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Proteomic identification of Reticulocalbin 1 as potential tumor marker in Renal Cell Carcinoma

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Abbreviations: RCC, renal cell carcinoma; IHC, immunohistochemistry; RCN1, Reticulocalbin 1; WB, Western Blotting; DAB, 3,3'-diaminobenzidine.

ABSTRACT

Renal cell carcinoma (RCC) biomarkers are necessary for diagnosis and prognosis. They serve to monitor therapy response and follow-up, as drug targets, and therapy predictors in personalized treatments. Proteomics is a suitable method for biomarker discovery. Here we investigate differential protein expression in RCC, and we evaluate Reticulocalbin 1 (RCN1) use as a new potential marker. Neoplastic and healthy tissue samples were collected from 24 RCC patients during radical nephrectomy. Seven specimens were firstly processed by proteomic analysis (2-DE and MALDI-TOF) and 18 differentially expressed proteins from neoplastic and healthy renal tissues were identified. Among them, RCN1 was over-expressed in all cancer specimens analyzed by proteomics. Consequently RCN1 use as a potential marker was further evaluated in all 24 donors. RCN1 expression was verified by Western Blotting (WB) and immunohistochemistry. WB analysis confirmed RCN1 over-expression in 21 out of 24 tumor specimens, whereas immunohistochemistry displayed focal or diffuse expression of RCN1 in all 24 RCC tissues. Thus RCN1 appears as a potential marker for clinical approaches. A larger histopathological trial will clarify the prognostic value of RCN1 in RCC.

1. Introduction

Renal cell cancer (RCC) is the most frequent neoplasm of the kidney. This disease accounts for approximately 2–3% of all cancers. RCC is a heterogeneous disease, comprised of different histological variants with a distinct clinical course, genetic changes and response to systemic treatment. The categorization of RCC includes different subtypes based on morphology, including clear cell, papillary, chromophobe, granular, spindle cell, cyst-associated, translocation and collecting-duct carcinomas. The most common subtype is clear cell carcinoma, accounting for 75% of cases, papillary follows with 10%, chromophobe 5% and undifferentiated represent approximately 10% of cases [1]. RCC incidence varies substantially worldwide [2]. Nonetheless, several studies have shown evidence of an increasing number of RCC cases. Apparently, this was associated with a larger number of early-stage diagnoses - presumably due to improved use of imaging techniques - but also with an increasing incidence of late-stage tumors [3]. Even though imaging examination such as ultrasonography represents a major tool for diagnosis and screening of renal masses, complementary research methods should not be neglected, and several authors have recently suggested new potential RCC markers [4-7].

Indeed, RCC biomarkers are useful not only in diagnosis but also to determine the prognosis, function as drug targets, monitor follow-up and therapy response, and choose therapy in personalized treatments [4-7]. To date, a quite large number of molecules (including cytokeratins, vimentin, PAX2, PAX8, CD10, E-cadherin, kidney-specific cadherin, parvalbumin, claudin-7, claudin-8, α-methylacyl coenzyme A racemase, CD117, TFE3, thrombomodulin, uroplakin III, p63, CD57, and carbonic anhydrase IX) are under evaluation as RCC markers, and some of them are used in pathological diagnosis through immunohistochemical (IHC) techniques [8]. Some major issues calling for IHC include differential diagnosis of renal *versus* non-renal neoplasms, histologic sub-typing of RCC,

diagnosis of renal neoplasms in small core-biopsy specimens, diagnosis of possible metastatic renal carcinomas, and less frequently, molecular prognostic prediction [8-9]. Notable, proteomics is a major approach used for biomarker discovery, since it allows examination of either biological fluids or tissues [10] and comparison of protein expression between cancer patients and normal subjects [11].

In this study, we investigated the presence of abnormally expressed proteins in RCC tissues from a small cohort of patients, through an already described proteomic approach [12]. The most significantly differentially expressed protein was Reticulocalbin 1 (RCN1), which underwent additional investigation through IHC and Western Blotting (WB), in order to confirm data from proteomics and support its putative use as RCC biomarker.

2. Materials and methods

2.1 Materials

Unless otherwise stated, all materials were from Sigma-Aldrich, Milano, Italy. TissueLyser was from Qiagen, Milano, Italy; Standard RNA Releaser was from Nurex S.r.l., Sassari, Italy; DC Protein Assay Kit, IPG strip gels, Protean IEF cell apparatus, Bio-Rad XI cell, PDQuest software (version 7.2), Immun-Star HRP chemiluminescence kit, and Mini Protean system were from Bio-Rad, Segrate (MI), Italy; Immobilon-P Transfer Membranes were from Merck Millipore, Milano, Italy; anti-RCN1 rabbit polyclonal antibodies (only for WB use) were from Bethyl Laboratories, Montgomery, TX, USA; EnVision system and 3,3'-diaminobenzidine (DAB) were from DakoCytomation, Glostrup, Denmark.

2.2 Tissue collection

From October 2010 to February 2012, patients with a renal mass were enrolled in this study

before undergoing radical nephrectomy; patients with a histological diagnosis other than RCC were subsequently excluded. The study was approved by the local research Ethical Committee and was conducted according to Helsinki Declaration principles. All subjects included in the protocol signed a declaration of informed consent. The specimens of RCC and macroscopically normal tissue distant from tumor mass were obtained from 24 RCC patients (Table 1). Renal tissue cold sections were obtained during radical nephrectomy, immediately after kidney removal. Almost 5-10 mg of both malignant and normal tissues were collected . Tissue specimens were immediately frozen in liquid nitrogen and stored at -80 °C before use.

2.3 Protein extraction

Neoplastic and normal renal tissues were disrupted through TissueLyser in 1 ml of Standard RNA Releaser, followed by the addition of 0.1 ml of chloroform and proteins were extracted as previously described [12]. Briefly, the homogenate was kept for 5 min in wet ice and then was centrifuged (12,000 g for 15 min). The upper aqueous phase was discarded, then 0.3 ml of 100% ethanol was added to the lower organic phase for DNA precipitation. After centrifugation (2000 g at 4 °C for 5 min), the supernatant was used for protein purification by adding 1 ml of isopropanol. The mixture was incubated for 5 to 10 min at room temperature and was centrifuged (12,000 g for 10 min). The pellet was washed twice for 20 min at room temperature in 0.3 M guanidine hydrochloride (dissolved in 95% of ethanol). After centrifugation (7500 g at 4 °C for 5 min), 2 ml of ethanol were added to the protein pellets and samples were incubated overnight at -20 °C. After discarding the ethanol, the protein pellets were suspended in solubilization buffer (8 M urea, 2% [w/v] Chaps, 40 mM Tris, 1 mM PMSF, 1 mM EDTA), then sonicated for 10 min in a water bath, and incubated at 4°C for 24 h. After protein centrifugation, the supernatants were collected and stored at -20 °C before use. Protein concentration was quantified through a DC Protein Assay Kit, using bovine

serum albumin as a standard, according to manufacturer's instructions.

2.4 Two-dimensional polyacrylamide gel electrophoresis

2-DE was performed using IPG strip gels (17 cm IPG strips, pH 3–10NL). Each protein sample (1.2 mg) was loaded onto an IPG gel through overnight in-gel rehydration. Isoelectric focusing was carried out in a Protean IEF cell apparatus as previously described [12]. Briefly, focusing commenced at 50 V, and the voltage was increased to 9000 V until a maximum of 60,000 V/h was reached. Focusing was performed at 18 °C with a limit of 50 A per strip. The IPG strips were equilibrated under continuous shaking for 15 min in equilibration buffer no. 1 (6 M urea, 3% [w/v] SDS, 0.375 M Tris–HCl [pH 8.8], 30% [v/v] glycerol, 1% [w/v] dithiothreitol) and for 12 min in equilibration buffer no. 2 (6 M urea, 3% [w/v] SDS, 0.375 M Tris–HCl [pH 8.8], 30% [v/v] glycerol, 2.5% [w/v] iodoacetamide). For the second dimension protein separation, 12% acrylamide gels were run on a Bio-Rad XI cell.

2.5 Gel staining and image analysis

Gels were stained with colloidal Coomassie (18% [v/v] ethanol, 15% [w/v] ammonium sulfate, 2% [v/v] phosphoric acid, 0.2% [w/v] Coomassie G-250) for 48 h and destained with water. Gel images were obtained by scanning through a Chemidoc MP Bio-Rad. 2-DE image analysis was performed using PDQuest software (version 7.2) according to manufacturer's instructions. Normalization of each individual spot was performed according to total quantity of the valid spots in each gel, after subtraction of background values. Spot volume was used as an analytical parameter to quantify protein expression.

2.6 Sample preparation for mass spectrometry analysis

Coomassie G-stained spots underwent excision from 2-DE gels, and proteins were digested with trypsin. Each spot was destained with 100 µl of 50% (v/v) acetonitrile in 5 mM ammonium bicarbonate and dried with 100 µl of acetonitrile. Each dried gel piece was rehydrated for 40 min at 4 °C in 10 µl of digestion buffer (5 mM ammonium bicarbonate; 10

ng/l trypsin). Digestion was allowed to proceed overnight at 37 °C, and peptide mixtures were stored at 4 °C before use.

2.7 MALDI-MS and peptide mass Fingerprinting

MS analysis of peptides was performed using a MALDI-TOF spectrometer (MALDI micro MX) equipped with a delayed extraction unit, according to manufacturer's tuning procedures, operating on reflectron mode as previously described [13]. Briefly, samples were loaded onto the MALDI target using 1.5 µl of the tryptic digest mixed 1:1 with a saturated α-cyanohydroxycinnamic acid (10 mg/ml) solution in 40% v/v acetonitrile, 60% v/v trifluoroacetic acid 0.1%. The MALDI-TOF was calibrated with a mix of PEG (PEG 1000, 2000 and 3000 with the ratio 1:1:2) and mass spectra were acquired in the positive-ion mode. Peak lists were generated by ProteinLynx Global Server 2.2.5 (Waters, Milford, MA, USA) data preparation using the following parameters: external calibration with lock mass using a mass of 2465.1989 Da for ACTH (adrenocorticotropic hormone), background subtract type adaptive combining all scans, and deisotoping with a threshold of 1%. The 25 most intense masses were used for database searches against SWISSPROT database (Release 2011_12 of 14-Dec-11) using free search program MASCOT 2.3.02 (http://www.matrixscience.com). The following parameters were used in the searches: taxa Homo sapiens, trypsin digest, one missed cleavage by trypsin, carbamidomethylation of cysteine as fixed modification, methionine oxidation as variable modifications and 100 ppm as maximum error allowed. Only proteins with a Mascot score > 56 were considered.

2.8 Western Blotting

After adding Laemmli buffer [14], samples were boiled for 5 min, and 30 µg of each protein

sample were run on 10% SDS-polyacrylamide gel on a Mini Protean system. Proteins resolved by electrophoresis were blotted onto Immobilon™-P polyvinylidene fluoride membranes. After blotting, membranes were probed using anti-RCN1 rabbit polyclonal antibodies (diluted 1:3000 in PBS-TWEEN 1% non-fat dry milk) and anti-β-actin mouse monoclonal antibodies (diluted 1:500 in PBS 1% BSA) for 1 h. After washing, they were incubated for 1 h with horseradish-peroxidase-labeled anti-rabbit (diluted 1:10000 in PBS-TWEEN 1% BSA) or anti-mouse (diluted 1:10000 in PBS-TWEEN 1% non-fat dry milk) antibodies. The immunoreactivity was detected by using a Immun-Star HRP chemiluminescence kit. Densitometric analysis of the bands was performed using free ImageJ software (version 1.44).

2.9 Immunohistochemistry

Formalin-fixed paraffin-embedded selected blocks from 24 RCC specimens were cut into 4 μm thick sections and collected onto charged slides for IHC staining. After de-paraffination and rehydration through graded alcohols and PBS (pH 7.5), the endogenous peroxidase activity was blocked by incubation with absolute methanol and 0.3% hydrogen peroxide for 15 minutes. Antigen retrieval was performed by sterilizing the section in 10 mmol/L citrate buffer (pH 6.0) at 98 °C for 40 minutes. Sections were incubated at the optimal conditions with anti-RCN1 rabbit polyclonal antibodies (1:1000). Immunoreaction was revealed by a dextran-chain (biotin-free) detection system (EnVision), using DAB as a chromogen. The sections were lightly counterstained with hematoxylin. Negative control reactions were obtained by omitting primary antibodies, whereas neuroblastoma cell line blocks were used as positive control. A pathologist (LD) interpreted the results from immunohistochemical analysis unaware of the concurrent results obtained from investigation by histopathology and proteomics.

2.10 Statistical analysis

Statistical significance, calculated by a two-sided Student's *t* test, a chi-square test and ANOVA was set at p values < 0.05. In 2-DE experiments, proteins were classified as differentially expressed when spot intensity ratios between neoplastic and normal tissues were greater than 1.5-fold (over-expressed proteins) or lower than 0.5-fold (down-expressed proteins). The relationship among TNM staging, Fuhrman grading, histotype and RCN1 expression (both by WB and IHC) were also explored.

3. Results

3.1 Renal tissue proteomic analysis

Seven tissue specimens of RCC and corresponding normal tissue distant from neoplastic lesion were comparatively analyzed. Figure 1 shows two representative 2-DE gel images of normal and neoplastic renal tissues stained by colloidal Comassie. Table 2 shows MS identification of differentially expressed proteins listed by frequency. For each identified protein, the average ratios of protein expression in cancer *versus* normal tissues and corresponding *p* values are also shown. Results showed 18 differentially expressed proteins in neoplastic tissues. Seven of them (corresponding to spots 1-9) were found over-expressed in neoplastic specimens. In particular, spot 1 (corresponding to RCN1) was over-expressed in all analyzed neoplastic tissues.

3.2 RCN1 expression analysis by WB in RCC and normal tissues

Since RCN1 was over-expressed in all RCC tissues analyzed by MS, results were validated by performing anti-RCN1 WB and subsequent densitometry on a larger cohort of patients (24

RCC patients, including the seven cases previously analyzed by proteomics). RCN1 protein was over-expressed in 21 out of 24 RCC tissues. Among these 21 cases, RCN1 was found to be induced *de novo* in 9 neoplastic tissues, whereas in the remaining 12 patients RCN1 protein levels from RCC tissues were significantly enhanced compared to normal renal tissues. Such an enhancement was measured by calculating densitometric RCN1 protein ratios between RCC and normal tissues (mean value ± SD: 3.28 ± 2.76, $p<0.01$). Eight RCC tissues appeared weakly enhanced (mean value ± SD: 1.57 ± 0.35, $p<0.001$), whereas 4 RCC tissues were intensely enhanced (mean value ± SD: 6.70 ± 2.07, $p<0.05$). Figure 2 shows a representative blot obtained from three RCN1-positive RCC patients in which RCN1 was induced *de novo* or intensely/weakly enhanced. As already showed by Cooper et al. [15] two bands are detected by the antibody and both are RCN1. The analysis of histopathological data and WB results did not show any statistically significant relationship between RCN1 expression and tumor stage (TNM) and grade (G).

3.3 RCN1 expression analysis by IHC in RCC and normal tissues

All 24 RCC/normal tissues, including those displaying low RCN1 expression after WB analysis, were additionally tested for RCN1 protein levels by IHC. Normal renal tissues adjacent to lesions showed a positive and uniform staining of the tubules but not of the glomerulus (Figure 3, Panel A). On the contrary, all RCC specimens displayed either focal or diffuse expression of RCN1 protein, with stain intensity varying from weak to strong depending on patients. Figure 3 shows a representative IHC image from 2 RCC patients out of 24 analyzed in which RCN1 was weakly or intensely expressed. The degree of expression did not appear to correlate to stage (TNM) and grade in a statistically significant manner, even though most of the low T stage cancers had a weak staining while most of the high T stage cers had a strong degree (p=0,19).

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4. Discussion

Molecular biomarkers are relevant for a large kaleidoscope of applications in clinical practice, including diagnosis, outcome prediction, drug targeting, monitoring of response to therapy, and development of personalized treatments. In recent years, proteome analysis revealed itself as a useful approach to identify differentially expressed proteins as possible new biomarkers. Therefore, the present work aimed at finding new biomarkers for RCC - a life-threatening disease characterized by high incidence in Western countries - by performing differential proteomic analysis of neoplastic and normal renal tissues obtained from a small cohort of RCC patients (n=7).

Results showed 18 differentially expressed proteins in RCC tissues. Seven proteins were over-expressed in RCC tissues in comparison with healthy tissues, whereas 11 proteins appeared down-regulated. Over-expressed proteins included molecules related to glucose metabolism (α-enolase, phosphoglycerate kinase 1 and triose phosphate isomerase), carrier proteins (transthyretin and retinol binding protein-4), a member of the small heat shock protein family (α-crystallin B) and a calcium-binding protein (RCN1). Down-regulated proteins included molecules related to metabolism (ATP synthase, NADH dehydrogenase flavoprotein 2, electron-transfer-flavoprotein, alcohol dehydrogenase, inorganic pyrophosphatase) or signal transduction (WD40-repeat-containing gene 25, phosphatidylethanolamine-binding protein 1), stress proteins (HSP β-1 and Protein DJ-1) and ion binding proteins (myosin and ester hydrolase C11orf54).

Interestingly, some of the identified proteins have been previously associated to renal cancer (α-enolase, triosephosphate isomerase, α-crystallin B, RCN1, HSP beta-1, ATP synthase, phosphatidylethanolamine-binding protein 1) [11, 16-19] or to other renal diseases (retinol-binding protein-4) [20]. A possible role for HSP beta-1 and triosephosphate isomerase as prognostic/diagnostic markers was validated by Valera and collegues by IHC [17]. However,

any data confirming the possible use of these proteins in clinical practice is not available to date.

Notable, our results from proteomic analysis showed a significant over-expression of RCN1 protein in all RCC tissues. RCN1 is a Ca(2+)-binding protein discovered two decades ago and shown to be stored in endoplasmic reticulum [21]; a recent study displayed RCN1 additional localization at the surface of bone endothelial cells and prostate cancer cells [22]. Moreover, RCN1 is a component of CREC molecules (acronym for: Cab45, Reticulocalbin, ERC-45, Calumenin), a family of multiple (up to seven) EF-hand proteins involved in secretory pathways of mammalian cells and associated with pathological activities such as malignant cell transformation, mediation of the effects of snake venom toxins and putative participation in amyloid formation. Nevertheless, the role of RCN1 in malignant transformation is largely unknown. In some tissues, RCN1 was observed to interact with SEC63p, a protein related to autosomal polycystic liver and kidney disease, that should operate in protein translocation and quality control pathways in the endoplasmic reticulum [23, 24]. It was also proposed that loss of the RCN1 gene might compromise cell survival [25].

According to literature data, broad expression of RCN1 was found by IHC analysis in a large number of endocrine and exocrine organs, apart from thyroid gland cells. However, RCN1 expression appears heterogeneous, depending on which specialized cells of different organs it belongs to. Increased RCN1 expression concurrent with inflammation was observed both in epithelial and non-epithelial cells [26]. Notable, epithelial cells generally display strong RCN1 staining, except for squamous cells; on the other hand, strong staining is also found in non-epithelial cells, including testicular germ, neuronal, vascular endothelial, follicular dendritic and plasma cells [25]. Up-regulation of RCN1 protein or gene was detected also in a number of cancerous cell lines, including breast [27], colorectal [28] and liver [29]. In non-small cell

lung cancer patients, RCN1 was proposed as a prognostic factor to identify post-operative adjuvant chemotherapy responders [30].

To validate our proteomic data, and to confirm the involvement of RCN1 in renal cancer, we set up a small pilot study by collecting surgical samples from a more extensive cohort of RCC patients, and RCN1 protein levels were evaluated by WB and IHC. Interestingly, IHC confirmed RCN1 over-expression in RCC tissues of all examined patients, displaying weak protein expression in healthy renal tissues only in correspondence to the renal tubule section. On the other hand, WB analysis showed over-expressed RCN1 protein in 21 RCC patients out of 24. This data indicates a possible use of RCN1 as a new marker in renal cancer and indicates the proximal convoluted renal tubule as a putative origin point for RCC. This evidence is consistent with results by Fukuda and colleagues [25], suggesting that RCN1 may be helpful in establishing cellular origin of neoplasms in some organs. Therefore, IHC for RCN1 could be employed in clinical practice in order to distinguish between RCC and other tumors not originating from renal tubule, provided that a number of different histological types of renal cancer will be tested in order to confirm it. Since IHC staining displayed different grades of intensity in tested tissues, RCN1 could also be employed as a prognostic marker or as a response predictor for RCC-targeted therapy. The analysis of correlation of IHC staining and TNM stage, grade and histotype did not show any statistically significant results because of the small population of the study. Nevertheless, a correlation trend between tumor staining degree and T stage was observed (p=0,19). As far as histotype is concerned, only three non-clear cell cancers were included (1 cromophobe and 2 papillary) and we cannot formulate any hypothesis on different RCN1 expression in different histotypes, even though one can expect that a different kind of RCC could have a larger or smaller involvement of RCN1 molecular paths. To test the correlation of IHC staining with TNM stage, Fuhrman grade, histotype, response to therapy and survival, a larger retrospective trial

on paraffin-embedded tissues obtained from radical or partial nephrectomy of RCC patients is planned to be performed by our group.

In conclusion, in the present study a preliminary proteomic approach, performed on a small cohort of 7 RCC patients, identified 18 differentially expressed proteins. Among them, RCN1 was significantly over-expressed in all seven samples. Further investigation on RCN1 protein expression performed either by IHC or by WB on a larger cohort (24 patients) confirmed previous results from proteomics. Therefore, data from the present pilot study supports the potential use of RCN1 as a new marker in renal cancer, encouraging future large-scale studies to be performed.

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REFERENCES

[1] Vera-Badillo FE, Conde E, Duran I. Chromophobe renal cell carcinoma: a review of an uncommon entity. Int J Urol 2012;19:894-900.

[2] Chow WH, Dong LM, Devesa SS. Epidemiology and risk factors for kidney cancer. Nat Rev Urol 2010;7:245–257.

[3] Kane CJ, Mallin K, Ritchey J, Cooperberg MR, Carroll PR. Renal cell cancer stage migration: analysis of the National Cancer Data Base. Cancer 2008;113:78–83.

[4] Bukowski RM. Biomarkers in renal cell carcinoma: what next? Curr Oncol Rep 2011;13: 87–89.

[5] Bukowski RM. Prognostic factors for survival in metastatic renal cell carcinoma: update. 2008. Cancer 2009;115:2273–2281.

[6] Shah JB, Margulis V. In search of a better crystal ball: recent advances in prognostic markers for clear-cell renal cell carcinoma. Expert Rev Anticancer Ther 2010;10:837–842.

[7] Cairns P. Renal cell carcinoma. Cancer Biomark 2010;9:461–473.

[8] Shen SS, Truong LD, Scarpelli M, Lopez-Beltran A. Role of immunohistochemistry in diagnosing renal neoplasms: when is it really useful? Arch Pathol Lab Med 2012;136:410–417.

[9] Netto GJ, Cheng L. Emerging critical role of molecular testing in diagnostic genitourinary pathology. Arch Pathol Lab Med 2012;136:372–390.

[10] Sandim V, Pereira DA, Ornellas AA, Alves G. Renal cell carcinoma and proteomics. Urol Int 2010;84:373–77.

[11] Okamura N, Masuda T, Gotoh A, Shirakawa T, Terao S, Kaneko N, Suganuma K, Watanabe M, Matsubara T, Seto R, Matsumoto J, Kawakami M, Yamamori M, Nakamura T, Yagami T, Sakaeda T, Fujisawa M, Nishimura O, Okumura K. Quantitative proteomic

352 analysis to discover potential diagnostic markers and therapeutic targets in human renal
353 cell carcinoma. Proteomics 2008;8:3194–3203.
354 [12] Barbero G, Carta F, Giribaldi G, Mandili G, Crobu S, Ceruti C, Fontana D, Destefanis P,
355 Turrini F. Protein/RNA coextraction and small two-dimensional polyacrylamide gel
356 electrophoresis for proteomic/gene expression analysis of renal cancer biopsies. Anal
357 Biochem 2006;349:62–71.
358 [13] Mandili G, Marini C, Carta F, Zanini C, Prato M, Khadjavi A, Turrini F, Giribaldi G.
359 Identification of phosphoproteins as possible differentiation markers in all-trans-retinoic
360 acid-treated neuroblastoma cells. PLoS One 2011;6:e18254
361 [14] Laemmli UK. Cleavage of structural proteins during the assembly of the head of
362 bacteriophage T4. Nature 1970;227:680–685.
363 [15] Cooper CR, Graves B, Pruitt F, Chaib H, Lynch JE, Cox AK, Sequeria L, van Golen KL,
364 Evans A, Czymmek K, Bullard RS, Donald CD, Sol-Church K, Gendernalik JD, Weksler
365 B, Farach-Carson MC, Macoska JA, Sikes RA, Pienta KJ. Novel surface expression of
366 reticulocalbin 1 on bone endothelial cells and human prostate cancer cells is regulated by
367 TNF-alpha. J Cell Biochem 2008;104:2298-309.
368 [16] Junker H, Venz S, Zimmermann U, Thiele A, Scharf C, Walther R. Stage-related
369 alterations in renal cell carcinoma--comprehensive quantitative analysis by 2D-DIGE and
370 protein network analysis. PLoS One. 2011;6:e21867.
371 [17] Valera VA, Li-Ning-T E, Walter BA, Roberts DD, Linehan WM, Merino MJ. Protein
372 Expression Profiling in the Spectrum of Renal Cell Carcinomas. J Cancer 2010;1:184–
373 196.
374 [18] Seliger B, Dressler SP, Wang E, Kellner R, Recktenwald CV, Lottspeich F, Marincola
375 FM, Baumgärtner M, Atkins D, Lichtenfels R. Combined analysis of transcriptome and
376 proteome data as a tool for the identification of candidate biomarkers in renal cell

carcinoma. Proteomics 2009; 9:1567–1581.

[19] Siu KW, DeSouza LV, Scorilas A, Romaschin AD, Honey RJ, Stewart R, Pace K, Youssef Y, Chow TF, Yousef GM. Differential protein expressions in renal cell carcinoma: new biomarker discovery by mass spectrometry. J Proteome Res 2009;8: 3797–3807.

[20] Christou GA, Tselepis AD, Kiortsis DN. The metabolic role of retinol binding protein 4: an update. Horm Metab Res 2012;44:6-14.

[21] Ozawa M, Muramatsu T. Reticulocalbin, a novel endoplasmic reticulum resident Ca(2+)-binding protein with multiple EF-hand motifs and a carboxyl-terminal HDEL sequence. J Biol Chem 1993;268:699–705.

[22] Cooper CR, Graves B, Pruitt F, Chaib H, Lynch JE, Cox AK, Sequeria L, van Golen KL, Evans A, Czymmek K, Bullard RS, Donald CD, Sol-Church K, Gendernalik JD, Weksler B, Farach-Carson MC, Macoska JA, Sikes RA, Pienta KJ. Novel surface expression of reticulocalbin 1 on bone endothelial cells and human prostate cancer cells is regulated by TNF-alpha. J Cell Biochem 2008;104:2298–2309.

[23] Honoré B. Vorum H. The CREC family, a novel family of multiple EF-hand, low-affinity Ca(2+)-binding proteins localised to the secretory pathway of mammalian cells. FEBS Lett 2000;466:11–18.

[24] Honoré B. The rapidly expanding CREC protein family: members, localization, function, and role in disease. Bioessays 2009;31:262–277.

[25] Kent J, Lee M, Schedl A, Boyle S, Fantes J, Powell M, Rushmere N, Abbott C, van Heyningen V, Bickmore WA. The Reticulocalbin gene maps to the WAGR region in human and to the Small eye Harwell deletion in mouse. Genomics 1997;42:260–267.

[26] Fukuda T, Oyamada H, Isshiki T, Maeda M, Kusakabe T, Hozumi A, Yamaguchi T, Igarashi T, Hasegawa H, Seidoh T, Suzuki T. Distribution and variable expression of secretory pathway protein reticulocalbin in normal human organs and non-neoplastic

pathological conditions. J Histochem Cytochem 2007;55:335–345.

[27] Liu Z, Brattain MG, Appert H. Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem Biophys Res Commun 1997;231:283–289.

[28] Nimmrich I, Erdmann S, Melchers U, Finke U, Hentsch S, Moyer MP, Hoffmann I, Müller O. Seven genes that are differentially transcribed in colorectal tumor cell lines. Cancer Lett 2000;160:37–43.

[29] Yu LR, Zeng R, Shao XX, Wang N, Xu YH, Xia QC. Identification of differentially expressed proteins between human hepatoma and normal liver cell lines by two-dimensional electrophoresis and liquid chromatography-ion trap mass spectrometry. Electrophoresis 2000;21:3058–3068.

[30] Hirano T, Kato H, Maeda M, Gong Y, Shou Y, Nakamura M, Maeda J, Yashima K, Kato Y, Akimoto S, Ohira T, Tsuboi M, Ikeda N. l. Identification of postoperative adjuvant chemotherapy responders in non-small cell lung cancer by novel biomarker. Int J Cancer 2005;117:460–468.

FIGURE LEGENDS

Fig. 1 - 2-DE proteomic analysis of differentially expressed proteins in RCC tissue. Neoplastic and normal tissues from 7 RCC patients were analyzed by 2-DE proteomics. Data are shown as representative gels obtained from one patient out of seven showing almost consistent results (Panel A: normal Tissue; Panel B: RCC tissue). Black circled spots indicate the position of identified proteins (listed in Table 2) differentially expressed in RCC and normal tissues.

Fig. 2 - RCN1 protein is over-expressed in RCC tissues: WB analysis. Paired biopsies of neoplastic and normal tissues (30 µg proteins for each tested sample) from 24 RCC patients were analyzed for RCN1 protein expression by WB. Results are shown as a representative blot (upper Panel) of RCN1 protein levels in normal (lanes A, C, E) and neoplastic (lanes B, D, F) renal tissues from three of 21 RCC patients in the cohort identified as RCN1-positive. House-keeping β-actin protein levels are also shown (lower Panel).

Fig. 3 - RCN1 protein is over-expressed in RCC tissues: IHC analysis. Paired biopsies of neoplastic and normal tissues from 24 RCC patients underwent hematoxylin/eosin staining and IHC reaction with anti-RCN1 antibodies. Results are shown as representative IHC images (20X magnification, all Panels) from 2 RCC patients out of 24 analyzed. Panel A: IHC reaction with anti-RCN1 antibodies on normal renal tissue; Panels B-C: hematoxylin/eosin staining (B) and IHC reaction with anti-RCN1 antibodies (C) on weakly RCN1-positive neoplastic renal tissue; Panels D-E: hematoxylin/eosin staining (D) and IHC reaction with anti-RCN1 antibodies (E) on strongly RCN1-positive neoplastic renal tissue.

Table 1 - Summary of clinicopathological features of the renal cancer patients

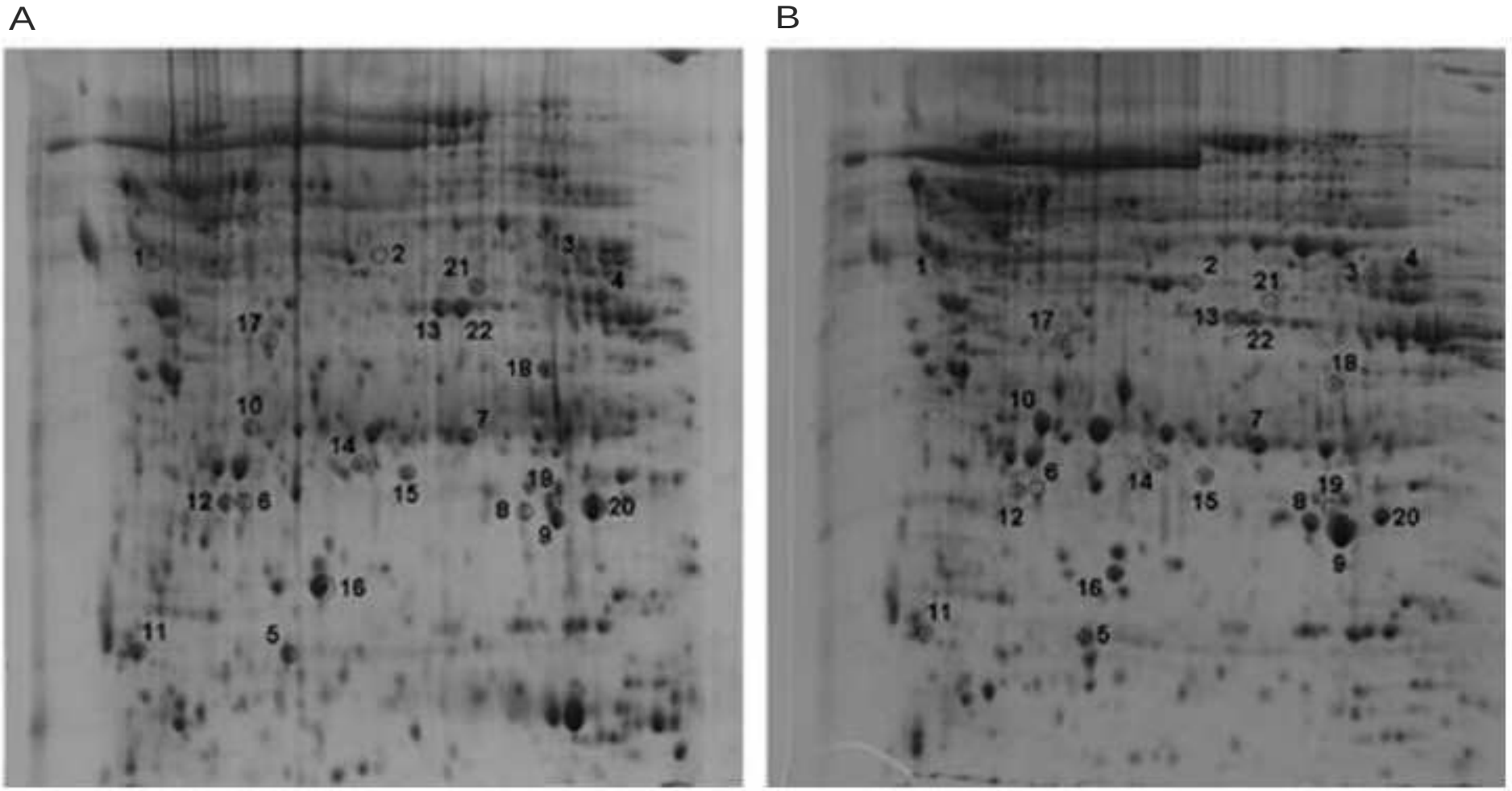
Characteristic	
Patients, no.	24
Age, years, mean (range)	63.17 (42-79)
Sex, no., male/female	13/11
Kidney cancer histology, no. (%)	
Clear cell (cRCC)	21 (87,5%)
Papillary (pRCC)	2 (8,33%)
Chromophobe (chRCC)	1 (4,17%)
Pathological T stage no. (%)	
T1a	3 (12.50%)
T1b	7 (29.17 %)
T2a	1 (4.17%)
T2b	2 (8.33%)
T3a	6 (25.00%)
T3b	5 (20.83%)
Pathological N stage no. (%)	
N0	14 (58.33%)
Nx	9 (37.5%)
N+	1 (4.17%)
Pathological M stage no. (%)	
M0	4 (16.17%)
Mx	18 (75.00%)
M+	2 (8.33%)
Pathological G stage no. (%)	
G1	1 (4.17%)
G2	12 (50.00%)
G3	6 (25.00%)
G4	5 (20.83%)

Table 2

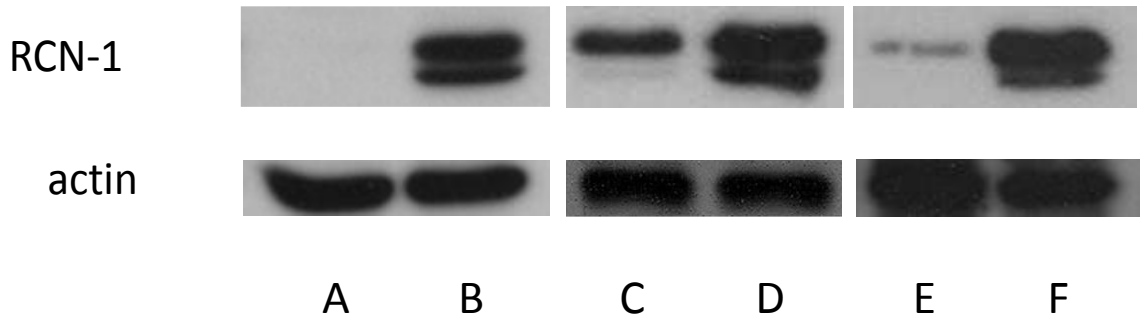
Table 2 - List of identified differentially expressed proteins sorted by frequency between normal and RCC tissues obtained after 2-DE coupled with MALDI-TOF mass spectrometry analysis. [a]Frequency: number of samples in which a protein is altered more than 1.5-fold (over-expressed proteins) or less than 0.5-fold (down-expressed proteins) in RCC tissues. Total 7 samples. [b]Spots numbers relatives to Figure 1, B. [c]Average ratio of differential expression (≧ 1.5-fold increase or ≦ 0.5-fold decrease) between RCC and normal tissues. [d]*p* values corresponding to average ratios.

Frequency[a]	Spot No.[b]	Protein name	Accession No.	matched/unmatched peptides	coverage	MASCOT score	Average Ca/N ratio[c]	Ca/N *p* value[d]
7	1	Reticulocalbin-1 [Precursor]	Q15293	10/25	33%	102	2.92	<0.05
6	2	Alpha-enolase	P06733	10/25	28%	100	3.11	<0.01
5	3	Phosphoglycerate kinase 1	P00558	7/25	26%	62	2.82	<0.01
3	4	Phosphoglycerate kinase 1	P00558	9/25	26%	67	2.40	<0.001
4	5	Transthyretin [Precursor]	P02766	5/25	48%	73	1.94	<0.001
3	6	Retinol-binding protein 4 [Precursor]	P02753	8/25	40%	96	2.19	<0.01
3	7	Triosephosphate isomerase	P60174	12/25	66%	171	2.23	<0.01
3	8	Alpha-crystallin B chain	P02511	7/25	40%	77	2.86	<0.01
3	9	Alpha-crystallin B chain	P02511	9/25	52%	125	2.41	<0.01
4	10	Heat shock protein beta-1	P04792	7/25	32%	86	0.45	<0.001
4	11	Myosin light polypeptide 6	P60660	10/25	47%	115	0.35	<0.001
4	12	ATP synthase subunit d, mitochondrial	O75947	8/25	46%	98	0.41	<0.001
4	13	Ester hydrolase C11orf54	Q9H0W9	10/25	26%	77	0.30	<0.001
4	14	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial [Precursor]	P19404	8/25	32%	66	0.40	<0.001
4	15	Protein DJ-1	Q99497	7/25	37%	70	0.45	<0.001
4	16	WD repeat-containing protein 25	Q64LD2	7/25	23%	72	0.46	<0.01
5	17	Inorganic pyrophosphatase	Q15181	12/25	44%	156	0.33	<0.001
5	18	Electron transfer flavoprotein subunit alpha, mitochondrial [Precursor]	P13804	9/25	43%	104	0.39	<0.001
5	19	Phosphatidylethanolamine-binding protein 1	P30086	9/25	54%	131	0.13	<0.001
6	20	Phosphatidylethanolamine-binding protein 1	P30086	10/25	54%	132	0.37	<0.001
5	21	Alcohol dehydrogenase [NADP+]	P14550	14/25	41%	181	0.23	<0.001
6	22	Ester hydrolase C11orf54	Q9H0W9	9/25	20%	78	0.34	<0.001

Giribaldi et al. Figure 1



Giribaldi et al. Figure 2



Giribaldi et al. Figure 3