

# New monoclonal antibodies against bilitranslocase as a diagnostic tool in determining the progress of clear cell renal cell carcinoma

Novi monoklonski protitelesi proti bilitranslokazi kot diagnostično orodje pri ugotavljanju napredovanja svetloceličnega karcinoma ledvičnih celic

Alexandra Bogožalec Košir,<sup>\*1,3</sup> Tjaša Lukan,<sup>\*1,3</sup> Mateja Kukovec,<sup>2</sup> Vivijana Snoj,<sup>2</sup> Sendi Montanič,<sup>1</sup> Vladka Čurin Šerbec,<sup>1</sup> Uroš Rajčević<sup>1</sup>

## Abstract

**Background:** Monoclonal antibodies (mAbs) are an important tool in diagnostics and research, especially when we are dealing with a protein marker of unknown primary structure as in the case of bilitranslocase (BTL). BTL is also expressed on kidney cells, where it acts as an organic anion transporter. We have shown earlier that there are differences in bilitranslocase expression in normal kidney cells versus early grade kidney cancer.

**Methods:** We developed monoclonal antibodies against extra- and intra-cellular domains of bilitranslocase protein model. To also gain a deeper insight in bilitranslocase expression in clinical samples, we assessed BTL expression in different grades of clear cell kidney cell carcinoma (ccRCC).

**Results:** Both new monoclonal antibodies bind to a protein in UOK171 cells but not in the negative control. Binding of mAb is specific. mAb produced by cell line 2A9/2E9 (peptide 298–310; intracellular domain) is more suitable for immunohistochemical analyses as it gives stronger intensity of binding than mAb produced by cell line 11C9/2G9 (peptide 235–246; extracellular domain). Antibody 2A9/2E9 stains bilitranslocase in proximal renal tubules of normal kidneys but not in the surrounding stroma. Staining decreases in grade I compared to normal kidney, gradually increases in grades II and III, and decreases again in grade IV of ccRCC tissue.

**Conclusions:** Our results show that these antibodies can be used in different immunoassays. Furthermore, specificity and affinity of our mAbs allowed us to use them in the analysis of progressive grades of clear cell renal cell carcinoma in a limited number of patients. Thus, mAbs developed here can be used as a diagnostic tool that could help distinguish between early and late grades of clear cell renal cell carcinoma.

## Izveček

**Uvod:** Monoklonska protitelesa (mAbs) so pomembno orodje v diagnostiki in raziskovanju, še posebej kadar obravnavamo proteinski označevalec neznane primarne strukture, kot je v primeru bilitranslokaze (BTL). BTL se izraža tudi na celicah ledvic, kjer deluje kot prenašalec organskih anionov. V prejšnjih študijah smo ugotovili, da se bilitranslokaza izraža različno v normalnih ledvičnih celicah v primerjavi z zgodnjimi fazami ledvičnega raka.

<sup>1</sup> Department for the Production of Diagnostic Reagents and for Research, Blood Transfusion Center of Slovenia, Ljubljana

<sup>2</sup> Department of Pathology, Izola General Hospital, Izola

<sup>3</sup> Department of Systems Biology and Biotechnology, National Institute of Biology, Ljubljana

\* Authors contributed equally to this work, Ljubljana

## Korespondenca/ Correspondence:

Uroš Rajčević,  
uros.rajcevic@ztm.si

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**Metode:** Razvili smo monoklonska protitelesa proti zunaj- in znotrajceličnim domenam proteinskega modela bilitranslokaze. Da bi pridobili globlji vpogled v izražanje bilitranslokaze tudi v kliničnih vzorcih, smo ugotavljali izražanje bilitranslokaze tudi pri različnih gradusih svetloceličnega karcinoma ledvičnih celic (ccRCC).

**Izsledki:** Obe novi monoklonski protitelesi se vežeta na protein v celicah UOK171, ne pa tudi v negativnih kontrolah. mAb, ki ga proizvaja celična linija 2A9/2E9 (peptid 298–310; znotrajcelična domena), je bolj primeren za imunohistokemijske preiskave, ker daje večjo intenziteto vezave kot mAb, ki ga proizvaja linija 11C9/2G9 (peptid 235–246; zunajcelična domena). Protitelo 2A9/2E9 obarva bilitranslokazo v proksimalnih ledvičnih tubulih, ne pa v okoliški stromi. Obarvanje se zmanjšuje v gradusu I v primerjavi z normalno ledvico, postopno naraste v gradusih II in III ter ponovno pade v gradusu IV ccRCC.

**Sklepi:** Naši rezultati kažejo na to, da se lahko ta protitelesa uporabijo pri različnih imunskih testih. Poleg tega sta nam specifičnost in afiniteta naših mAb omogočila njihovo uporabo pri analizi napredovalih gradusov svetloceličnega karcinoma ledvičnih celic pri omejenem številu bolnikov. mAb, razvita v tej študiji, se lahko uporabijo kot diagnostično orodje za razlikovanje med zgodnjimi in poznimi gradusi svetloceličnega karcinoma ledvičnih celic.

## 1 Introduction

In this work, we describe the use of anti-peptide monoclonal antibodies against bilitranslocase as a diagnostic tool. Bilitranslocase is a membrane protein localized in hepatocyte plasma membrane, basolateral domain of proximal tubules in the kidney, gastric epithelia and endothelial cells. Its main known biological purpose is to transfer organic anions from blood to hepatocytes (1,2,3).

Although the primary structure of bilitranslocase was predicted over a decade ago (4), a model of three-dimensional structure has been proposed only recently (5), due to the low ability of membrane proteins to form a crystal structure and inability of isotopic reorientation in solution that might prevent structure solving using X-ray crystallography or NMR (5).

Until recently, the most widely used antibody targeting bilitranslocase was an anti-peptide polyclonal antibody produced by immunization of rabbit with a multi-antigen peptide, corresponding to segment 65–75 of the primary bilitranslocase structure (4). In one of our recent studies monoclonal antibodies against segment 65–75 were described (6) and

in the present research monoclonal antibodies against segments 235–246 and 298–310 are characterized. According to the three-dimensional structure, both segments 65–75 and 235–246 belong to extracellular domains of bilitranslocase, crucial for its transport function, while segment 289–310 is an intracellular one and its function is yet unknown.

In our previous research we were able to show that expression of bilitranslocase is down-regulated in grade I clear cell renal cell carcinoma (ccRCC), using monoclonal antibodies against peptide 65–75 (6).

Renal cell carcinoma (RCC) represents 2 % of all cancers and is the seventh most commonly diagnosed cancer in men and the ninth most commonly diagnosed cancer in women (7). It usually occurs in people between the ages of 50 and 70, where men are more exposed than women (8). Its incidence worldwide is about 300 000 new cases per year, a quarter of them present with advanced disease. RCC arises from the proximal renal tubular epithelium (9) and is a heterogeneous disease made up of a number of different histological types of can-

cer (10) that occur as an inherited and as a sporadic, non-inherited cancer (11). The most common histological diagnosis is a clear cell renal cell carcinoma (ccRCC) which represents 85 % of kidney tumors (12,13). The most studied form of inherited ccRCC is the one associated with von Hippel-Lindau (VHL) syndrome (11). Affected individuals are at risk for developing tumors in many organs, including the kidney. In VHL syndrome, one VHL allele, located on chromosome 3p25 and encoding for tumor suppressor protein is inherited with a mutation, when the other, wild-type VHL allele, is inactivated or silenced, and ccRCC arises. Defects in the VHL gene also appear to be responsible for a high percentage of tumors in patients with sporadic ccRCC (14,15).

Recent high throughput sequencing projects have identified driver genes in ccRCC beyond VHL (16,17,18). These studies identified frequently mutated tumor suppressors, including PBRM1, BAP1 and SETD2, all of which function as chromatin and/or histone modifiers and intriguingly map to the frequently lost 3p21 locus. New reports also suggest that additional pathogenic mechanisms are at play and suspect that ccRCC is a metabolic disease (19), with the results potentially yielding novel therapeutic insights for the care of kidney cancer.

Prognosis is closely related to the stage of disease (9). The average life expectancy for patients whose disease is already advanced (locally invasive or metastatic renal-cell carcinoma) is about 13 months (9). Mortality rates of RCC are rising with its incidence worldwide of about 102 000 deaths per year (7). Therefore it is crucial to detect disease before it spreads, which is a major problem, since in the early development the disease is characterized only by nonspecific symptoms (9,12,20). More specific

symptoms such as flank pain, hematuria, and a palpable abdominal mass are signs of advanced disease (9).

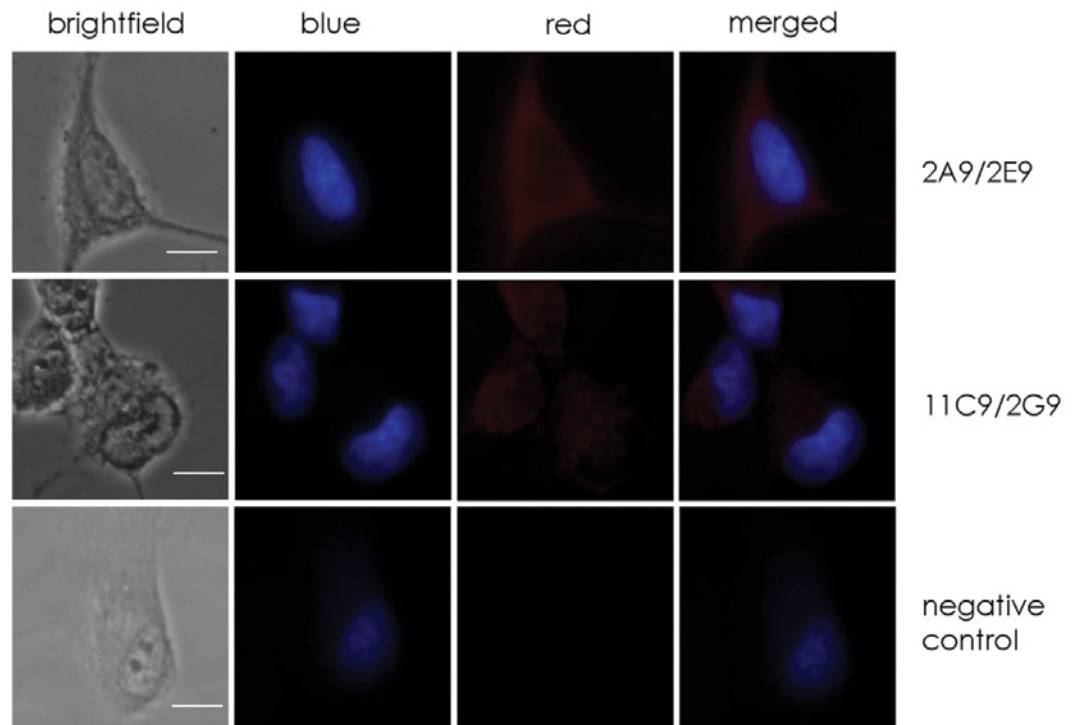
## 2 Materials and methods

### 2.1 Cell culture

Human clear cell renal carcinoma UOK 171 cell line was obtained at NIH-NCI, Bethesda MD, USA. Cells were grown in Dulbecco modified Eagle's medium (MP Biomedicals), supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) glutamine, 0.34 % (v/v) penicillin (Sigma) and 0.34 % (v/v) streptomycin (Sigma) in a humidified incubator at +37 °C and 5 % CO<sub>2</sub>. Cells were seeded to 6-well plates ( $1 \times 10^5$ – $4 \times 10^5$  cells per well) with round coverslips (2r = 13 mm), treated with Poly-L-Lysine (Sigma). Next day cells were ready for the immunocytochemistry assay.

### 2.2 Immunocytochemistry

Cells were rinsed with PBS and fixed with ice-cold 70 % methanol (Sigma) in PBS for 20 min at +4 °C. They were then permeabilized with 0.2 % Triton (Technicon) in PBS for 10 min at room temperature (RT) and blocked with 4 % FCS in PBS for 15 min at RT. Samples were incubated with primary antibodies for 2h at RT. Cells were then incubated with appropriate fluorescent secondary antibodies Alexa Flour 555 Goat anti-Mouse IgG (Life Technologies), at concentration 2 µg/ml in 4 % FCS and 0.1 % Triton in PBS for 1h at RT. Cell nuclei were stained by Hoechst 33258, pentahydrate (bis-benzimide) FluoroPure™ grade stain (Invitrogen), diluted 1:1000 in the same buffer as antibodies and incubated for 1 h at RT. Cells were washed three times with PBS between all incubation steps. After the final three washes with 4 %



**Fig. 1:** Detection of bilitranslocase in UOK171 cells by monoclonal antibodies against peptides B (extracellular) and C (intracellular). Specificity of binding of mAbs 2A9/2E9 (peptide C) and 11C9/2G9 (peptide B) to bilitranslocase is confirmed by using negative control. Magnification bars: 10  $\mu$ m.

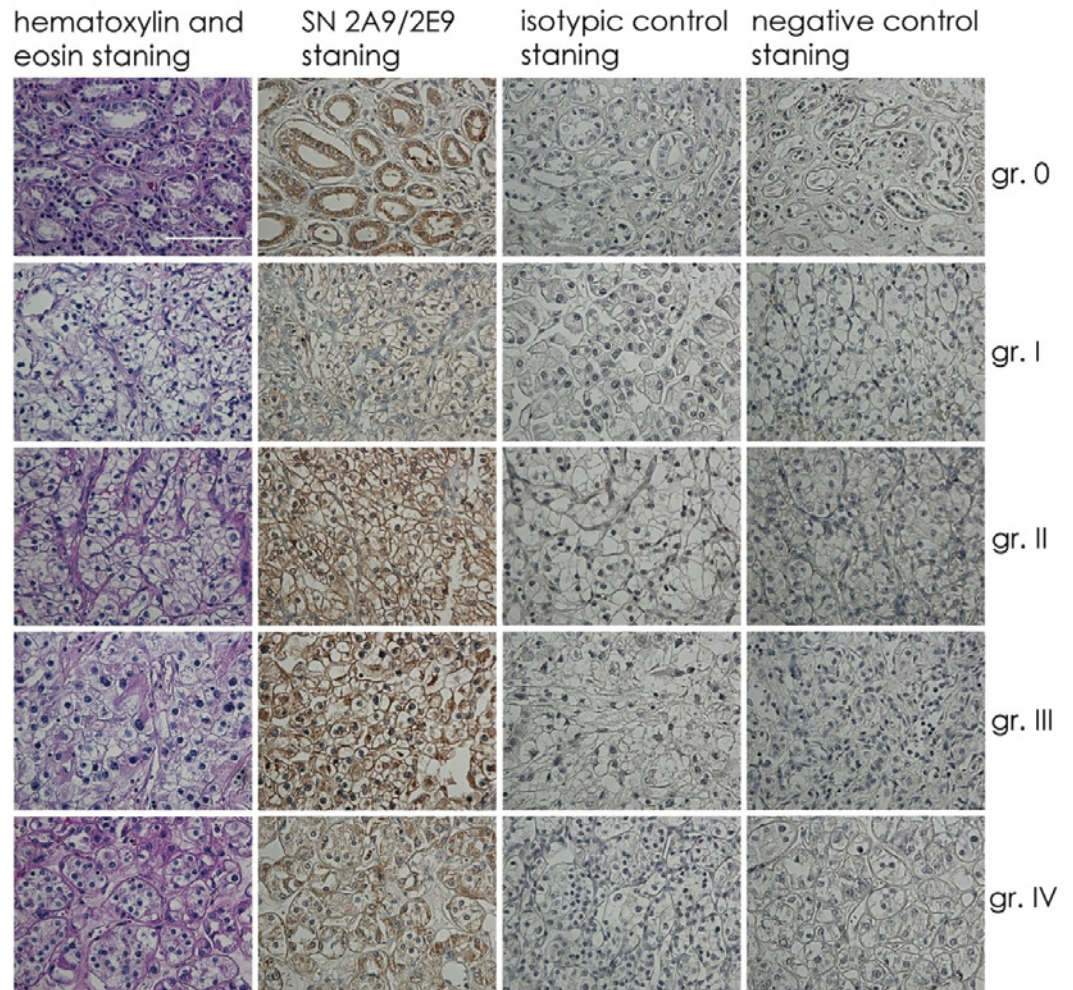
FCS and 0.1 % Triton in PBS, slides were mounted in Fluoromount (Sigma). Negative controls were treated identically with omission of the primary antibody. The cells were visualized by fluorescent microscopy (Nikon, Eclipse Ti-E).

### 2.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin embedded, archived tissue sections of ccRCC and normal human kidney tissue sections. Immunohistochemical analysis was performed on five samples (n=5) in triplicates for each grade (21) of carcinoma, except grade 1, where only three samples (n=3) in triplicates were used. We also tested normal kidney samples. Two samples in five parallels were used. Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol and treated with sodium citrate buffer (20 minutes at 95–100°C) for antigen retrieval.

After washing in water and PBS, incubation with undiluted primary antibodies in supernatant was performed at RT for 2h. Tissue slides were washed with PBS and treated with 2 % hydrogen peroxide (Sigma) in PBS for 8 min to block endogenous peroxidases, followed by incubation with secondary goat anti-mouse IgM antibodies (Sigma), conjugated with HRP (diluted 1:1000) for 2h at RT. After washing with PBS, reaction products were visualized by diaminobenzidine (DAB) as chromogen. Slides were rinsed with water. Mayer's hematoxylin (Sigma) and Scott's buffer (0.2 % (w/v) sodium bicarbonate, 2 % (w/v) magnesium sulfate) were used as a counterstain. Sections were dehydrated in graded ethanol followed by xylene before they were mounted with Eukitt mounting agent (Sigma) and inspected by pathologist for staining intensity, staining area, background and ccRCC grading. Negative controls were treated identically with omission of the





**Fig. 2:** Staining of bilitranslocase in four grades of human clear cell renal cell carcinoma (ccRCC) and normal human kidneys using mAb 2A9/2E9. Magnification bar: 100  $\mu$ m.

primary antibody. Isotype controls were carried out by using monoclonal antibodies of the same antibody class against blood group antigen A.

#### 2.4 IHC Statistics

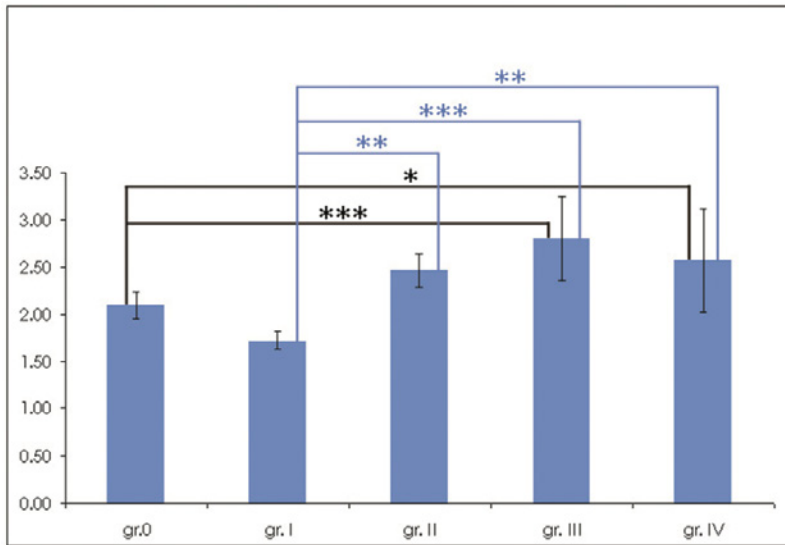
All diagnostic stainings were performed in triplicate. Negative controls were repeated five times. Pathologist inspected the IHC preparations for staining intensity, staining area, background and ccRCC grading all by grading from 0–3. Mean and standard deviation of intensity values were calculated. Null hypothesis that there were no differences

in mean values of staining intensities between ccRCC grades, and ccRCC grades and normal kidney was tested by unpaired (homoscedastic) two-tailed student's t-test. The null hypothesis was rejected with  $p < 0.05$ .

### 3 Results

#### 3.1 Binding of mAbs to BTL

Antibodies were tested for their ability to bind bilitranslocase in UOK171 cell line by fluorescent immunocytochemistry (Fig. 1). The results show that both monoclonal antibodies bind to a protein



**Fig. 3:** Staining intensities of the specimens of all four grades of ccRCC (gr. I–IV) and normal human kidney tissue (gr. 0). Black lines above the histogram connect normal kidney with significantly differential grades in ccRCC. Blue lines above the histogram connect grade I with II–IV ccRCC. Asterisks above the connecting lines indicate the level of significance between the tested pairs.

in UOK171 cells but not in the negative control. The binding of monoclonal antibodies is specific.

### 3.2 Applications of mAbs

Based on the results of a pilot IHC test we concluded that mAb produced by cell line 2A9/2E9 (peptide 298–310; intracellular domain) is more suitable for immunohistochemical analyses as it gives stronger intensity of binding than mAb produced by cell line 11C9/2G9 (peptide 235–246; extracellular domain) and no background staining. Hence, this antibody was tested by immunohistochemical staining of bilitranslocase in all four grades of human clear cell renal cell carcinoma (ccRCC) and normal human kidneys (Fig. 2). The results showed that our monoclonal antibody 2A9/2E9 stains bilitranslocase in proximal renal tubules of normal kidneys but not in the surrounding stroma. Figure 2 also shows that staining decreases in grade I, gradually increases in grades II and III,

but then decreases again in grade IV of ccRCC tissue.

To confirm the above-mentioned results, we performed a statistical analysis of staining intensities in different grades of ccRCC and normal kidney (Fig. 3),  $n=5$  for each grade, except grade 1 ( $n=3$ ). Compared to normal kidney, bilitranslocase staining intensity decreased in grade I ccRCC tissue as we reported before (6). The difference however was not found to be significant ( $p = 0.086$ ). Staining increases from grade I on and there is a significant difference between grades III and IV and normal kidney tissue. There is also a significant difference between grades II, III and IV and grade I of ccRCC.

## 4 Discussion

For potential diagnostic purposes we selected two anti-peptide monoclonal antibodies against bilitranslocase (2A9/2E9 and 11C9/2G9, respectively). Antibodies are specific for extracellular domain crucial for BTL transport function and C-terminal intracellular domain, respectively. They specifically bind both domains in the protein (Fig. 1, 2), enabling us to follow the expression of bilitranslocase in different tissues as well as in different cell lines according to the proposed model of BTL (5). Specificity of antigen-antibody binding was characterized by different immunoassays (Fig. 1, 2). Moreover, to make sure our antibodies are specific for the targeted antigen, we introduced a set of isotype monoclonal antibodies (monoclonal antibodies of the same class, against another antigen) as a control, which confirmed our results as specific (Fig 1, 2).

BTL expression in kidney cells was previously described in animal models (23) where it acts as a transporter of

flavonoids from the diet. BTL expression was also demonstrated in normal human kidney and kidney cancer (6,23), where it was localized in distal tubules in normal kidney (6) and proximal and distal renal tubules while it was absent in renal cortical tumors (23). ccRCC originates in the epithelium of the proximal convoluted tubule, which transports glomerular filtrate from the glomerulus to the descending part of the nephron. In our study, we detected BTL expression in the epithelium of proximal tubules in normal kidneys but not in the surrounding stroma (Fig 2). We have shown earlier that BTL expression decreases in early grade ccRCC compared to normal kidneys (6) in a limited number of patients. To get a better insight in BTL expression in ccRCC and to demonstrate our novel monoclonal antibodies against bilitranslocase as a tool in research and possibly in the diagnostics of this potential new biomarker, we performed this study on a larger number of kidney tumor samples from patients with progressive grades of ccRCC (stages I-IV) and normal kidney controls. We are able to confirm the decrease in BTL expression from normal kidney where it stained the proximal tubules to grade I ccRCC (Fig. 2, 3). The observed difference in BTL expression was statistically insignificant (Fig. 3). However, we were able to demonstrate that significant differences in bilitranslocase exist as the cancer progresses from stage I towards stage IV (Fig. 3). There is also a significant difference between bilitranslocase expression in normal kidney cells and grades III and IV of ccRCC. This way, based on BTL expression, we were able to distinguish between normal kidneys and high grades (III-IV) and low- versus high-grade tumors (I vs. II-IV), while it was impossible to distinguish between grades II-IV based on

BTL expression. Why it appears that the BTL expression decreases in low-grade ccRCC compared to high-grade ccRCC remains elusive. The fact that ccRCC is suspected to be a metabolic disease (19) and that different genetic changes seem to be directed toward metabolic pathways in our opinion this may be related to the disrupted transport and perhaps metabolism of antioxidants, where bilitranslocase plays a role (22). The exact role of bilitranslocase in the progression of ccRCC will have to be revealed by additional testing.

## 5 Conclusions

In this work, specific monoclonal antibodies 2A9/2E9 and 11C9/2G9, of extracellular and intracellular C-terminal domains, respectively, of predicted primary structure of rat bilitranslocase were produced and characterized. Described monoclonal antibodies could in future be used as a diagnostic tool that could help distinguish between early and late stages of clear cell renal cell carcinoma.

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## References

- Elias MM, Lunazzi GC, Passamonti S, Gazzin B, Miccio M, Stanta G, et al. Bilitranslocase localization and function in basolateral plasma membrane of renal proximal tubule in rat. *Am J Physiol Renal Physiol* 1990;259(4 Pt 2):F559–64.
- Nicolin V, Grill V, Micali F, Narducci P, Passamonti S. Immunolocalisation of bilitranslocase in mucosecretory and parietal cells of the rat gastric mucosa. *Journal of Molecular Histology*. 2005;36(1–2):45–50.
- Maestro A, Terdoslavich M, Vanzo A, Kuku A, Tramer F, Nicolin V, et al. Expression of bilitranslocase in the vascular endothelium and its function as a flavonoid transporter. *Cardiovascular Research*. 2009;85(1):175–83.
- Battiston L, Passamonti S, Macagno A, Sottocasa GL. The Bilirubin-Binding Motif of Bilitranslocase and Its Relation to Conserved Motifs in Ancient Biliproteins. *Biochemical and Biophysical Research Communications*. 1998;247(3):687–92.
- Perdih A, Roy Choudhury A, Župerl Š, Sikorska E, Zhukov I, Solmajer T, et al. Structural Analysis of a Peptide Fragment of Transmembrane Transporter Protein Bilitranslocase. *PloS one*. 2012;7(6):e38967.
- Montanic S, Terdoslavich M, Rajcevic U, De Leo L, Department of Medical Sciences US, Curin Serbec V, et al. Development and characterization of a novel mAb against bilitranslocase—a new biomarker of renal carcinoma. *Radiology and Oncology*. 2013;47(2).
- Gupta K, Miller JD, Li JZ, Russell MW, Charbonneau C. Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): A literature review. *Cancer Treatment Reviews*. 2008;34(3):193–205.
- Rajer M. Kidney cancer. *Radiology and Oncology*. 2007;41(2).
- Cohen HT, McGovern FJ. Renal-Cell Carcinoma. *New England Journal of Medicine*. 2005;353(23):2477–90. Linehan WM, Vasselli J, Srinivasan R, Walther MM, Merino M, Choyke P, et al. Genetic basis of cancer of the kidney: disease-specific approaches to therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2004;10(18 Pt 2):6282s–9s.
- Linehan WM. Identification of the von Hippel-Lindau (VHL) Gene. *Jama*. 1995;273(7):564.
- Motzer RJ, Agarwal N, Beard C, Bolger GB, Boston B, Carducci MA, et al. NCCN clinical practice guidelines in oncology: kidney cancer. *Journal of the National Comprehensive Cancer Network : JNCCN*. 2009;7(6):618–30.
- DeVita TV, Hellman S, Rosenberg AS, editors. *Principles and practice of Oncology 7th edition*. Philadelphia: Lippincott Williams & Wilkins; 2005.
- Kim WY, Kaelin WG. Role of VHL Gene Mutation in Human Cancer. *Journal of Clinical Oncology*. 2004;22(24):4991–5004.
- Iliopoulos O, Levy AP, Jiang C, Kaelin WG, Goldberg MA. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proceedings of the National Academy of Sciences*. 1996;93(20):10595–9.
- Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* 2011;469(7331):539–42.
- Dalgliesh GL, Furge K, Greenman C, Chen L, Bignell G, Butler A, et al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. *Nature*. 2010;463(7279):360–3.
- Hakimi AA, Pham CG, Hsieh JJ. A clear picture of renal cell carcinoma. *Nature Genetics*. 2013;45(8):849–50.
- Linehan WM, Srinivasan R, Schmidt LS. The genetic basis of kidney cancer: a metabolic disease. *Nature Reviews Urology*. 2010;7(5):277–85.
- Čufer T. Rak ledvic. *Onkologija*. 2005;9:76–9.
- Ljungberg B, Hanbury DC, Kuczyk MA, Merseburger AS, Mulders PFA, Patard J-J, et al. Renal Cell Carcinoma Guideline. *European Urology*. 2007;51(6):1502–10.
- Vanzo A, Terdoslavich M, Brandoni A, Torres AM, Vrhovsek U, Passamonti S. Uptake of grape anthocyanins into the rat kidney and the involvement of bilitranslocase. *Molecular Nutrition & Food Research*. 2008;52(10):1106–16.
- Golijanin DJ, Marshall J, Cardin A, Singer EA, Wood RW, Reeder JE, et al. Bilitranslocase (BTL) is immunolocalised in proximal and distal renal tubules and absent in renal cortical tumors accurately corresponding to intraoperative near infrared fluorescence (NIRF) expression of renal cortical tumors using intravenous indocyanine green (ICG). *The Journal of Urology*. 2008;179(4):137.