Reduced expression of E-cadherin and increased sialylation level in clear cell renal cell carcinoma

Małgorzata Borzym-Kluczyk1,2, Iwona Radziejewska2, Marzanna Cechowska-Pasko1 and Barbara Darewicz3

1Department of Pharmaceutical Biochemistry; 2Department of Medical Chemistry; 3Department of Urology, Medical University of Białystok, Białystok, Poland

Cancer cells are characterized by an aberrant increase in protein N-glycosylation and by disruption of E-cadherin-mediated adherens junctions. However, the relationship between alterations in N-glycosylation process and loss of E-cadherin adhesion in cancer remains unclear. The mechanisms of altered expression of adhesive glycoproteins in cancer cells have not been fully elucidated. Thus, the aim of this study was to examine the expression of E-cadherin and sialyl Lewisα antigen; NeuAcα2-3Gal, NeuAcα2-6Gal/GalNac structures in the normal renal tissue and intermediate and cancerous tissues from patients with clear cell RCC. Moreover, we attempted to correlate the E-cadherin expression with some specific sugar residues of renal cancer tissue glycoproteins. The expression of E-cadherin was analysed using ELISA test and immunoblotting. Oligosaccharide structures and sialylation level were detected with ELISA test using specific biotinylated lectins or antibodies. A significant decrease of E-cadherin expression as well as a significant increase in sialylated oligosaccharides level in intermediate zone and renal cancer tissue in comparison to normal renal tissue are reported. Significant decrease in expression of cadherins and increase in sialylation of oligosaccharide structures in renal cancer tissue in comparison to normal renal tissue, and in renal cancer tissue in comparison to intermediate zone of renal tissue, are important for the future research concerning detection and quantification of cadherins and sialylated oligosaccharide structures in urine and cells of urinary sediment as possible non-invasive marker of early RCC.

Key words: clear cell renal cell carcinoma; E-cadherin; lectins; sialyl Lewis antigens

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. It has been reported that, in the USA, RCC accounts for approximately 3% of adult malignancies and 90–95% of neoplasms originating from the kidney (Gupta & Spiess, 2013). Despite the development of a number of modern diagnostic methods, the RCC is usually diagnosed after invasion into surrounding tissues and organs. The invasion of RCC includes cells decompaction and migration. Renal cells are compacted mostly by homophilic adhesion mediated by classical cadherins of adherens junction or by non-classical adherins (desmogleins and desmocollins) of desmosomal junctions that bind renal cells together, and by integrins that bind renal cells to extracellular matrix (Alberts et al., 2008; Syed et al., 2002; Stemmler, 2008). It was revealed that antibodies against E-cadherin blocked compaction, whereas antibodies against other cell-surface proteins did not. The cadherin superfamily in humans includes more than 180 Ca2+-dependent, membrane anchored, external, heavily N-glycosylated glycoproteins (Syed et al., 2002; Pinho et al., 2011; Paulson et al., 2014). While extracellular N-terminal tip of cadherin molecules on neighboring renal cells mediate highly selective homophilic Velcro type of adhesion in these cells, cytoplasmic tail of classical adherins binds to actin in adherenrs junctions and cytoplasmic tails of non-classical adherins bind to intermediate filament (Leckband & de Rooij, 2014; McEwen et al., 2012). It was reported that mutations, disrupting the production or function of cadherins, facilitated invasion of the cancerous cells into the neighboring tissues, because mutated cancer cells had both E-cadherin-mediated adhesion and desmosomal junctions disrupted (Leckband & Israelevich, 2001; Berx & van Roy, 2009; Giepmans & van Ijzendoorn 2009). The separation of renal cells may be facilitated by increased surface glycan sialylation, that increases repulsion induced by negative charge of dissociated carboxyl groups of sialic acids. Increased branching of N-glycans on the surface of the cells with decreased fucosylation are among important factors determining metastatic potential of the cells (Varki & Freeze, 2009; Lange et al., 2014; Zhao et al., 2008). However, the relationship between altered N-glycosylation, as well as sialylation and loss of E-cadherin adhesion in RCC remains unclear. Thus, the aim of this study was to examine the expression of E-cadherin and sialyl Lewisα, sialyl Lewisα, NeuAcα2-3Gal, NeuAcα2-6Gal/GalNac structures in normal renal tissue and intermediate and cancerous tissue from patients with clear cell RCC. Moreover, we made the attempt to correlate the expression of E-cadherin with sialic acid-containing structures of the glycoproteins in above-mentioned three areas of renal tissue.

MATERIALS AND METHODS

Ethical approval. All procedures performed during the studies involving human participants were in accord-
Table 1. Fuhrman nuclear grade, TNM stage and size of examined tumors

<table>
<thead>
<tr>
<th>Patients</th>
<th>n=35</th>
</tr>
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<tbody>
<tr>
<td>Fuhrman Nuclear Grade</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>4</td>
</tr>
<tr>
<td>G2</td>
<td>22</td>
</tr>
<tr>
<td>G3</td>
<td>4</td>
</tr>
<tr>
<td>G4</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TNM Stage, Size</th>
<th>T1-17</th>
<th>T2-14</th>
<th>T3-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-7 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7≥10 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All tumors extended into major veins or peripheral tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor invaded beyond Gerota’s fascia</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Nodes</th>
<th>N0</th>
<th>N0</th>
<th>N0</th>
<th>N1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastases</td>
<td>MX</td>
<td>MX</td>
<td>MX</td>
<td>MX</td>
</tr>
</tbody>
</table>

Table 2. Binding specificity of lectins and monoclonal antibodies

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Binding preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maackia amurensis (MAA)</td>
<td>NeuAca2-3Gal</td>
</tr>
<tr>
<td>Sambucus nigra (SNA)</td>
<td>NeuAca2-6Gal/GalNac</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Binding preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sialyl Lewisα</td>
<td>NeuAca2-3Gal</td>
</tr>
<tr>
<td>sialyl Lewisα</td>
<td>NeuAca2-3Gal</td>
</tr>
</tbody>
</table>

Preparation of tissue extract. The 10% (w/v) tissue homogenates were prepared in 0.1 M citric buffer, pH 4.3 supplemented with protease inhibitors (Sigma, St Louis, MO, USA) using Ultra-Turrax T8 homogenizer (2 min, 4°C) and centrifuged (20 min, 10000×g, 4°C). Supernatants, after concentration in Centripreps C30 concentrators (Amicon, Millipore, Bedford, MA, USA), were applied on Sephadex G-10 column and eluted with water. Collected 2 mL-fractions containing protein (absorbance at 280 nm) were collected and re-concentrated by few centrifugations using Centripreps C30 (60 min 1500×g). The supernatants containing 40–60 mg of protein/mL were further analyzed.

Sialylated sugar determination with lectins. To analyze the sialylation level of glycoproteins in renal cell extracts, ELISA test with biotinylated lectins (Vector, Burlingame, USA) was performed as previously described (Borzym-Kluczyk et al., 2015). The binding specificity of lectins is presented in the Table 2. Briefly, samples were diluted with phosphate-buffered saline, pH 7.4 (PBS) to protein concentration 5 μg/mL. Microtiter plates (NUNC F96; Maxisorp, Roskilde, Denmark) were coated with samples (50 μL/well), overnight, at room temperature. All the following steps were performed at room temp. The plates were washed 3 times with 100 μL of PBS, containing 0.05% Tween 20 (PBS-T), between all ensuing steps. Unbound sites were blocked with 100 μL of 1% blocking reagent (Roche Diagnostics, Mannheim, Germany) for 1 h and incubated for 1 h with 100 μL/well of biotinylated lectins (0.5 μg/mL in PBS-T containing 1% bovine serum albumin (PBS-T-BSA), (BSA – Sigma, St Luis, MO, USA)). Lectin solutions were supplemented: for SNA lectin with 0.1 mmol/L CaCl₂; for MAA lectin with 0.01 mmol/L CaCl₂ and 0.01 mmol/L MnCl₂. After washing, microtiter plates were incubated with 100 μL/well of horseradish peroxidase-conjugated avidin D (Vector, Burlingame, CA, USA) (1:2500) in PBS-T-BSA, for 1 h. After washing 4 times with PBS colored reaction with 100 μL/well of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St Luis, MO, USA) was developed for 45 min and spectrophotometric measurements were performed at 405 nm, using an Infinite M200 microplate reader (Tecan, Salzburg, Austria) with control samples containing BSA instead of tissue extracts.

Protein concentration assay. Protein concentrations were determined using the bicinechonic acid (BCA) test according to the method (Smith et al., 1985). Bovine serum albumin was used as a standard.

E-cadherin and Lewis antigens determination. The expression of E-cadherin and Lewis antigens of glycoproteins in renal cell extracts in normal, intermediate...
E-cadherin and sialyl Le antigens of renal cancer

were analyzed using ELISA test. The binding specificity of applied monoclonal antibodies is presented in Table 2. The sources and dilutions of applied antibodies are presented in Table 3.

Preparation of microtiter plates including coating with tissue extracts, washing and blotting was performed as above. Then the microtiter plates were incubated for 1 h with 100 μL/well of primary monoclonal or polyclonal antibody (0.5 μg/mL in PBS-T) (Table 3), washed, and incubated with horseradish peroxidase–conjugated secondary antibody: anti-mouse IgG (for anti-sialyl Lewis a), anti-mouse IgM (for anti-sialyl Lewis x) or anti-rabbit IgG (for anti-E-cadherin). After washing the colored reaction was developed as described above.

Polyacrylamide gel electrophoresis (SDS/PAGE). Samples of renal tissue extracts, containing 15 μg of protein, were subjected to SDS-PAGE, as described by Laemmli (1970). The electrophoresis was run for 50–60 minutes. In each experiment 7.5% polyacrylamide gel and constant current (25 mA) were used.

Immunoblotting. After electrophoresis samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA), and nonspecific binding was blocked with 5% solution of milk (Sigma, St. Louis, MO, USA). Membranes were probed with primary rabbit anti-E-cadherin antibody (1:500) for 16 h at 4°C. Next, the membranes were immersed in alkaline phosphatase-conjugated antibody against whole molecule of rabbit IgG (1:10000) in TBS-T, for 1 h, with shaking, washed with TBS-T (5× for 5 min) and exposed to BCIP/NBT reagent (Sigma-Fast). Densitometric analysis of protein bands on nitrocellulose membrane was performed using GeneTools image software (Syngene).

Statistical analysis. Statistical analyses were performed using Statistica 10 (StatSoft, Cracow, Poland) using Student’s test, ANOVA and post hoc test. Results are expressed as means ± S.D. Statistical significance was defined as \( p \leq 0.05 \). The correlation between E-cadherin and all examined sugar structures levels in the three analyzed areas (normal, intermediate and cancer) of renal tissue were also calculated. To express correlation evaluated by Pearson’s coefficient we used the following degrees: \( r=1 \) perfect correlation; \( 0.75 \leq r < 1 \) high degree of correlation; \( 0.30 \leq r < 0.75 \) moderate degree of correlation; \( r<0.1 \) weak degree of correlation. Statistical significance at \( \* p<0.05 \).

RESULTS

Figure 1 shows E-cadherin polyclonal antibody reactivity with extracts of normal, intermediate and cancer renal cortex/medulla tissues. We observed a significant decrease of E-cadherin expression in intermediate zone and in renal cancer tissue in comparison to normal renal tissue, and in renal cancer tissue in comparison to intermediate zone of renal tissue.

Figure 2 shows Western blot analysis (A) and densitometric analysis (B) of E-cadherin expression in extracts of normal, intermediate and cancer renal cortex/medulla tissue. We observed a decrease in E-cadherin expression in extracts of intermediate and cancer tissues in comparison to normal renal tissue.

Figure 3 shows the expression of oligosaccharide structures on glycoproteins of renal tissue extracts in normal, intermediate and cancer tissue. We revealed significantly higher expression of NeuAcα2-3Gal structures (detected by MAA lectin) in cancer and intermediate renal tissue, as compared to normal tissue. The expression

![Figure 1. Expression of E-cadherin in extracts of normal (N), intermediate zone adjacent to tumor (I) and renal cancer tissue (C).](image)

![Figure 2. Western blot (A) and densitometric analysis (B) of E-cadherin expression in extracts of normal (N), intermediate (I) and cancer (C) renal cortex/medulla tissue.](image)
of NeuAcα2-6Gal/GalNAc (detected by SNA lectin) and NeuAcα2-3Galα1-3(Fucα1-4)GlcNAcβ2- (detected by sialyl Lewisx antibody) was significantly higher in extracts of cancer tissue in comparison to normal and intermediate renal tissues. The expression of NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ2- structures (detected by sialyl Lewisx antibodies) was significantly higher in cancer tissue in comparison to normal tissue.

Table 4 shows correlation coefficients of E-cadherin expression versus expression of sialylated oligosaccharide structures on glycoproteins in renal tissue extracts in normal, intermediate and cancer tissue. We observed significant, moderate negative correlations between E-cadherin in intermediate renal tissue and expression of NeuAcα2-3Gal structures in cancer tissues and sialyl Lewisx in normal tissues.

**DISCUSSION**

Most RCC develop from the epithelium of renal tubules. The possibilities and results of surgical RCC treatment depend on the stage of malignancy. The tumors at I and II stage, limited to the kidney are relatively easy to remove via surgical eradication, but it is much more difficult to cure the RCC metastases. In our research we found significant decrease of E-cadherin expression in intermediate tissue as compared to normal tissue, and in cancerous tissue as compared to normal and intermediate renal tissue. The abovementioned results were confirmed with Western blot and qualitative analysis of E-cadherin expression, which strongly support the thesis considering the decompaction as a preliminary step in RCC invasion.

Other authors revealed significance of E-cadherin expression level as a potential factor that could be used in cancer diagnosis (Truong & Shen, 2011). Gervais et al., 2007 observed the decrease of the protein expression in cancerous tissues suggesting its application as potential valuable prognostic marker in clear cell RCC. However, there are also contradictory reports. Ronkainen et al., 2010 stated, that the nuclear or membranous expression of E-cadherin was not a prognostic factor for RCC-dependent survival. It was reported that mutations that disrupt the production or function of E-cadherin are often found in cancer cells and promote the development of malignancy (Varelas et al., 2014).

Renal cell compaction depends not only on cadherins, but also on their sugar structures carrying a plethora of information, due to a variety of sugar composition, branching, location and anomeric forms of cognate sugars (Thedieck et al., 2005; Langner et al., 2004). It is well established that many human tumor-associated antigens are the result of aberrant glycosylation of cell surface glycoproteins (Durand & Seta, 2000; Hakomori, 1989). Increase in self-repelling properties of two cancerous cells may be caused by negatively charged sialic acids that frequently occupy the terminal, non-reducing position on glycan chains on membrane glycoproteins and glycolipids forming so called tumor associated carbohydrate antigens (TACAs). TA-
CAs found in both N- and O-linked oligosaccharides of glycoproteins can be divided into subgroups e.g.: sialyl Lewis° (NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ-) and sialyl Lewisx (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ-) (Hakomori, 1984). In our studies we demonstrated an increase in sialylated Lewis antigens as well as NeuAcα2-6Gal/GalNAc and NeuAcα2-3Gal structures in cancerous and intermediate zones in comparison to normal tissues. In our previous works we revealed similar, increasing tendencies of the expression of some specific sugar antigens, e.g. Lewis blood group antigens or MUC1 mucin (Borzym-Kluczyk & Radziejewska, 2013; Borzym-Kluczyk et al., 2015; Borzym-Kluczyk et al., 2012).

Cancer cells often exhibit sialyl Lewisx and other selectin ligands on their surfaces. It is sought that these ligands play a role in the invasion and metastasis of cancer cells. Obtained values of Pearson's coefficient support the general tendency emerging from our results: whereas E-cadherin expression in RCC decreases, the sialylation level rises.

It has to be emphasized, that our results should be treated as preliminary due to limited number of evaluated cases. On the basis of observation that renal tubular cells exfoliate into urine, we suggest that our results may direct future research to the detection and quantification of cadherins in concentrated urine and/or urine sediment, as possible non-invasive marker of early cc RCC.

Figure 4. Pearson coefficient of statistically significant variation between the expression of E-cadherin and NeuAcα2-3Gal structures in cancer tissues (A) and between expression of E-cadherin and sialyl Lewisx structure in normal renal tissue (B).
Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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