved by tumour. Expression of sialic acid, fucose, mannose, T and Tn antigen, sugars of which levels have been shown to be changed in different cancers, was estimated in tissue extracts.

Material and methods

The study was performed on renal tissues taken from 15 patients with clear cell renal cell carcinoma kidney cancer, hospitalised in the Department of Urology, Medical University of Białystok, Poland. Diagnosis was confirmed by histopathological assessment in the Department of Pathological Anatomy, Medical University of Białystok. The age of patients ranged from 51 to 72 years (mean age 60, seven females and eight males). Kidneys were subjected to complete nephrectomy, and then sectioned in such a way as to have a cross-section plane passing through the centre of the tumour and its long axis. Tumour size, Fuhrman nuclear

grade, and TNM stage are presented in Table 1. Tissue samples ($2 \times 2 \times 2$ cm) were taken from the tumour (C), an intermediate zone which was adjacent to tumour tissue (I), and normal (N) renal cortex/medulla which was uninvolved by tumour. Samples were rinsed with saline, dried with blotting paper and stored at -70°C .

Renal tissues were thawed and immersed in 0.1 M citric buffer pH 4.3 in order to prepare 10% (w/v) tissue suspensions. Then, tissues were homogenised in Ultra-Turrax T8 homogeniser for 2 min. The homogenate was centrifuged at 10,000 g at 4°C for 20 min. The supernatants were concentrated in Centriprep C30 concentrators (Amicon, Millipore, Bedford, MA, USA). Concentrated samples were applied on a Sephadex G-10 column and eluted with water. Collected 2 ml fractions were assayed for absorbance at 280 nm. Samples containing protein were collected and concentrated in Centripreps C30. Aliquots were centrifuged several times at 1,500 g for 60 min and supernatants were taken for further analyses. The concentration of proteins in the samples was 40–60 mg protein/mL. Sugar structures of isolated proteins were analysed using ELISA-like test with biotinylated lectins (Vector Laboratories, Burlingame, CA, USA). The binding specificity of lectins is presented in Table 2. Samples of renal tissue were diluted to a protein concentration of $5 \mu\text{g/mL}$ in PBS buffer (Phosphate-Buffered Saline) and aliquots ($50 \mu\text{l}$) were coated onto microtiter plates (NUNC F96; Maxisorp, Roskilde, Denmark) at room temperature overnight. The microtiter plates were washed three times with PBS-T washing buffer (PBS, 0.05% Tween (PBS-T), pH 7.4) between all ensuing steps. Unbound sites were blocked with $100 \mu\text{l}$ of 3% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) in washing buffer for 1 hour. Then the plates were incubated with $100 \mu\text{l}$ of biotinylated lectins (diluted to $0.5 \mu\text{g/mL}$ in PBS-T, 1% BSA with $0.1 \text{ mmol/L CaCl}_2$; *Maackia amurensis* (MAA) and *Vicia villosa* (VVA) were also supplemented with $0.01 \text{ mmol/L MnCl}_2$) for 1 hour. Then plates were incubated with $100 \mu\text{l}$ of horseradish peroxidase avidin D (Vector Laboratories, Burlingame, CA, USA) (1:2,500) in PBS-T, 1% BSA for 1 hour. Next, plates were washed four times in PBS and the colour reaction was developed by incubation with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) liquid substrate for horseradish peroxidase (Sigma, St Louis, MO, USA). Spectrophotometric measurements were performed after 45 min at 405 nm using an Infinite M200 mi-

Table 2. Lectins used in the study and their binding specificity

Lectin	Binding preference
<i>Maackia amurensis</i> (MAA)	NeuAc α 2–3Gal
<i>Sambucus nigra</i> (SNA)	NeuAc α 2–6Gal/GalNAc
<i>Aleuria aurantia</i> (AAA)	Fuc α 1–6GlcNAc
<i>Ulex europaeus</i> (UEA)	Fuc α 1–2GlcNAc
<i>Tetragonolobus purpureus</i> (LTA)	Fuc α 1–3GlcNAc
<i>Galanthus nivalis</i> (GNA)	Man α 1–3
<i>Narcissus pseudonarcissus</i> (NPA)	Man α 1–6
<i>Vicia villosa</i> (VVA)	antigen Tn (GalNAcO-Ser/Thr)
<i>Arachis hypogaea</i> (PNA)	antigen T (Gal β 1–3GalNAcO-Ser/Thr)

croplate reader (Tecan, Salzburg, Austria). The protein content was determined using the bicinchoninic acid (BCA) test according to the method of Smith et al. [10].

Statistical analysis. Statistical analyses were done using Statistica 9.0 (StatSoft, Krakow, Poland) according to ANOVA and post hoc test. Pearson's correlation coefficients were used to determine the association between two variables. For nonparametric statistics, we used Friedman ANOVA test. Spearman's rank correlation coefficients were used to study the associations between variables. Results are expressed as means \pm SD. Statistical significance was defined at $p < 0.05$. The study was approved by the Institutional Ethical Committee and conducted according to the principles of the Declaration of Helsinki.

Results

Sugar structures of glycoproteins isolated from the studied renal tissues were examined by ELISA-like test with biotinylated lectins.

We observed a significant increase ($p < 0.05$) in the amount of fucose combined with 1,2,3 and 6 carbons of GlcNAc: Fuc α 1-2GlcNAc, Fuc α 1-3GlcNAc, Fuc α 1-6GlcNAc in oligosaccharide chains of glycoproteins of tumour (C) and intermediate renal tissue (I) compared to normal renal tissue (N, cortex/medulla uninvolved by tumour). This was revealed by increases in reactivity with UEA, LTA and AAA lectins (Figure 1A). We found positive correlations between all examined types of renal tissues for AAA and LTA lectin. Correlations for UEA were negative or very low (Table 3).

The analysis of interactions of MAA and SNA with glycoproteins of all the tissues showed a higher amount of bindings for NeuAc α 2-6 than NeuAc α 2-3. Both linkages showed significantly higher levels in cancer tissues compared to healthy and intermediate tissue (I) (Figure 1B). Significant differences for MAA lectin were observed for renal cortex/ medulla uninvolved by tumour (N) and cancer tissue (C) and also for intermediate tissue (I) and cancer tissue (C). For both lectins, positive correlations between all examined renal tissue regions were assessed (Table 3).

Oligosaccharide chains of renal glycoproteins of intermediate (I) and tumour (C) tissues showed significant increases ($p < 0.05$) in the amount of disaccharide core structures of O-glycans represented by T antigen (Gal β 1-3GalNAcO-Ser/Thr) compared

Table 3. Pearson's and Spearman's coefficients calculated for three variables

Lectins used for determination of specific sugar structures	Pearson's(*) and Spearman's(**) coefficients values (r)		
	Normal (N) v. Intermediate tissue (I)	Normal (N) v. Cancer tissue(C)	Intermediate (I) v. Cancer tissue(C)
AAA(*)	0.40	0.26	0.66
UEA(**)	-0.11	0.57	0.03
LTA(**)	0.63	0.71	0.74
MAA(**)	0.48	0.55	0.52
SNA(*)	0.71	0.67	0.75
PNA(**)	0.13	0.22	0.66
VVA(**)	0.03	0.01	0.40
GNA(**)	0.55	0.57	0.47
NPA(**)	-0.06	0.16	0.43

Degrees of correlation coefficients: $r = 1$, perfect correlation; $0.75 \leq r < 1$, high degree; $0.25 \leq r < 0.75$, moderate degree; $0 < r < 0.25$, low degree of correlation; $-1 \leq r \leq 0$, lack of correlation

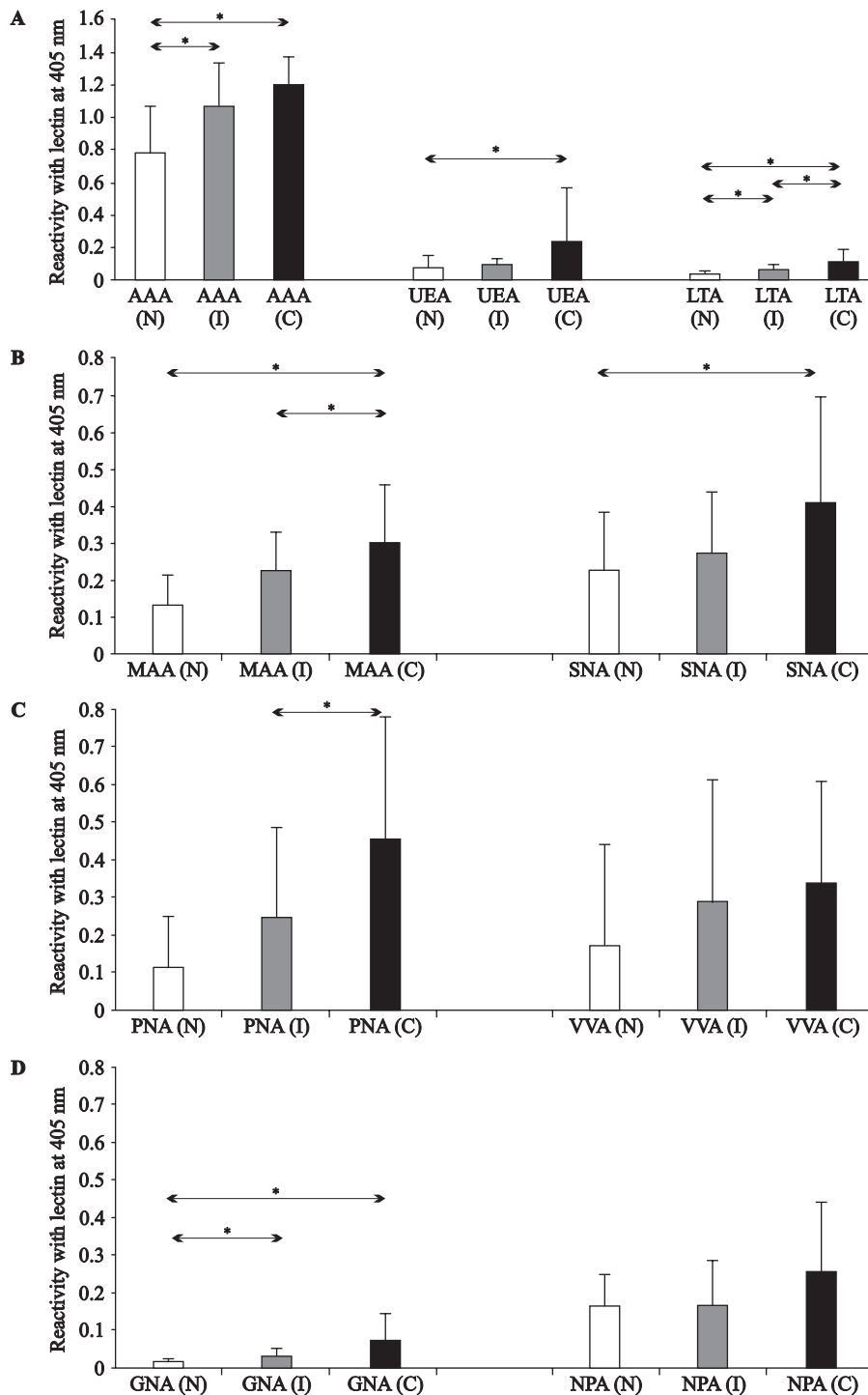


Figure 1. Reactivity of normal (N), intermediate (I) and ccRCC (C) renal tissue extracts with the studied lectins. (A) Reactivity with lectins AAA from *Aleuria aurantia*, UEA from *Ulex europaeus*, LTA from *Tetragonolobus purpureus*; (B) lectins MAA from *Maackia amurensis* and SNA from *Sambucus nigra*; (C) lectins PNA from *Arachis hypogaea* and VVA from *Vicia villosa*; (D) Reactivity with lectins GNA from *Galanthus nivalis* and NPA from *Narcissus pseudonarcissus*. Mean values and standard deviations are presented, n = 15. *p < 0.05

to the renal cortex/medulla uninvolved by tumour (N), as was revealed by PNA binding (Figure 1C). It is worth noting that there were no significant changes in the amount of simple structured, one saccharide core struc-

ture of O-glycans represented by Tn antigen (GalNAc-O-Ser/Thr), as revealed by VVA lectins (Figure 1C). Statistically significant differences were observed only for PNA lectin (p < 0.05) (Figure 1C and Table 3).

Residues of α 1-6 mannose detected by NPA were revealed in higher amounts than α 1-3 residues recognised by GNA. We observed an increased level of α 1-6 linkages isolated from cancer tissues compared to healthy ones (Figure 1D). Significant differences were revealed only for α 1-3 linkage. In all examined tissues, we found positive correlations for GNA, but not for NPA.

Discussion

Glycosylation of proteins can influence many functions of cells, including their roles in tumour progression and metastases formation.

In our study, we characterised some specific glycoforms present on proteins in three locations of ccRCC cells: tumour, intermediate zone adjacent to the tumour, and normal kidney cortex. We showed the presence of all studied structures (specified in Table 2) in examined tissues and found differences in their content dependent on the location of the tissue. We observed a higher amount of sialic acid linked by α 2-6 and α 2-3 bonds in cancer tissue compared to healthy and intermediate tissue. Fucose was the most abundant in cancer cells, which is in accordance with the general tendency for all examined sugar structures. Our results support the observations of other authors that a high expression of sialic acid is a typical phenomenon for different cancer cells [9, 11]. The amount of sialic acid can influence the adhesion of cells. Increased amounts of this acid connected by α 2-6 binding in breast cancer cells caused a decrease of adhesion between cells and increased invasiveness [12]. The same tendency was observed for colon cancer [13]. Sialic acid and fucose are components of sialo Lewis^{x/a} antigens present on the surface of cancer cells and involved in metastases formation [14, 15]. The molecular mechanism of this process has been only partially explored. It has been suggested that sialo Lewis^{x/a} structures on cancer cells in the blood could serve as ligands for selectins present on blood vessels endothelium. This could to some degree be responsible for metastases formation because of direct contacts of cancer cell glycoproteins with endothelial E and P selectins [16, 17].

Tn (GalNAc-Ser/Thr) antigen expression and increased amount of disaccharide structure of T (Gal α 1-3 GalNAc-Ser/Thr) antigen is connected mainly with cancer diseases [18]. Tn and T structures represent incomplete forms of O-glycosidic sugar chains and are classified as oncofetal antigens. Tn and T antigens on normal cells and tissues are in covered forms because they are hidden by peripheral sugars or sugar structures of neighbouring glycoconjugates

[19]. High expression of T antigen was found using natural anti-T antisera in breast cancer endothelial cells, where it is a component of mucins. In normal mammary gland epithelium or metaplastic lesions of the breast, T antigen has been demonstrated at a low level using monoclonal antibodies [20]. Our results support the thesis about the elevated level of T and Tn antigens in malignant tissues because both antigens were revealed in higher level in cancer tissue compared to healthy and intermediate ones. Sialylated forms of Tn and T epitopes including sialyl-Tn (STn; NeuAc α 2,6GalNAc-Ser/Thr), sialyl-3T (S-3T; NeuAc α 2,3Gal β 3GalNAc-Ser/Thr), sialyl-6T (S-6T; Gal β 3(NeuAc α 2,6)GalNAc-Ser/Thr) and disialyl-T (NeuAc α 2,3- Gal β 3(NeuAc α 2,6)GalNAc-Ser/Thr) were increased in O-glycans from mucins in colon and breast cancer [21, 22]. Ours is one of the first reports documenting increased content of glycoconjugates in ccRCC.

In the examined samples of ccRCC and normal renal tissue, mannose bound by a 1-3 and a 1-6 linkages was observed in a very low amount. This observation could suggest, that examined glycoproteins possess mainly O-glycosidic bonds. The expression of mannose at cell surface structures seems to be only slightly influenced by cancer development. In colon and breast cancer, increased N-glycan branching was observed, whereas mucin-type O-glycans became shorter (Gal β 3GalNAc-Thr/Ser) [22].

The results of our experiments, which revealed differences in glycosylation between healthy, intermediate and cancer renal cells, confirm the importance of glycosylation in cancer development.

Several studies of human kidneys have shown that the binding sites for certain lectins are strictly confined to various parts of the nephron [23]. Studies of sugar structures presented on proteins of cancer cells have revealed that there are different types of glycans on them compared to normal cells.

In conclusion, lectin histochemistry may be a useful means of estimating the characteristics of renal tumours and elucidating their histogenesis.

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