Histol Histopathol (2017) 32: 1029-1040

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

GLUT1, MCT1/4 and CD147 overexpression supports the metabolic reprogramming in papillary renal cell carcinoma

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Summary. Papillary Renal Cell carcinoma (pRCC) is the second most common type of RCC, accounting for about 15% of all RCCs. Surgical excision is the main treatment option. Still, 10-15 % of clinically localized tumours will recur and/or develop metastasis early after surgery, and no reliable prognostic biomarkers are available to identify them. It is known that pRCC cells rely on high rates of aerobic glycolysis, characterized by the up-regulation of many proteins and enzymes related with the glycolytic pathway. However, a metabolic signature enabling the identification of advanced pRCC tumours remains to be discovered.

The aim of this study was to characterize the metabolic phenotype of pRCCs (subtypes 1-pRCC1 and 2-pRCC2) by evaluating the expression pattern of the glucose transporters (GLUTs) 1 and 4 and the monocarboxylate transporters (MCTs) 1 and 4, as well as their chaperon CD147. We analysed the clinico-pathological data and the protein and mRNA expression of GLUT1, GLUT4 and MCT1, MCT4 and CD147 in tumours from Porto and TCGA series (http://cancer genome.nih.gov/), respectively.

With the exception of GLUT4, plasma membrane expression of all proteins was frequently observed in pRCCs. GLUT1 and MCT1 membrane overexpression was significantly higher in pRCC2 and significantly associated with higher pN-stage and higher Fuhrman grade.

Overexpression of GLUT1, MCT1/4 and CD147, supports the metabolic reprograming in pRCCs. MCT1 expression was associated with pRCC aggressiveness, regardless of the tumour histotype.

Key words: Papillary renal cell carcinoma pRCC1, pRCC2, Glucose Transporter, Monocarboxylate transporter

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DOI: 10.14670/HH-11-863

Introduction

Renal cell carcinoma (RCC) comprises a heterogeneous group of tumours arising from the epithelium of the renal tubules and accounts for >90% of all adult renal carcinomas (Chow et al., 2010; Fernandes and Lopes, 2015). RCC originates from a diverse set of genetic abnormalities, presenting diverse histologic features, distinct biologic behaviour, variable responses to therapy and variable clinical outcomes (Lam et al., 2005; Wang et al., 2014; Fernandes and Lopes, 2015).

According to the WHO classification, the most common adult RCC subtypes are: clear cell renal cell carcinoma (ccRCC; 65-70%) followed by papillary renal cell carcinoma (pRCC; up to 18.5%), and chromophobe renal cell carcinoma (chRCC; 5-7%) (Moch et al., 2016).

pRCCs are a heterogeneous group of tumours, composed of epithelial cells with papillary or tubulopapillary architecture, traditionally subdivided in two subtypes (pRCC1 and pRCC2), on the basis of

distinct morphological and behavioural features that may influence therapeutic options (Yang et al., 2013; Moch et al., 2016). pRCC1 is characterized by the presence of cuboidal or columnar neoplastic small cells, with nuclei aligned in a single layer and a scanty and pale cell cytoplasm, covering fibrovascular cores or stalks. pRCC2 displays large epithelial cells lining fibrovascular cores, showing pseudostratification or irregular stratification of nuclei, often presenting abundant cytoplasm and moderate to marked nuclear pleomorphism usually with prominent nucleoli. pRCC subtyping is considered to be of prognostic significance, having type 1 tumours a better prognosis than those with type 2 morphology (Delahunt et al., 2001; Alomari et al., 2015; Fernandes and Lopes, 2015; Moch et al., 2016).

Remarkably, available evidence shows that, like other human cancer cells, pRCC cells display high glycolytic rates and uncoupled oxidative phosphorylation, even in the presence of oxygen (Warburg effect) (Shuch et al., 2013). One of the first consequences of the cell commitment to a glycolytic phenotype is the upregulation of glucose transporters (GLUTs) and monocarboxylate transporters (MCTs). The increased glycolytic flux starts with increased glucose uptake (with consequent glucose transporters upregulation), which is oxidised to pyruvate and further converted into lactate in the cytosol. The excess of produced lactate is then extruded to the surrounding milieu by monocarboxylate transporters (Halestrap and Price, 1999; Le Floch et al., 2011).

GLUTs are a family of facilitative sugar transporters (GLUT1-14), encoded by the *Solute Carrier 2* (*SLC2*) gene family, which transport glucose across the plasma membrane by diffusion gradient, exhibiting different substrate specificities, kinetic properties and tissue expression profiles (Zhao and Keating, 2007). GLUT1 and GLUT4 are the most studied glucose transporters in human tumours, and their upregulation has been associated with tumour growth, invasiveness and metastasis (Wang et al., 2014). The monocarboxylate transporter (MCT) family is composed of 14 members with distinct transport properties and tissue distribution. MCTs are encoded by the superfamily of Solute *Carrier Genes 16* (*SLC16*) which is conserved among species (Halestrap and Price, 1999). Like GLUTs, MCTs, namely MCT1 and MCT4 have been found upregulated in several types of tumours (Pinheiro et al., 2011, 2012). The importance of MCTs for tumour growth has been reported by various authors either by using MCT inhibitors and proliferation studies (Halestrap, 2013) or by combining the silencing of MCT1 or MCT4 and CD147 [also called Basigin (BSG) or EMMPRIN], demonstrating that MCT impairment leads to a significant reduction of the glycolytic flux and cell proliferation (Le Floch et al., 2011). CD147 is a highly glycosylated transmembrane protein member of the immunoglobulin superfamily of receptors, being encoded by the BSG gene (Biswas et al., 1995). CD147 is a chaperon required for MCT1 -3 and -4 cell membrane expression and function, via the formation of heterodimeric complexes. CD147 has been associated with the regulation of the trafficking and anchoring of MCT1 and MCT4 to different cell surfaces of polarized cells (Le Floch et al., 2011; Pinheiro et al., 2012; Halestrap, 2013) and has been reported to be overexpressed in many cancers, including RCC (Jin et al., 2006; Dang et al., 2008; Han et al., 2010; Pinheiro et al., 2010; Rademakers et al., 2011; Huang et al., 2013, 2014a,b; Sato et al., 2013; Monteiro et al., 2014).

One of the most common diagnostic problems in pRCC is the identification of the 10-15 % of clinically localized tumours that will recur and/or develop metastasis early after surgical excision. While these tumours share morphological features with those that are successfully treated, they should have distinct biological properties, constituting a challenge for the prognosis and management of RCC in general and pRCC in particular due to the inexistence of reliable prognostic biomarkers (Osunkoya et al., 2009; Wang et al., 2014).

The aim of this study was to assess the expression of GLUT1, GLUT4, MCT1, MCT4, and the chaperon CD147, in a series of pRCCs, and to evaluate its clinicopathological significance. Furthermore, we questioned whether such biomarkers may contribute to the identification of a distinct metabolic phenotype of pRCCs and help in distinguishing pRCC1 from pRCC2, ultimately contributing to the development of therapeutic strategies directed to particular onco-driven stages. In an attempt to validate our results, we used a series of pRCC derived from The Cancer Genome Atlas (http://cancer genome.nih.gov/).

Materials and methods

Sample characterization: clinical and pathological data

A total of 51 consecutive (from January 1998 to July 2013) pRCC were identified in the files of the Department of Pathology of Centro Hospitalar de São João and re-evaluated by two pathologists (JML and RS). All patients were submitted to surgical resection by either radical (n=37; 72.5%) or partial (n=14; 27.5%) nephrectomy. Clinical parameters were annotated from the patients' records and the oncology registries from Centro Hospitalar de São João and Oncology Registry of North Region (RORENO).

Demographic, clinical and pathologic data include: gender, age, tumour size, TNM stage and Fuhrman grade. Tumours were classified according to the tumour-node-metastasis (TNM) cancer staging system, corresponding to the 7th edition of the AJCC Cancer Staging Manual (Edge and Compton, 2010) and the Fuhrman grading systems (Fuhrman et al., 1982). The tumours were grouped into low (pT1-pT2) or high (pT3-4) stage and low (G1-G2) or high (G3-G4) Fuhrman grade.

This study was approved by the Local Ethical Committee of Centro Hospitalar de São João, (Porto,

Portugal)/Medical Faculty (Porto, Portugal) and is in accordance with the National Ethical rules.

Evaluation of protein expression levels by immunohistochemistry (IHC)

The expression of GLUT1, GLUT4, MCT1, MCT4, and CD147 was assessed in all tumours and matched adjacent kidney parenchyma. In fourteen (27.5%) cases [12 (26.6%) pRCC1 and 2 (33.3%) pRCC2] adjacent kidney parenchyma was excluded from IHC evaluation due to the presence of distorted tubules, several cysts and thyroid like structures (data not shown).

Serial tissue sections (3μ thick) of representative tumour samples and normal kidney were used for IHC. The IHC for GLUT1, GLUT4, MCT1, MCT4 and CD147 was performed according to the manufacturer's protocols with the following primary antibodies: GLUT1 (ab15309, Abcam, UK), GLUT4 (ab33780, Abcam, UK), MCT1 (EMD Millipore Corporation, USA), MCT4 (sc-50329, Santa Cruz Biotechnolgy, INC., CA, USA) and CD147 (1.BB.218; sc-71038, Santa Cruz Biotechnolgy, INC., CA, USA). The tissue sections were deparaffinised followed by a sequential hydration. Antigen retrieval was performed at 98°C, with citrate solution (pH=6) for GLUT1, GLUT4, MCT1, and MCT4 and with EDTA (pH=8) for CD147 (Table 1). After peroxidase and avidin-biotin blocking, all sections were treated with Lab Vision™ UltraVision™ Large Volume Detection System: anti-Polyvalent, HRP (Thermo Scientific). All reactions were revealed with diaminobenzidine (DAB) chromogen (Dako Carpinteria, CA, USA) and counterstained with haematoxylin and eosin. All protocols included positive and negative controls. In negative controls the primary antibodies omitted and replaced by the antibody dilution reagent. The external positive controls included known positive tissues for the specific antibody. Kidney/adjacent tissue surrounding the tumour was used as internal control.

The expression of MCT1, MCT4, GLUT1, GLUT4 and CD147 in tumour tissue was evaluated according to an immune reactive staining (IRS) score adapted from Fonteyne et al. (2009) and Couto et al. (2012). Briefly, the extension (E) of immunostaining for each antibody was evaluated using a score from 0 to 4 (0: <10% cells; 1: 11% - 25%; 2: 26% - 50%; 3: 51% - 75%; and, 4: >75% cells) and the immunostaining intensity (I) was evaluated using a score from 0 to 3 [(0=absent; 1=weak (+); 2=moderate (++); 3=strong (+++)]. The IRS score was calculated by multiplying E score by I score (IRS=E x I). Membrane IRS (mIRS) score, indicating protein expression in cell membrane, was calculated (Gould and Holman, 1993, Halestrap and Price, 1999). Cases showing an IRS score 1 or above were considered positive.

In order to validate our results, we analysed the TCGA database for mRNA expression of GLUT1, GLUT4, MCT1, MCT4 and CD147 i.e., Solute Carrier Family 2, Member 1 (SLC2A1) and Member 4 (SLC2A4), Solute Carrier Family 16, member 1 (SLC16A1) and Member 3 (SLC16A3), and Basigin (BSG), since, in this series, we did not have access to tissue protein expression.

Statistical analyses

Statistical analysis was performed using IBM SPSS Statistics Version 22. Fisher's exact test was used to validate differences in frequencies of clinical and pathological parameters, as well as protein expression scores (IRS), comparing pRCC1 and pRCC2. Spearman's rank correlation test was used to assess the correlation between evaluated parameters (categorical variables). Kruskal-Wallis test was used to assess and validate differences in mIRS (Porto series) and in mRNA expression (TCGA series) for comparing pRCC1 and pRCC2. Univariate survival analyses were performed using the Kaplan-Meier method with log-rank test. Results were considered statistically significant when $p \leq 0.05$.

Results

Patient and tumour features

The tumours were classified as pRCC1 (n=45) and pRCC2 (n=6) and the ratio female/male was 1:5.4 (43 men and 8 women). Table 2 summarises the main features observed in pRCC from the Porto series.

The clinic-pathologic data from the 163 primary pRCC retrieved from the TCGA database are summarized in Table 3. The cases were classified as pRCC1 (n=77) and pRCC2 (n=86) and the ratio female/male was 1:2.5.

Table 1. Antigen retrieval and antibody incubation conditions.

Primary Antibodies	Antigen Retrieval		Primary Ab dilution	Primary Ab incubation
GLUT1 (0,2 mg/mL)	Citrate, pH 6	Water bath, 98°C, 10 min.	1/400	1H, RT
GLUT4 (0,3 mg/mL)	Citrate, pH 6	Water bath, 98°C, 20 min.	1/400	ON, RT
MCT1 (1.0 mg/mL)	Citrate, pH 6	Water bath, 98°C, 20 min.	1/100	ON, RT
MCT4 (H-90) (200 μg/mL)	Citrate, pH 6	Water bath, 98°C, 20 min.	1/1000	2H, RT
CD147 (200 μg /mL)	EDTA, pH 8	Water bath, 98°C, 20 min.	1/300	ON, RT

Ab, antibody; ON, overnight; H, hour; RT, room temperature.

Table 2. Patient data and clinical-pathological features - Porto series.

Porto - series	pRCC	pRCC1	pRCC2	p
Total: n (%)	51 (100.0)	45 (88.2)	6 (11.8)	
Gender: men n (%) | women n (%)	43 (84.3) | 8 (15.7)	37 (82.2) | 8 (17.8)	6 (100.0) | 0 (0.0)	p=0.572
Age: Median | Mean (years)	58.0 | 59.8±13.6	59.0 | 59.4±13.8	63.5 | 62.8±12.9	
Tumour Size: Median | Mean (cm)	3.5 | 3.9± 2.2	3.5 | 3.7± 1.6	4.6 | 5.4± 4.5	
pT Total: n (%):	51 (100.0)	45 (88.2)	6 (11.8)	p=0.036*
pT1: n (%)	44 (86.3)	41 (91.1)	3 (50.0)	
pT2: n (%)	2 (3.9)	2 (4.4)	0 (0.0)	
pT3: n (%)	4 (7.8)	2 (4.4)	2 (33.3)	
pT4: n (%)	1 (2.0)	0 (0.0)	1 (16.7)	
pN Total: n (%)	51 (100.0)	45 (88.2)	6 (11.8)	p=0.224
pN: n (%)	0 (0.0)	0 (0.0)	0 (0.0)	
pM Total: n (%)	51 (100.0)	45 (88.2)	6 (11.8)	p=0.224
pM: n (%)	2 (3.9)	1 (2.2)	1 (16.7)	
Fuhrman Nuclear Grade Total: n (%)	51 (100.0)	45 (88.2)	6 (11.8)	p=0.041*
G1: n (%)	2 (3.9)	2 (4.4)	0 (0.0)	
G2: n (%)	18 (35.3)	18 (40.0)	0 (0.0)	
G3: n (%)	30 (58.8)	25 (55.6)	5 (83.3)	
G4: n (%)	1 (2.0)	0 (0.0)	1 (16.7)	

p, based on Fisher's exact test; *p≤0.05.

Table 3. Patient data and clinical-pathological features - TCGA series.

TCGA - series	pRCC	pRCC1	pRCC2	
Total: n (%)	163 (100.0)	77 (47.2)	86 (52.8)	
Gender: men n (%) | women n (%)	117 (71.8) | 46 (28.2)	56 (72.7) | 21 (27.3)	61 (70.9) | 25 (29.1)	p =0.469
Age: Median | Mean | (years)	61.0 | 61.3±12.2 |	59.0 | 58.1±11.2 |	65.0 | 64.2±12.4	
Tumour size: Median | Mean (cm)	4.2 | 5.0±3.1 |	4.0 | 4.4±2.3 |	4.7 | 5.4±3.5	
pT Total: n (%)	163 (100.0)	77 (47.2)	86 (52.8)	p =0.033*
pT1: n (%)	113 (69.4)	50 (64.9)	63 (73.2)	
pT2: n (%)	17 (10.4)	13 (16.9)	4 (4.7)	
pT3: n (%)	31 (19.0)	14 (18.2)	17 (19.8)	
pT4: n (%)	2 (1.2)	0 (0.0)	2 (2.3)	
pN Total: n (%)	162 (100.0)	77 (47.2)	85 (52.8)	p =0.002*
pN: n (%)	18 (11.1)	2 (2.6)	16 (18.8)	
pM Total: n (%)	162 (100.0)	74 (47.1)	83 (52.9)	p =0.088
pM: n (%)	3 (1.9)	0 (0.0)	3 (3.6)	
Fuhrman Nuclear Grade Total: n (%)	117 (100.0)	56 (47.9)	61 (52.1)	p <0.001**
G1: n (%)	17 (14.5)	14 (25.0)	3 (4.9)	
G2: n (%)	42 (35.9)	34 (60.7)	8 (13.1)	
G3: n (%)	55 (47.0)	8 (14.3)	47 (77.0)	
G4: n (%)	3 (2.6)	0 (0.0)	3 (4.9)	

p, based on Fisher's exact test; *p≤0.05; **p≤0.001.

Table 4. GLUT1, GLUT4, MCT1, MCT4 and CD147 membrane expression (Porto series).

Protein	pRCC			pRCC1			pRCC2			
	Total n	Neg n (%)	Pos n (%)	Total n	Neg n (%)	Pos n (%)	Total n	Neg n (%)	Pos n (%)	p
GLUT1	51	20 (39.2)	31 (60.8)	45	20 (44.4)	25 (55.6)	6	0 (0.0)	6 (100.0)	0.044*
GLUT4	51	46 (90.2)	5 (9.8)	45	40 (88.9)	5 (11.1)	6	6 (100.0)	0 (0.0)	1.000
MCT1	51	42 (82.4)	9 (17.6)	45	41 (91.1)	4 (8.9)	6	1 (16.7)	5 (83.3)	<0.001**
MCT4	51	3 (5.9)	48 (94.1)	45	3 (6.7)	42 (93.3)	6	0 (0.0)	6 (100.0)	0.227
CD147	51	20 (39.2)	31 (60.8)	45	18 (40.0)	27 (60.0)	6	2 (33.3)	4 (66.7)	0.126

p, based on Fisher's exact test; n, sample size; *p≤0.05; **p≤0.001.

Patients with pRCC2 tumours had worse overall survival in both series (Fig. 1).

GLUT1, GLUT4, MCT1, MCT4 and CD147 expression

In the normal kidney, GLUT1 and MCT4 had a similar expression, being localised in the cytoplasm and basolateral membrane of distal tubules (although MCT4 was also observed in the cytoplasm of proximal tubules). MCT1 and CD147 were only expressed in the basolateral membrane, the former being limited to proximal tubules, and the latter present in proximal and distal tubules. GLUT4 was limited to the cytoplasm of proximal and distal tubules, not being observed in the membrane (Fig. 2).

Data concerning membrane (m) GLUT1, GLUT4, MCT1, MCT4 and CD147 expression in tumour tissues are summarised in Figure 2 and in Tables 4-5. Membrane expression in the pRCC series was high for MCT4 (94.1%), GLUT1 (60.8%) and CD147 (60.8%), and low for MCT1 (17.6%) and GLUT4 (9.8%). When comparing the two subtypes, differential membrane expression was observed for mGLUT1 [55.6% in pRCC1 and 100% in pRCC2 ($p=0.044$)] and notably for mMCT1 [8.9% in pRCC1 and 83.3% in pRCC2 ($p<0.001$)].

When using the membrane IRS (mIRS), which represents not only the intensity of the staining, but also the extent of the respective staining, the results described above were confirmed (Table 5): GLUT1 and MCT1 mIRS means were significantly higher in pRCC2 than in pRCC1 tumours ($p=0.004$ and $p<0.001$, respectively), while GLUT4, MCT4 and CD147 mIRS means were comparable in pRCC1 and pRCC2 tumours (Table 5).

Analysing mRNA expression data from the TCGA series, the same tendency was observed: GLUT1 and MCT1 mRNA mean expression levels were significantly higher in pRCC2 than in pRCC1 ($p=0.005$ and $p<0.001$, respectively) while GLUT4, MCT4 and CD147 mRNA mean expression levels were identical (Table 6).

Correlation between membrane GLUT1, GLUT4 and CD147 with membrane MCT1 and MCT4 expression in tumour cells

Since one of the aims of our work was to verify if pRCCs display increased glycolytic rate, we assessed the

Table 5. GLUT1, GLUT4, MCT1, MCT4 and CD147 membrane (mIRS) (Porto series).

Protein	pRCC Mean±SD	pRCC1 Mean±SD	pRCC2 Mean±SD	p
GLUT1	2.76±3.03	2.27±2.63	6.50±3.50	0.004*
GLUT4	0.29±1.06	0.33±1.13	0.00±0.0	0.619
MCT1	0.67 ±1.99	0.24±0.80	8.00±4.49	<0.001**
MCT4	6.00±3.52	6.16±3.50	8.40±3.92	0.348
CD147	3.18±3.32	2.87±2.97	9.40±4.97	0.198

Kruskal-Wallis test, p, based on Fisher's exact test; *p≤0.05; **p≤0.001.

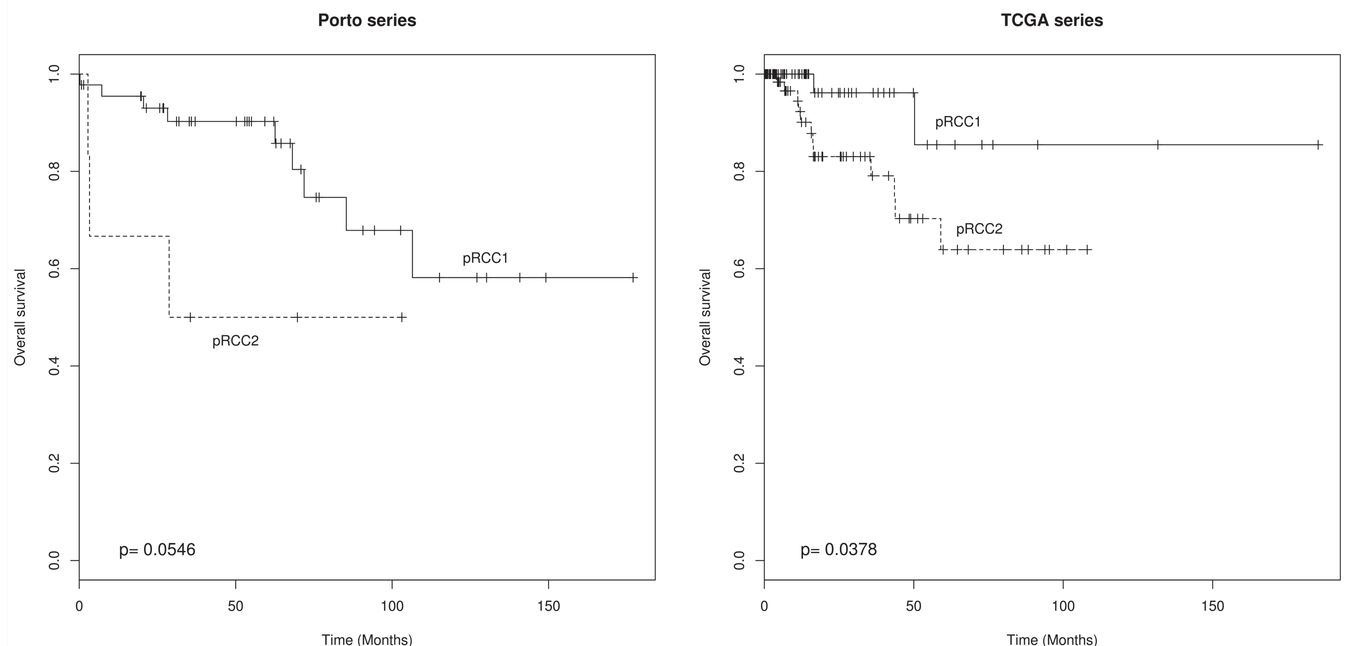

Fig. 1. Kaplan - Meier survival analyses in patients from Porto and TCGA series with pRCC1 and pRCC2 tumours. Patients with pRCC2 tumours had shorter overall survival.

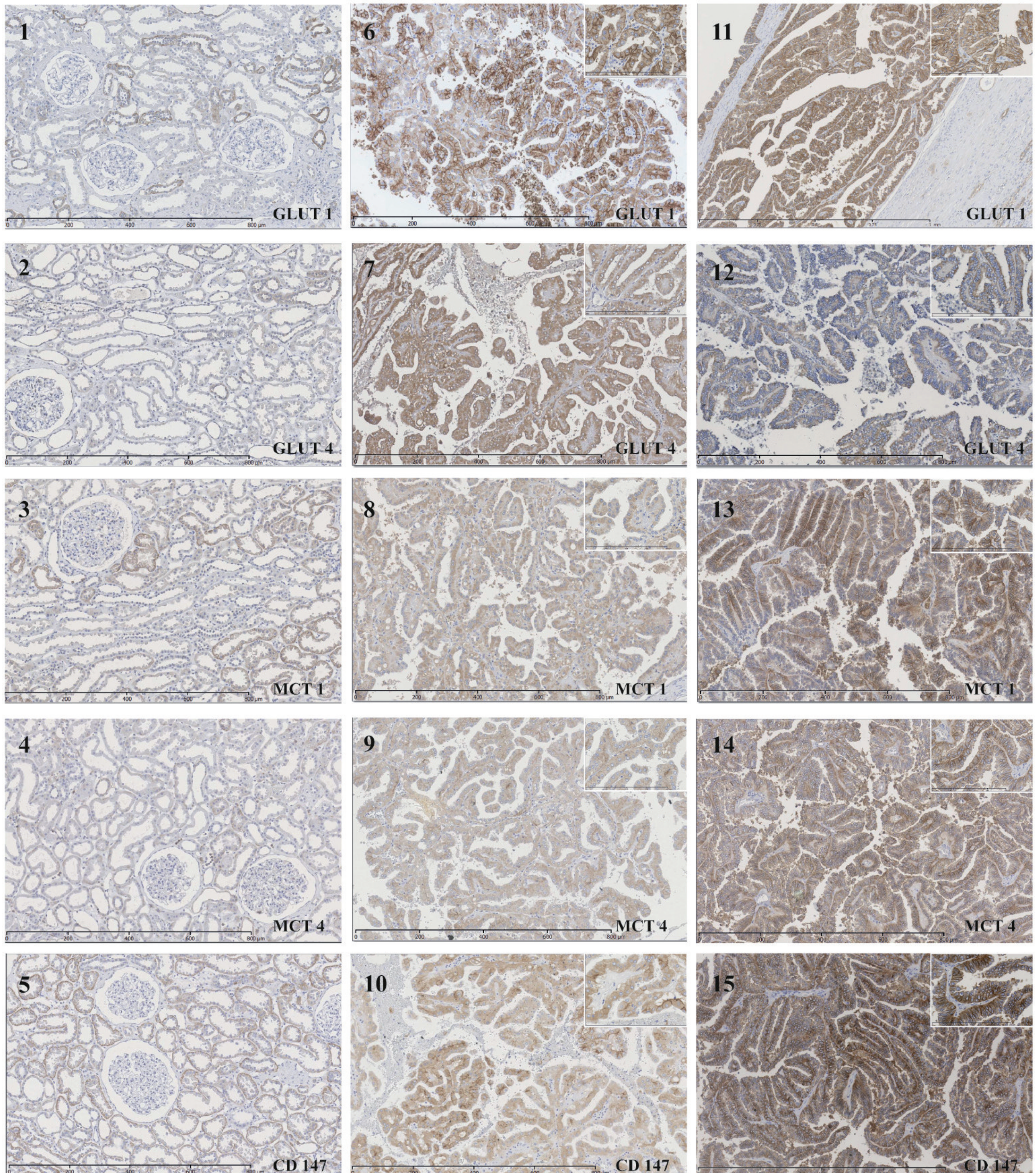

Fig. 2. Immunohistochemical expression of GLUT1 **(1, 6, 11)**, GLUT4 **(2, 7, 12)**, MCT1 **(3, 8, 13)**, MCT4 **(4, 9, 14)**, and CD147 **(5, 10, 15)** in the normal kidney **(1-5)**, in the pRCC1 **(6-10)** and in the pRCC2 **(11-15)** tumours. Scale bars: 1-15, 800 µm; insets, 200 µm.

Table 6. GLUT1, GLUT4, MCT1, MCT4 and CD147 mRNA expression (TCGA series).

mRNA	pRCC Mean±SD	pRCC1 Mean±SD	pRCC2 Mean±SD	P
GLUT1/ SLC2A1	2248.64±4953.67	2122.79±5446.57	2361.31±4496.31	0.005*
GLUT4/ SLC2A4	80.84±134.68	75.47±72.06	85.65±172.86	0.510
MCT1/ SLC16A1	457.10±628.14	258.16±502.21	635.22±677.14	<0.001**
MCT4/ SLC16A3	3412.20±3900.77	3184.85±2626.39	3615.76±4769.02	0.455
CD147/ BSG	29840.58±11165.48	9433.01±10605.80	30205.49±11693.76	0.735

Kruskal-Wallis test, p, based on Fisher's exact test; *p≤0.05; **p≤0.001.

Table 7. Correlation between GLUT1, GLUT4, MCT1, MCT4 and CD147 expression in pRCC: A - protein (mIRS), Porto series; B - mRNA, TCGA series.

	pRCC	
A) Protein	MCT1 p | r	MCT4 p | r
GLUT1	<0.001** | 0.528	0.002* | 0.423
GLUT4	NS	NS
CD147	0.001** | 0.469	0.001** | 0.449
B) mRNA	MCT1 p | r	MCT4 p^a | r
GLUT1	<0.001** | 0.250	<0.001** | 0.525
GLUT4	0.270 | -0.087	0.991 | -0.001
CD147	0.677 | -0.033	0.035* | -0.165

p based on Spearman's rank correlation test; NS, no statistical significance; r, correlation value; *p≤0.05; **p≤0.001.

correlation between glucose transporters (GLUT1 and GLUT4) and lactate transporters (MCT1 and MCT4) mIRSs. Additionally, and assuming that CD147 functions as a MCT1 and MCT4 chaperon, we analysed the correlation between CD147 mIRS and MCT1 and MCT4 mIRS, in the Porto series (Table 7A). We observed significant correlations between GLUT1 and MCT1 mIRS (p<0.001); GLUT1 and MCT4 mIRSs (p=0.002); CD147 and MCT1 mIRS (p=0.001); and CD147 and MCT4 mIRS (p=0.001). GLUT4 mIRS do not correlate with any of the lactate transporters (MCT1 and MCT4) mIRS (Table 7A).

In the TCGA series, GLUT1 mRNA expression was significantly correlated with MCT1 (p=0.001) and MCT4 (p<0.001), although the association between CD147 and MCT1 could not be established (p=0.677), a negative correlation between CD147 and MCT4 was observed (p=0.035) (Table 7B).

Association between MCT1, MCT4, GLUT1, GLUT4 and CD147 expression with patient and tumour clinical and pathological features

In the Porto series, GLUT1 mIRS was significantly higher in tumours with higher pN stages (p=0.042) and high nuclear grade (p<0.001), whereas MCT1 and CD147 mIRSs were significantly higher in tumours with higher nuclear grade (p=0.033 and p=0.017, respectively). GLUT4 and MCT4 mIRSs did not correlate with any clinico-pathological features (Table 8A).

Concerning the TCGA series, GLUT1 mRNA expression was significantly higher in tumours from women (p=0.003), higher pT (p<0.001), higher pN (p=0.001) and higher nuclear grade (p=0.014); GLUT4 mRNA expression was higher in tumours from women; MCT1 mRNA expression was increased in tumours with higher pT stages (p<0.001) and higher nuclear grade (p=0.014); MCT4 mRNA expression was increased in tumours with higher pT stages (p=0.048); and CD147 mRNA expression was higher in tumours from younger (p=0.018) and men (p<0.001) patients (Table 8B).

Discussion

In the present study we analysed the expression of glucose transporters (GLUT1 and GLUT4), lactate transporters (MCT1 and MCT4) and the MCT1/MCT4 chaperon (CD147), in two different series (Porto and TCGA) of pRCCs, each of them providing complementary information. The Porto series, although fewer in cases than the TCGA series, allowed the analysis of GLUT1, GLUT4, MCT1, MCT4 and CD147 protein expression in pRCC tumours. The TCGA series, which resulted from a collection of various centres worldwide with a centralized revision panel, allowed the analysis of GLUT1 (SLC2A1), GLUT4 (SLC2A4), MCT1 (SLC16A1), MCT4 (SLC16A3) and CD147 (BSG) mRNA expression in a larger series of pRCCs.

To the best of our knowledge, our study is the first to assess the co-expression of GLUT1, GLUT4, MCT1, MCT4 and CD147 in pRCC tumours. We observed that the glycolytic markers GLUT1, MCT4 and the chaperon CD147 were highly expressed in pRCCs and may play an important role in the metabolic remodelling of pRCCs, as observed in other types of tumours (Izumi et al., 2011; Pertega-Gomes et al., 2011, 2015; Pinheiro et al., 2011, 2012, 2014; Baek et al., 2014; Ohno et al., 2014; Zhu et al., 2014). The high number of cases showing membrane expression (the cellular compartment where those proteins are thought to exert their function) of such markers, indicates a significant role of glycolysis in pRCC, thus supporting their functional role in the metabolic remodelling of pRCC

cells (Gould and Holman, 1993; Halestrap and Price, 1999).

Interestingly, we observed a positive correlation between GLUT1 expression (membrane and mRNA), and MCT1 and MCT4 expression (membrane and mRNA), in Porto and TCGA series, respectively. These data substantiate the likelihood of increased glycolytic rate in pRCCs (assuming it as an indicator of increase of glycolysis rate), which is probably due to concurrent expression of GLUT1 and MCT4, since MCT1 was expressed in a smaller subset of cases (see below). Nonetheless, further studies are needed to validate this assumption. The lack of correlation observed between membrane GLUT4 and MCT1 and MCT4 expression, suggests that GLUT4 may not have a major role in the glycolytic phenotype in pRCCs.

We also observed a correlation between membrane CD147 and membrane MCT1 and MCT4, in Porto series, confirming that the role of CD147 as a chaperon of MCT1 and MCT4, being important for their plasma membrane localization in RCCs (Kim et al., 2015), as described in other models (Pinheiro et al., 2008, 2009; Le Floch et al., 2011; Pertega-Gomes et al., 2011; Choi et al., 2014; Simões-Sousa et al., 2016).

In Porto series, we observed that membrane GLUT1 and MCT1 mIRS mean levels were significantly higher in pRCC2 than in pRCC1. Remarkably, GLUT1 and MCT1 mean mRNA expression levels in the TCGA series, were also significantly higher in pRCC2 than in pRCC1. Our results suggest that pRCC2 are more glycolytic than pRCC1, and that GLUT1 and MCT1 are probably the glucose and lactate transporters, which most contribute to these differences. This needs to be functionally confirmed, since it has been advanced that MCT1 are mainly involved in lactate influx and MCT4 in lactate efflux, meaning that the MCT4 isoform is a better marker for increased glycolysis in tumour cells (Witkiewicz et al., 2012). However, in our series this seems not to be true, since MCT1 positive cells were also MCT4 positive.

To confirm our assumptions, it would be interesting to include the Fludeoxyglucose (FDG) uptake data, by Positron emission tomography (PET) with 2-deoxy-2-[fluorine-18] fluoro-D-glucose (18F-FDG) integrated with computed tomography (18F-FDG PET/CT), but this test is not commonly used in the routine in our hospital at the time of collection of our series. However, data from other studies indicate, without specifying the subtype of tumour (i.e., pRCC1 or pRCC2), that pRCCs usually display higher standardized uptake value (SUVs) of 18F-FDG PET/CT (Yamasaki et al., 2011; Takahashi et al., 2015). Interestingly, overexpression of GLUT1 and MCT1 were also observed in other models and associated to adverse prognosis. (Pinheiro et al., 2011, 2015, 2016).

Despite the small number of cases, all pRCC2 cases displayed GLUT1 membrane expression, and most of them MCT1 expression. The fact that only about 9% of pRCC1 and more than 83% of pRCC2 expressed MCT1 in the plasma membrane leads us to suggest that membrane MCT1 expression may be a pRCC2 useful biomarker. Moreover, MCT1 may be a putative predictor of increased pRCC aggressiveness, since it was significantly associated with increased tumour grades, irrespectively of the subtype (Hong et al., 2016).

An interesting finding was that all pRCC2 tumours displayed GLUT1 membrane expression, and 5 out of 6 expressed MCT1, MCT4 and CD147 (data not shown) suggesting a marked metabolic shift in this pRCC subtype. If confirmed in a larger series of pRCC2 cases, this metabolic phenotype (GLUT1, MCT1, MCT4 and CD147 co-expression) may substantiate the therapeutic

Table 8. Correlation between clinical-pathological features and mIRS and mRNA expression of GLUT1, GLUT4, MCT1, MCT4 and CD147 in pRCC by subtype: A - Porto series; B - TCGA series.

	pRCC				
A) Protein/ Parameter	GLUT1 p | r	GLUT4 p | r	MCT1 p | r	MCT4 p | r	CD147 p | r
Age	NS	NS	NS	NS	NS
Gender	NS	NS	NS	NS	NS
pT	NS	NS	NS	0.058 | 0.268	NS
pN	0.042* | 0.285	NS	NS	NS	NS
pM	NS	NS	NS	NS	NS
Fuhrman Nuclear Grade	<0.001** | 0.507	NS	0.033* | 0.300	NS	0.017* | 0.333
B) mRNA/ Parameter	GLUT1 p | r	GLUT4 p | r	MCT1 p | r	MCT4 p | r	CD147 p | r
Age	NS	NS	NS	NS	0.018* | 0.187
Gender	0.003* | 0.233	0.002* | -0.239	NS	NS	<0.001** | -0.283
pT	<0.001** | 0.365	NS	<0.001** | 0.281	0,048* | 0,155	NS
pN	0.001** | 0.237	NS	NS	NS	NS
pM	NS	NS	NS	NS	NS
Fuhrman Nuclear Grade	0.014* | 0.226	NS	0.014* | 0.226	NS	NS

p, based on Spearman's rank correlation test; r, correlation; NS, no statistical significance; *p≤0.05; p ≤0.001.

targeting of lactate transporters. Interestingly, the combined administration of biguanidines (an inhibitor of the mitochondrial complex I) and MCT1, MCT4 or CD147 inhibitors was shown to cause a synergistic anti-cancer effect in tumours co-expressing MCT1, MCT4 and CD147 (Hong et al., 2016; Selwan et al., 2016).

It is important to point out that in the Porto series we evaluated protein expression (IHC), while in the TCGA series mRNA expression was assessed. Although a relationship between mRNA and protein expression has been identified for MCT1, in MCT4 this correspondence is less likely to occur due to post-translational modification (PTM) mechanisms. In fact, Bonen and collaborators (2000), found a significant correlation between MCT1 protein and mRNA levels, in rat hindlimb muscles, but did not observe any relationship between MCT4 protein and mRNA levels (Bonen et al., 2000). Similar results were observed in breast tumours and cell lines by Hong and collaborators (Hong et al., 2016).

We assessed, for the first time, the correlation between the expression levels of GLUT1, GLUT4, MCT1, MCT4 and CD147 in pRCC1 and pRCC2, and clinical and pathological features accepted with prognostic significance in pRCC (Alomari et al., 2015; Cornejo et al., 2015; Moch et al., 2016).

The association of GLUT1 expression with higher grade tumours (G3-4) and the presence of nearby lymph nodes involvement (pN) in both series, (also with higher T stage, in TCGA series), together with the association of MCT1 expression with higher grade tumours (G3-4) in both series (also with higher T stage in TCGA series), suggests that GLUT1 and MCT1 may be markers of tumour aggressiveness and thus potential prognostic biomarkers in pRCCs, as suggested in other tumours types (Pinheiro et al., 2008a,b, 2011, 2014; Izumi et al., 2011; Pertega-Gomes et al., 2011, 2015, Baek et al., 2014; Ohno et al., 2014; Zhu et al., 2014).

Interestingly, pRCC2 tumours showed characteristics of poor prognosis (data not shown), as well as higher expression of GLUT1 and MCT1, when compared with pRCC1 tumours, reinforcing the hypothesis that GLUT1 and MCT1 can serve as biomarkers for tumour aggressiveness. Remarkably, MCT1 membrane expression was found only in 4 pRCC1 cases, 3 of them being high grade tumours (G3). Our results confirm the data previously published in other human tumour models, where MCT1 expression associates with poor prognosis (Pinheiro et al., 2008a,b, 2009, 2011, 2015, 2016; Rademakers et al., 2011; de Oliveira et al., 2012; Mogi et al., 2013; Granja et al., 2015,).

Similarly to GLUT1 and MCT1, CD147 expression was also higher in higher grade tumours, in the Porto series. The association of CD147 expression with an aggressive phenotype, such as increased proliferation, migration, invasion, metastization and poor survival, has already been described in other models (Dang et al., 2008, Rademakers et al., 2011, Huang et al., 2013, 2014a,b, Monteiro et al., 2014) and also with poor prognosis and decreased overall survival in RCC (Jin et al., 2006, Han et al., 2010, Sato et al., 2013). In ccRCC, CD147 overexpression correlated with high Fuhrman nuclear grade, presence of necrosis and larger tumour size (Kim et al., 2015). In our series, we also found an association between MCT4 expression and necrosis (data not shown). Since pRCCs often display extensive areas of necrosis, it would be interesting to relate the presence of these necrotic areas with the metabolic shift of the tumour cells. In fact, these necrotic hypoxic regions are associated with altered cellular metabolism (Bertout et al., 2008).

High MCT4 expression was also higher in tumours with higher pT stages. High levels of MCT4 expression in tumour cell membrane were reported to be associated with increased cellular motility and invasive potential in in vitro models of breast and lung cancer (Gallagher et al., 2007, Izumi et al., 2011) and were associated with poor prognosis in several human cancer models (Pinheiro et al., 2008a,b; Pertega-Gomes et al., 2011, 2015; Martins et al., 2013; Bovenzi et al., 2015) including RCC (Kim et al., 2015).

In the Porto series, we observed that, independently of the tumour type, 2 tumours co-expressing GLUT1, MCT4 and CD147 presented with synchronous metastasis and 2 other tumours co-expressing GLUT1, MCT1, MCT4 and CD147 developed metachronous metastasis, in line with the recent meta-analysis led by Bovenzi and collaborators (2015), who concluded that the increased MCT4 expression in cancer cells was associated with decreased overall survival in various cancers such as breast, colorectal, hepatocellular, pancreas and oral squamous cell carcinoma (Bovenzi et al., 2015). Furthermore, Kim and collaborators (2015) verified that the co-expression of elevated MCT4 and CD147 was associated with poor prognostic parameters and that the co-expression of MCT1, MCT4 and CD147 predicts tumour progression in ccRCC (Kim et al., 2015).

Our data supports the idea that pRCCs tumours are glycolic tumours, and that this glycolytic phenotype plays a role in tumour aggressiveness. This hyper glycolytic phenotype in pRCC, is mainly supported by the increased expression of the glucose transporter GLUT1 and the lactate transporters MCT1 and MCT4, being exacerbated in pRCC2 tumours. Although further studies are needed, we have the indication that pRCC2 in general, and pRCC1 tumours with MCT1 expression should be looked at with greater attention.

It would be interesting to relate the expression levels of the markers used in this study, with the genetic alterations of the tumours, namely those in *MET* oncogene, *succinate dehydrogenase* (*SDH*) genes and *fumarate hydratase* (*FH*). In fact, we assessed the expression of c-MET in a subset of our tumour series, and both pRCC1 and pRCC2 tumours displayed similarly high levels of c-MET protein expression (data not shown), as previously described (Yin et al., 2015).

Our study provides novel evidence for the

involvement of GLUT1 and MCT1/MCT4 in pRCCs, which seem more evident in pRCC2, and thus may help in evaluating the metabolic status in pRCC subtypes. Differences in MCT1 expression between pRCC subtypes have not yet been reported, but a larger number of tumours are warranted to validate our results. We also believe that the study of complementary markers may help to clarify the metabolic status of pRCC, providing a more comprehensive picture of the metabolic pathways involved in pRCC progression.

Acknowledgements: Almeida, LMCA is financed by the FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Inovação, Grant - FRH/BD/87294/2012.
This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Inovação in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274).
Conflicts of Interest: The authors declare no conflicts of interest.

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Accepted December 28, 2016