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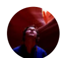

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SHORT COMMUNICATION

Comparison of protein isolation methods from clear cell Renal Cell Carcinoma tissue

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ABSTRACT

This study presents the comparison of three protein extraction methods in the investigations of clear cell Renal Cell Carcinoma tissue. For protein isolation, we applied: phenol extraction according to Hurkman and Tanaka (1986) protocol (method 1), whole tissue lysis in urea-containing buffer (method 2) and commercially available protein isolation kit (2-D Clean-up Kit) (method 3). Statistical analysis indicated that the additional preparation steps including extraction and purification of proteins by 2-D Clean-up Kit significantly increased the quality of obtained data. We believe that gathered information could be a valuable lead for researchers involved in proteomic studies of renal tissue.

Keywords: Biomarkers; Clear cell Renal Cell Carcinoma; Protein isolation; Proteomics; Renal tissue.

INTRODUCTION

Renal cell carcinoma (RCC) is still a major problem in modern oncology. Even though, it accounts for only 3% of cancer diagnosis, there are still more than 100 000 deaths per year worldwide [1]. Approximately 80% of RCCs are classified as clear cell carcinomas. Unfortunately, currently applied treatments are not efficient enough, especially in cases of advanced cancers. Early diagnosis of RCC significantly increases the 5-year survival rate (~85%) in comparison with those detected at metastatic stage (~9%). Thus, there is a large need for discovery of selective, sensitive and easily-accessible RCC biomarker [2]. Two-dimensional electrophoresis coupled with mass spectrometry is often applied strategy in biomarker studies in order to reveal potential disease-associated diagnostic proteins. Proteomics has shown great potential in renal research, however, it also has some serious limitations related to difficulties in identification of membrane and low abundance proteins, which possibly could serve as diagnostic biomarkers. In addition, currently used methods for protein isolation cause high protein loss, and therefore, proteome maps does not represent the whole set of renal proteins. Hence, optimization of the protocols for protein isolation, solubilization, purification and separation is crucial in increasing of protein yield, resolution of polyacrylamide gels and consequently the chances of finding a marker [3]. Our investigation has focused on the comparison of different protein isolation protocols in studies of RCC biomarkers. We applied 3 protocols: phenol extraction according to the Hurkman and Tanaka (1986) (method 1), whole tissue lysis in urea-containing buffer (method 2) and commercially available protein isolation kit (2-D

Clean-up Kit, GE Healthcare, Little Chalfont, UK) (method 3).

1 cm^3 block of normal renal cortex and adjacent cancerous tissue (without surrounding fibroadipose tissue) was removed *ex vivo* after radical nephrectomy and was examined by uropathologist according to Fuhrman grading system and American Joint Committee on Cancer clinical staging system. Final pathological report confirmed malignant character of tumor: clear cell RCC, Fuhrman III, pT1b (5.5 cm) with invasion of tumor beyond renal capsule into perinephric fat. The samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Frozen renal cortex was grounded in a mortar with liquid nitrogen and acquired powder was divided into 3 equal parts. First portion was extracted in accordance with the Hurkman and Tanaka protocol (method 1) [4]. The powdered tissue was solubilized in 500 µl of the extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 2% DTT, and 0.1 M KCl). The same amount of Tris/EDTA-saturated phenol was then added and the sample was incubated for 5 min at 4 °C. After that, the sample was vortexed by 10 min and centrifuged at 8 700 G for 30 min. The upper phenol phase was collected and re-extracted in 500 µl of the extraction buffer. After second centrifugation, 5 volumes of cold 0.1 M ammonium acetate in methanol was added to the phenol phase to precipitate the proteins. Sample was then incubated at -20 °C overnight and centrifuged at 20 500 G in 4 °C for 30 min. The precipitate was washed twice with the cold ammonium acetate in methanol and once in cold acetone, and dried. Obtained pellet was finally dissolved in 500 µl of the sample solution (7 M urea, 2 M thiourea, 2% NP-40, 2% IPG buffer pH range 3-10, 40 mM DTT).

Remained fractions were homogenized in 500 µl of the sample solution each, vortexed and sonicated for 10 min. The samples were centrifuged at 8 700 G in 4 °C for 30 min (method 2). Acquired supernatants were collected and placed into new tubes. Half of the sample was prepared using commercially available 2-D Clean-up Kit (GE Healthcare). Protein purification step was done according to procedure B from the protocol brought by manufacturer (method 3).

The concentration of proteins in samples was measured using the 2-D Quant Kit (GE Healthcare). 550 µg of the proteins was loaded onto 24 cm Immobiline DryStrip gels (GE Healthcare) with linear pH range 3-10 to perform isoelectrofocusing. The process was conducted in Ettan IPGphor II (GE Healthcare) using 2 µA per strip at 20 °C. Whole program was divided into 12 h of active rehydration (20 V) followed by 10 h of focusing (1st h - 500 V; 2nd-3rd h - 1 000 V; 4th-6th h - 8 000 V; 7th-10th h - 10 000 V). After equilibration, second dimension (SDS-PAGE) was performed on 13% polyacrylamide gels (1.5 × 255 × 196 mm) with Roti®-Mark PRESTAINED protein molecular weight marker (Roth, Karlsruhe, Germany). The electrophoresis was carried out on EttanDalt Six (GE Healthcare) for 5.5 h (0.5 h - 4 W per gel, 5 h - 17 W per gel). The protein spots were stained with colloidal Coomassie Brilliant Blue G-250 according to Neuhoff protocol [5].

The gels were scanned by ImageScanner III (GE Healthcare) and processed by LabScan 6.0 (GE Healthcare). Image Master 2-D Platinum software (GE Healthcare) was used to manually pick and count the number of spots on gels.

For Student's t-test, the means and standard deviations of the spot number from three independent replicates were compared between 3 gels of the ccRCC and 3 gels from the control for each isolation method by applying the standard algorithm [6]. The level of significance was set to $p < 0.05$ and data were compared by using Welch's t-test (because the variances of two populations are not equal). The differences between the methods were analyzed by one-way analysis of variance using appropriate tests [7] in STATISTICA (StatSoft, Inc., Tulsa, Oklahoma).

The comparison of three protein isolation methods was performed in order to assess their effectiveness in the studies of ccRCC proteome. Isolation and purification of proteins by 2-D Clean-up Kit led to the best clarity and resolution of the electrophoretic gels after 2DE. In contrast, the protein separation after lysis in urea-containing buffer provided less transparent gels (Fig. 1).

The statistical analysis revealed that in the investigation of healthy tissue, application of method 3 gave the highest number of spots on gels (~899), whereas method 2 provided the least spots (~694). One-way analysis of variances confirmed that differences between all three methods in the case of non-cancerous tissue were statistically significant (Fig. 2). Methods 1 and 3 showed high variation level of spot number between control tissue against sample tissue. Student's t-test confirmed the significance of these differences ($p < 0.05$). In turn, the gels from method 2 contained more protein spots on sample than on control (Fig. 3). However, the difference here was non-significant. Regarding to ccRCC samples, methods 2 and 3 gave similar yield, both significantly different from method 1 (Fig. 2).

The aim of this research was to compare the methods of protein isolation in proteomic studies of renal tissue based on 2DE separation. Efficient protein isolation and purification steps are crucial in future investigations, especially in the case of biomarker discovery research. Thus, selection of the most suitable method could significantly increase probability of success.

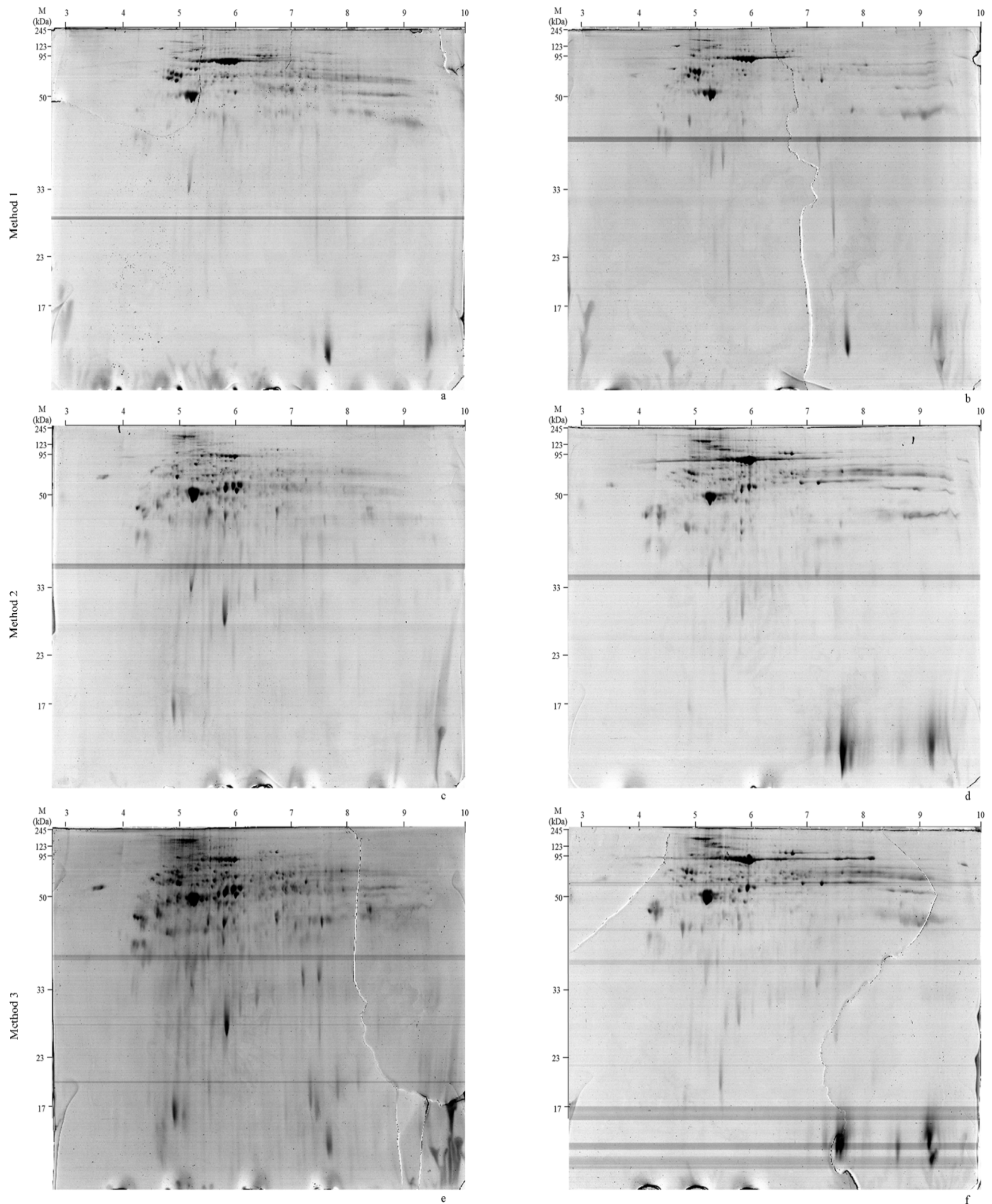

Figure 1. Representative 2-D protein maps obtained from healthy (left) and malignant renal tissue fragments (right) of patient with diagnosed ccRCC. The figure shows the differences in clarity and resolution of gels after 3 different protein isolation methods.

The comparison of 3 methods was performed to evaluate their usefulness in proteomic research of ccRCC biomarker exploration. The conclusions from this study are based on statistical analysis of spots as well as on visual assessment of gels. Previously described biomarker research were often conducted using whole cell lysate without any further isolation and purification steps [8-11]. However, our results indicate that

application of methods 1 and 3 are the best for comparative studies due to the high level of variation between healthy and cancerous tissue. Method 3 provided satisfactory number of spots on gels of both tissue types, most likely due to the lower protein loss. Our research demonstrated that isolation and purification of proteins using commercially available 2-D Clean-up Kit could improve the value of the obtained data and therefore increase chances of potential biomarker discovery.

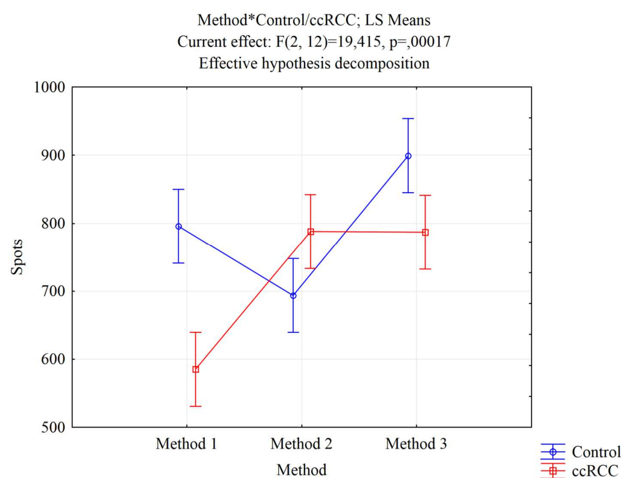

Figure 2. Analysis of variance (ANOVA) shows the average difference in the amount of spots between methods.

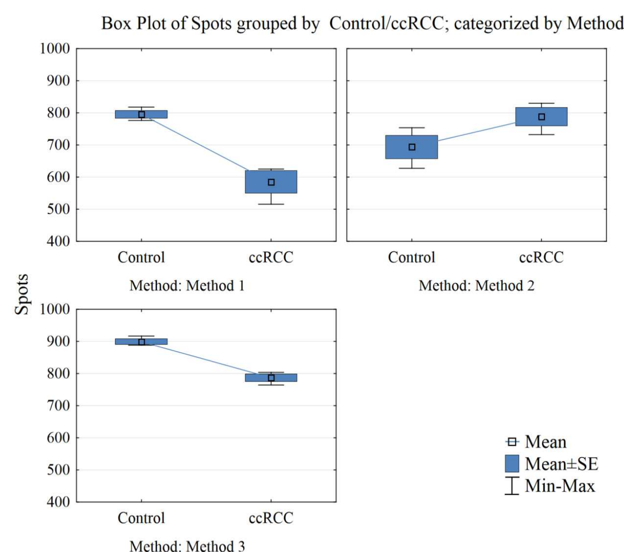

Figure 3. The comparison of differences in spot number between healthy (control) and malignant tissue (ccRCC) within each method.

The results also indicate that despite the high content of fat in ccRCC tumors [12], method 1, which is designated for the hydrophobic proteins, has not increased the amount of isolated protein. We expected that this method of extraction may allow for the isolation of proteins with less hydrophilic character, however this did not happen and the number of spots on the gels obtained from cancer tissue by this method was the lowest. This may indicate that the observed fat deposition acts rather as the cell insulator than as the integral organ tissue with its own protein profile.

In summary, our study provides data on protein isolation efficiency in the investigations of renal tissue based on 2DE. We believe that gathered information could be a valuable lead for researchers involved in such studies.

AUTHORS CONTRIBUTION

Conceptualization: AB, AŁ; Formal analysis: KKH, MJ, AB; Investigation: KKH, AB; Project administration: GG, ZD; Resources: KO, TO, AO, AB, AŁ, KKH; Supervision: AB, AŁ; Original draft preparation/review and editing: KKH, AB, AŁ, KO. The final manuscript has been read and approved by all authors.

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