Original Article

Multidrug Resistance Proteins in Renal Cell Carcinoma

(renal cell carcinoma / immunohistochemistry / P-glycoprotein / multidrug resistance-associated protein / lung resistance-related protein)

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Abstract. A large number of renal cancer patients show poor or partial response to chemotherapy and the precise mechanism has not been understood yet. MDR is the principal mechanism by which many cancers develop resistance to chemotherapeutic drugs and is associated with the elevated expression of MDR proteins. These are divided into two groups: ABC transporters and non-ABC transporters. The aim of our study was to determine the expression of MDR1/Pgp, MRP1 and LRP in 47 samples of renal cell carcinomas using immunohistochemical assay. Our results were analysed in relation to nuclear grade and other clinical and pathological parameters to see the possible correlation between the expression of MDR proteins and factors mentioned above. The majority of renal carcinoma specimens showed positivity for MDR proteins. In this regard, 21 % of samples revealed positive results for MDR1, 62 % for MRP1 and 76.6 % for LRP protein. Furthermore, our study displayed significant differences between MDR1, LRP and nuclear grade. On the other hand, no association was found between MRP1 and nuclear grade, as well as between the expression of three MDR proteins and other clinically relevant parameters.

Received: August 4, 2008. Accepted: November 27, 2008.

This study was supported partly by the Slovak Science and Technology Assistance Agency under the contract No. APVT-20-012104 and partly by VEGA 1/0388/08 and VEGA 1/3253/06.

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Abbreviations: DAB – 3.3'- diaminobenzidine tetrahydrochloride, LRP – lung resistance-related protein, MDR – multidrug resistance, MDR1/Pgp – P-glycoprotein, MRP1 – multidrug resistance-associated protein, MVP – major vault protein, RCC – renal cell carcinoma.

Introduction

The incidence of renal cell carcinoma (RCC) has been increasing steadily over the past decades. The diagnostic trend is mainly due to the widespread use of non-invasive abdominal imaging procedures, which detect incidental renal lesions (Pantuck et al., 2001). The majority of these incidentally detected tumours are at low stages and low grades and are amenable to curative surgical treatments; therefore, they carry a good prognosis (Tsui et al., 2000; Patard et al., 2002). However, a stable proportion of 20 % to 30 % of patients still present with metastatic disease, and 20 % to 30 % of the patients who undergo curative surgery will develop metastatic disease during follow-up (Zisman et al., 2002). RCC is characterized by poor prognosis because of its late presentation and/or high degree of intrinsic or acquired resistance to chemotherapy (Buzaid and Todd, 1989).

It is well known that renal cancer patients often show poor or partial response to chemotherapy and the mechanism is only partially explained. Multidrug resistance is the principal mechanism by which many cancers develop resistance to chemotherapeutic drugs. It affects patients with a variety of blood cancers and solid tumours, including breast, ovary, lung and gastrointestinal tract cancers. The resistance to therapy has been correlated to the presence of three molecular "pumps" that actively expel chemotherapeutics out of tumour cells: P-glycoprotein (MDR1/Pgp), multidrug resistance-associated protein (MRP1), and lung resistance-related protein (LRP) (Meijer et al., 1999; Hinoshita et al., 2000). In this regard, the most typical efflux pump in the cell membrane is represented by MDR1 transporting various xenobiotics out of cells by using ATP. Indeed, MDR1 is associated with resistance to anthracyclines, vinca alkaloids, colchicines, epipodophyllotoxins, and paclitaxel (Avedano and Menendez, 2002).

The next efflux pump of the mammalian cell membrane is represented by multidrug resistance-associated proteins (MRP) (Cole et al., 1992). These two men-

tioned resistant proteins belong to the ABC superfamily, which contributes to drug resistance via ATP hydrolysis. The structural similarities between MRP1 and MDR1 are parallel by an overlap in their drug resistance spectra, although taxanes are a notable exception as they are poor substrates for MRP1. Substrates of MRP1 include organic anions such as methotrexate. Non-anionic compounds may be transported as glutathione, glucuronide, or sulfate conjugates, or may be co-transported with glutathione without conjugation (Loe et al., 1998). Although frequently included in discussions of transporter-mediated resistance, lung resistance protein (LRP) is not an ABC transporter but is a major vault protein (MVP), found in the cytoplasm and nuclear membrane. It is thought to drive drugs away from the nucleus. The expression of LRP significantly correlated with *in vitro* resistance to various structurally and functionally unrelated drugs, including doxorubicin, etoposide, cisplatin, carboplatin, and melphalan (Izquierdo et al., 1996).

Our study was undertaken to investigate the expression of MDR1/Pgp, MRP1 and LRP/MVP proteins in renal cell carcinoma tissue samples. Furthermore, we decided to find out whether the grading of individual patients influences the expression of these multidrug resistance proteins in tumour specimens.

Material and Methods

Clinical samples: 47 samples of renal cell carcinoma were obtained from the Department of Pathological Anatomy, P. J. Šafárik University in Košice, Faculty of Medicine. All our patients were untreated by chemotherapy prior to the collection of samples. The samples were divided according to the histopathological type into two groups: 1. conventional type of RCC (34 samples of clear-cell type), and 2. other type of RCC (5 papillary type, 3 chromophobe type, 1 sarcomatoid type, 1 multilocular cystic type and 3 unclassified type). Patients and tumour characteristics are summarized in Table 1. All these samples were immunohistochemically analysed for MDR1/Pgp, MRP1 and LRP. We have distinguished four categories of quantity of these proteins: 3+ = high level (91–100 % of positive cells), 2+ = medium level (11–90 % of positive cells), 1+ = low level (up to 10 % of positive cells), – = negative cells (0 % of positive cells). For statistical analysis we considered as positive only samples with high-level [3+] and medium-level [2+] protein expression. Samples scored as [1+] or [–] were considered negative.

Table 1. Tumour characteristics of patients

Characteristics		No
All patients		47
Sex	Male	14
	Female	33
Age	≤ 50	10
	≥ 51	37
Histo-pathol. type	RCC – clear-cell type	34
	RCC – papillary type	5
	RCC – chromophobe type	3
	RCC – multilocular cystic type	1
	RCC – sarcomatoid type	1
	RCC – unclassified type	3
Nuclear grade (Fuhrman)	1	14
	2	21
	3	5
	Unknown	7
Pgp (MDR1)	Positive	10
	Negative	37
MRP1	Positive	29
	Negative	18
LRP	Positive	36
	Negative	11
Upper urinary tract	Without malign tumour lesions	45
	With malign tumour lesions	2
Structures of hilum	Without malign tumour lesions	40
	With malign tumour lesions	7

Immunohistochemical detection of MDR1, MRP1 and LRP: We used the indirect enzymatic immunohistochemical method. Formalin-fixed, paraffin-embedded tissue blocks were cut (7 µm) and attached to slides. The slides were processed for immunohistochemistry. Tissue sections were deparaffinized with xylene and rehydrated in decreasing ethanol to water concentrations. The slides were finally washed in phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tw), pH 7.6. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 30 min at room temperature. According to the analysed protein, sections were pretreated in citrate buffer solution in the microwave oven differently. The slides stained for MDR1 and LRP were pretreated in the microwave 2 x 5 min, MRP1 slides for 20 min. MDR1 and LRP staining procedure continued by blocking non-specific staining with milk buffer (5% dry milk in TRIS buffer) for 30 min at room temperature. In case of MRP1, blocking serum was omitted. The next step was application of primary antibodies. We used the following monoclonal antibodies: mouse anti-MDR1, clone C219 (Signet Laboratories, Inc., Dedham, MA), mouse anti-MRP1, clone MRPm6 (Chemicon International, Inc., Temecula, CA) and mouse anti-LRP, LRP56 (BD Transduction Laboratories, San Diego, CA). Primary antibodies were applied overnight in a humified chamber at 4 °C. After rinsing in PBS-Tw (3 x 5 min) the sections were subsequently incubated with the secondary antibodies: prediluted biotinylated horse antibody for MDR proteins (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. The slides were washed with PBS-Tw and submitted to application of peroxidase-conjugated streptavidine: prediluted R.T.U Vectastain for MDR proteins (Vector Laboratories) for 30 min at room temperature. The sections stained for MDR proteins were then visualized with 3.3'-diaminobenzidine tetrahydrochloride (DAB) at a concentration of 0.5 mg/ml in Tris buffer, pH 7.6, and

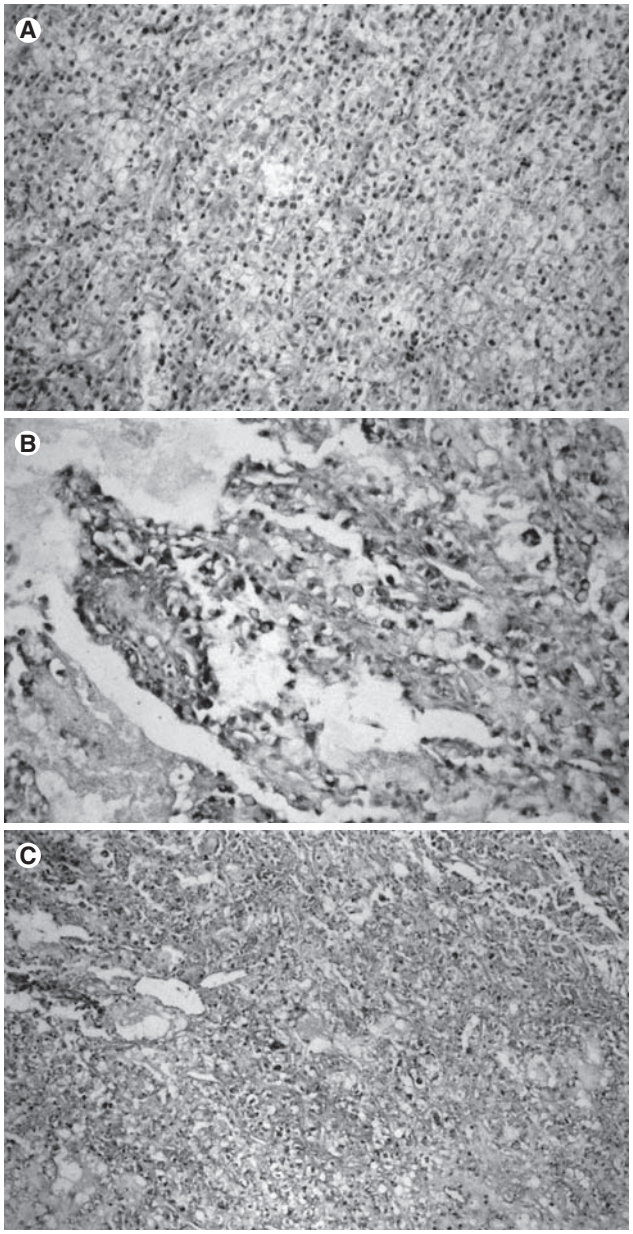

Fig. 1. MDR protein expression in RCC - conventional type was detected immunohistochemically employing monoclonal antibodies. For detection of MDR1 protein we used monoclonal antibody C 219 (A), monoclonal antibody MRPm6 for MRP1 (B), and monoclonal antibody LRP 56 for LRP protein (C).
Magnification: A – 10×10, B – 10×10, C – 4×10

0.015% H_2O_2. Slides were stream-rinsed with tap water, counterstained with haematoxylin for 2 min, washed in tap water, dried, mounted and coverslipped. Sections processed with omission of primary antibody served as a negative control of the immunohistochemical procedure.

Statistical analysis. Statistical evaluation was performed using χ^2 test. The value of $P < 0.05$ was considered to be significant.

Results

Using immunohistochemical assay we have analysed MDR1, MRP1 and LRP protein expression in 47 RCC samples (Table 1). In the renal carcinoma cells, we detected intracellular cytoplasmic localization of MDR1 (Fig. 1A). The MRP1 protein showed a similar cytoplasmic staining pattern (Fig. 1B). Furthermore, the LRP protein was also expressed in the cytoplasm close to the nuclear membrane (Fig. 1C).

MDR1/Pgp protein expression showed positivity in nine (26.5 %) cases of conventional and/or clear-cell RCC (first group) samples. The remaining samples of the first group did not show any MDR1 expression. Moreover, in other type of RCC (second group) samples only one case (8 %) revealed MDR1/Pgp positivity and the rest were considered as negative. Taken together, 10 cases or 21 % of RCC samples showed MDR1/Pgp positivity. On the other hand, MRP1 and LRP immunopositivity was observed in 21 (61.5 %) and 26 (76.5 %) cases of the first group and in eight (62 %) and 10 (77 %) cases of the second group of samples, respectively. In total, 62 % of samples showed MRP1 and 76.6 % LRP immunopositivity. For more details concerning the expression of MDR1, MRP1 and LRP see Table 2.

Comparison of MDR1, MRP1 and LRP expression with nuclear grade (grading) of RCC. Nuclear grade, a clinically relevant predictor parameter, was determined and compared with MDR1, MRP1 and LRP expression. The comparison of this parameter was evaluated in 47 clinical samples of all histo-pathological types of RCC.

A statistically significant difference was found in nuclear grade and MDR1 protein expression ($P < 0.01$). The number of samples with positive MDR1 expression decreased progressively with the increasing nuclear grade.

Table 2. Various levels of MDR1, MRP1 and LRP proteins in 47 samples of RCC tissue: 34 cases of conventional type (clear-cell) RCC and 13 cases of other type RCC

Quantity of expression	MDR1		MRP1		LRP	
	clear-cell	*other type*	*clear-cell*	*other type*	*clear-cell*	*other type*
3+	0 (0 %)	0 (0 %)	12 (35 %)	4 (31 %)	14 (41.2 %)	7 (54 %)
2+	9 (26.5 %)	1 (8 %)	9 (26.5 %)	4 (31 %)	12 (35.3 %)	3 (23 %)
1+	9 (26.5 %)	2 (15 %)	6 (17.5 %)	3 (23 %)	5 (14.7 %)	1 (7.7 %)
-	16 (47 %)	10 (77 %)	7 (21 %)	2 (15 %)	3 (8.8 %)	2 (15.3 %)
Number of positive samples	9 (26.5 %)	1 (8 %)	21 (61.5 %)	8 (62 %)	26 (76.5 %)	10 (77 %)
Number of negative samples	25 (73.5 %)	12 (92 %)	13 (38.5 %)	5 (38 %)	8 (23.5 %)	3 (23 %)

Table 3. Number/percentage of patients with positive and negative expression of MDR1/Pgp, MRP1 and LRP protein compared to different grading. Statistical analysis (χ^2 test)

MDR (number)	nuclear grade 1	nuclear grade 2	nuclear grade 3	unknown	χ^2 test
Pgp+ (10)	4 (40 %)	4 (40 %)	1 (10 %)	1 (10 %)	P < 0.01
Pgp- (37)	10 (27 %)	17 (45.9 %)	4 (10.8 %)	6 (16.2 %)	
MRP1+ (29)	9 (31.03 %)	13 (44.83 %)	3 (10.35 %)	4 (13.79 %)	P > 0.05
MRP1- (18)	5 (27.8 %)	8 (44.4 %)	2 (11.1 %)	3 (16.7 %)	
LRP+ (36)	9 (25 %)	18 (50 %)	4 (11.1 %)	5 (13.9 %)	P < 0.001
LRP- (11)	5 (45.4 %)	3 (27.3 %)	1 (9.1 %)	2 (18.2 %)	

Expression of MDR1 was positive in 40 % of grade 1, in 40 % of grade 2, and 10 % of grade 3 cases.

In comparison of LRP expression and nuclear grade we also found a statistically significant difference ($P < 0.001$). The number of samples with positive expression of LRP grew progressively with the increase of tumour grade. In LRP expression, 25 % of grade 1, 50 % of grade 2, and 11.1 % of grade 3 tumours were detected to be positive.

We did not find any statistically significant differences between MRP1 expression and nuclear grade ($P > 0.05$). The exact number of tissue tumoral samples and statistical correlations are shown in Table 3.

Discussion

The value of classical and modern prognostic factors in renal clear-cell carcinoma has been widely reported in the literature (Fukuda et al., 1998; Campbell, 2006). From this point of view, tumour stage is the most important independent prognostic factor. For each given tumour stage, the nuclear grade is the most reliable additional independent prognostic factor predicting patient survival (Campbell, 2006). Other potential prognostic factors, which are related to the patient (sex, age, serologic parameters), have lesser or no importance (Fukuda et al., 1998). Although the role of MDR proteins in the pathogenesis of drug resistance is clear, their role as a prognostic factor in RCC remains doubtful.

Considering RCC, there are still controversies about the use of particular markers as prognostic factors. Having outlined the difficulties in detecting MDR1/Pgp in cancer cells, it is not surprising that our knowledge about the expression of MDR1 protein from sample to sample may be incomplete. Despite this fact, cancers considered as primarily chemoresistant, such as renal cells, adrenocortical, colon, and hepatocellular cancers, have been shown to consistently demonstrate expression of MDR1 (Fojó et al., 1987). In our study, MDR1 expression was detected in 21 % of RCC tissue samples only. The statistical evaluation of immunohistochemical checking has been compared with patients and tumour characteristics. There was no statistically significant correlation between MDR1 expression and sex, age and histo-pathological type (data not shown). On the other hand, we have obtained a statistically significant difference between MDR1 expression and nuclear grade ($P < 0.01$). Indeed, the number of MDR1-positive renal carcinomas decreased with the growing of nuclear grade. Contrary to our results, Mignogna et al. (2006) have not found any correlation between MDR1 expression and nuclear grade. They only showed the highest Pgp protein expression in patients who died from RCC (Mignogna et al., 2006). These results indirectly confirmed those reported by Duensing et al. (1994) describing longer progression-free survival in patients with no or very few MDR1-positive tumour cells compared to the group of patients with higher MDR1 positivity. Many experimental evidences prove that MDR1, together with p53, plays a decisive role in chemoresistance (Bush and Li, 2002; Sakeda et al., 2002). The relationship between MDR1 and p53 is conditional, i.e. dependent on the cellular environment and drug used. Mutation of p53 induces MDR-1 promoter transactivation, resulting in an increased resistance to chemotherapy and radiation (Sampath et al., 2001).

The multidrug resistance proteins MRP1 and MRP2 are two integral membrane glycoproteins belonging to the ATP-binding cassette superfamily of transporters (Paulusma et al., 1996). The substrate specificities of MRP1 and MRP2 are similar. Despite the fact that both proteins share only 49% amino acid specificity (Keppler and König, 1997), MRP1 was found to be overexpressed in many types of human malignancies, e.g. ovarian carcinomas (Arts et al., 1999), acute myeloid leukaemia (Legrand et al., 1998), and human lung carcinomas (Rybárová et al., 2004). In normal renal tissue, MRP1 is localized in epithelial cells of proximal tubules. Basolateral localization of the protein is important for efflux of its substrates into the blood (Sarkadi et al., 2004). In our study, positive immunostaining of MRP1 was observed in 62 % of tissue samples, but MRP1 expression did not correlate with the nuclear grade of RCC. MRP1 expression correlated significantly with the survival of patients, indicating a prognostic value. These results are in accordance with investigations on other adult carcinomas, e.g. of the breast, lung and endometrium (Nooter et al., 1997; Oshika et al., 1998; Koshiyama et al., 1999). The only paper demonstrating MRP2 expression in RCC was published by Schaub et al. (1999), who showed its expression in 95 % of renal clear-cell patho-histology instances. In any case, our study is the first to show the expression of MRP1 in RCC tissue. Many recent studies have shown that p53 is also involved in the regulation

of MRP1. These findings demonstrated that wild-type p53 repressed endogenous *MRP1* gene expression at the mRNA and protein levels. The expression was lost with p53 inactivation (Wang and Beck, 1998; Hait and Yang, 2006).

The major vault protein LRP was studied in several human malignancies. Its expression is associated with the resistance to various anticancer drugs including melphalan, which increases the interest for the clinical outcome in patients with multiple myeloma (Filipits et al., 1999). Previously, the expression of LRP was studied in normal as well as in human renal cancer samples (Izquierdo et al., 1996). Moreover, the LRP protein was strongly expressed in urothelial carcinomas of renal pelvis and ureter, particularly in well-differentiated carcinomas (Kong et al., 2004). In this regard, Kong et al., (2004) have determined that the expression of LRP inversely correlated with nuclear grade in urothelial carcinomas. The analysis of LRP expression in our set of renal carcinomas revealed about 77% positivity in conventional as well as other type RCC. Next, we have revealed a statistically significant difference between LRP expression and nuclear grade ($P < 0.001$). Consequently, our results show that LRP-positive renal carcinomas increased from grade 1 to grade 2 in contrast to LRP-negative samples, which decreased from grade 1 to grade 3.

In conclusion, our data demonstrate different expression of three multidrug resistance proteins (MDR1/Pgp, MRP1 and LRP) in a representative group of RCC samples. The total number of MDR1 positively staining tumours was much lower in comparison with MRP1- and LRP-positive samples. This work will serve as the basis for our next experiments focused on the exact role of other mechanisms involved in the regulation of drug resistance.

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