Expression of the Multiple Drug Resistance Gene in Human Renal Cell Carcinoma Depends on Tumor Histology, Grade, and Stage

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ABSTRACT

Levels of mRNA expressed by the multidrug resistance gene MDRI were examined in 23 renal cell carcinoma samples and adjacent normal kidney cortex using reverse-transcription PCR. Comparison of MDRI levels between histological types revealed that there was on average significantly more MDRI in clear cell tumors than in oncocytomas (0.89 ± 0.10 versus 0.28 ± 0.20, ratio of MDRI in tumor cells to the drug-resistant cell line KB-8, P < 0.05). The mean MDRI level of all of the non-oncocytoma tumors was not significantly different from the mean MDRI level of normal adjacent kidney (0.89 ± 0.10 versus 1.11 ± 0.12, P = 0.07). However, the mean MDRI level of the more undifferentiated clear cell tumors was significantly lower than the mean MDRI level of adjacent normal kidney (0.74 ± 0.10 versus 1.11 ± 0.12, P < 0.05). MDRI levels in early stage, clear cell tumors (n = 14) were lower than in tumors that had spread into perinephric tissue or had metastasized (n = 6) (0.77 ± 0.08 versus 1.24 ± 0.30, P < 0.05). In conclusion, MDRI expression decreases in the more undifferentiated tumors, but still remains at levels high enough to be drug resistant. Higher MDRI expression in the invasive tumors compared with noninvasive tumors suggests that MDRI expression and invasiveness may be linked.

INTRODUCTION

P-gp, the product of the MDRI gene, is an ATP-dependent membrane transport pump capable of promoting efflux of a wide variety of lipophilic compounds. In normal human tissues, the highest p-gp levels are found on the luminal surface of the proximal tubules of the kidney, intestinal epithelium, bile duct canalicular cells, pancreas, and endothelial cells of the blood brain barrier (1, 2), where its function is thought to be protection of these cells and organs from as yet unidentified endogenous or exogenous toxins. Tumors with the highest levels of p-gp are those derived from p-gp-rich normal tissues, or those exposed to certain chemotherapeutic drugs such as the anthracyclins and Vinca alkaloids which are substrates for P-gp (3, 4). Overexpression of P-gp is felt to be a major cause of tumor resistance to these drugs by lowering their intracellular concentration (4). In several pediatric and adult malignancies, overexpression of P-gp or MDRI RNA, has been shown to correlate with resistance to chemotherapy and decreased survival (5, 6). Investigators have shown that drugs such as verapamil and cyclosporin which inhibit P-gp can overcome acquired drug resistance in vitro (7) as well as in vivo (8, 9). Tumors that overexpress P-gp are prime candidates for clinical trials combining a chemotherapy drug substrate for P-gp, with such an inhibitor, and many such trials are in progress.

RCC is one of the most drug-resistant tumors and expresses some of the highest levels of P-gp of any tumor (3). Kakemi et al. (10) found that the in vitro sensitivity of RCCs to vincristine was inversely correlated with MDRI RNA levels, while Mickisch et al. (11) showed that R-verapamil could reverse vincristine resistance in such tumors.

In the current study, MDRI levels in primary RCC tumors and adjacent normal kidney cortex were assessed by measuring mRNA levels by rt-PCR to determine whether there was a correlation between MDRI message expression and clinical outcome, or other indirect measures of tumor aggressiveness, such as histological subtype and grade. MDR2, which shares sequence homology with MDRI (12), was also measured by rt-PCR (12). Although the product of the MDR2 gene does not cause known drug resistance, its gene product is known to cross-react with the monoclonal antibody C219 (13), which is the antibody commonly used to assay P-gp levels immunohistochemically. Masking of the gene product of MDR1 by other proteins during immunodetection (14) may make rt-PCR the most accurate measure of the presence of this gene (15).

PATIENTS AND METHODS

Over a 12-month period, patients undergoing elective nephrectomy for RCC were identified through the operative list at three University of Toronto teaching hospitals. A minimum of 1 cm each of tumor and adjacent normal kidney cortex was selected separately immediately after tumor removal by nephrectomy and snap frozen in liquid nitrogen for semiquantitative rt-PCR analysis. Samples were taken, by the pathologist, from within non-necrotic tumor to avoid contamination by the surrounding normal tissue, and larger samples were taken from

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3 The abbreviations used are: P-gp, P-glycoprotein; RCC, renal cell carcinoma; rt, reverse transcription.
larger tumors to be more representative. The remaining kidney and tumor were submitted for routine histologic analysis including pathological staging. Samples were excluded from analysis if they were not confirmed as RCC. The slides were subsequently reviewed by a pathologist (W. H.) and classified as either clear cell with associated Fuhrman grade (higher being less differentiated) or oncocytoma. An investigator blinded to the pathology results reviewed the charts to determine the presence or absence of symptoms on presentation and the clinical tumor stage.

The tissue was pulverized in a bone crusher while frozen. Total RNA was extracted using a standard guanidium-cesium chloride procedure and then quantitated spectrophotometrically using the orcinol reaction (16). PCR was carried out as follows: 200 ng RNA were reverse transcribed using a random hexamer primer. One-half of the cDNA product was coamplified using primers specific for MDR1 (Ref. 17; 167 bp) and β2-microglobulin (Ref. 18; 123 bp, internal control) or MDR2 (147 bp) and β2-microglobulin. The entire PCR product was electrophoresed through a 12% or 15% polyacrylamide gel and stained with ethidium bromide. The gel was photographed using Polaroid type 665 film (positive/negative film), and the negative was scanned by laser densitometry to quantitate the levels of MDR1 and MDR2 expression relative to β2-microglobulin for each tumor and normal kidney sample. PCR results for seven patients are shown in Fig. 1.

Cell lines KB-3–1 (drug sensitive), KB-8, KB-8–5, and KB-8–5–11 (with increasing levels of MDR1 mRNA and all increasingly drug resistant; Ref. 19) were used as standards for calculating the relative MDR1 expression in normal tissue and tumor for each PCR assay. The parental KB-3–1 cell line expresses very low amounts of MDR1 mRNA and is drug sensitive (20). This cell line also represents the first step in selection of multidrug resistance from a drug-sensitive parent. Cell line KB-8–5 and the highly resistant KB-8–5–11 cell line are increasingly drug resistant and have been found to have progressively greater copies per cell of MDR1 mRNA (19).

Patient kidney tumor samples were each assayed three times for MDR1, and on each assay the four KB cell lines were run as standards. The ratio of MDR1 mRNA was calculated relative to that of β2-microglobulin mRNA in each reaction for the normal tissue, tumors, and KB cell lines. This ratio was normalized to the MDR1 expression level in the KB8 cell line, since the expression level of MDR1 in the normal tissue and tumors was similar to the MDR1 expression in KB8. As well, KB8 represents a quantifiable standard of low-level multidrug resistance. Similarly, the ratio of MDR2 mRNA was calculated relative to that of β2-microglobulin mRNA for each reaction, and this ratio was normalized to the expression level of MDR2 in the KB8–5 cell line. The expression level of MDR2 in the tumors and kidney samples was similar to that in the KB8–5 cell line. Since the MDR1 and MDR2 ratios were normalized to different cell lines, these values cannot be compared directly.

Results were expressed as the mean ± SE. Statistical analysis was performed using the SAS program (SAS Institute, Cary, NC). Comparison of MDR1 levels between tumor and normal kidney was performed with unpaired t tests. Comparison of MDR1 levels between tumors divided into groups based on stage, grade, or histology (oncocytoma versus non-oncocytoma) was performed with unpaired t tests. All values are given as mRNA copies per cell ± SEM.

RESULTS

Characteristics of patients, the histological features of the tumors, and the expression of MDR1 in tumor relative to the KB-8 cell line are found in Table 1. Fifteen of the 23 tumors were discovered incidentally on abdominal ultrasound examinations for unrelated reasons. Only one of these had spread and only to local nodes. Of the 23 tumors, 3 were oncocytomas, 2 were granular cell tumors (1 grade 2 and 1 grade 3), and the rest were clear cell tumors. The two granular cell tumors have been included in the analysis as clear cell.

Normal kidney cortex expressed levels of MDR1 averaging 1.11 ± 0.12 (ratio of MDR1 in normal kidney cortex to the cell line KB-8), indicating moderate expression of MDR1 and drug resistance, falling between the drug-resistant cell lines KB-8 and KB8–5. The cell line KB-8–5 expresses MDR1 at a ratio of 1.7 relative to cell line KB-8. Levels of MDR2 were also measured and in all but two cases (4th and 20th patient in Table 1) were closer to MDR2 expression in the KB-8–5 than the KB-8 cell line.

Comparison of MDR1 levels between histological types revealed that there were, on average, higher levels of MDR1 in clear cell tumors than in the three oncocytomas, although the oncocytomas were more differentiated histologically (0.89 ± 0.10 versus 0.28 ± 0.20, ratio of MDR1 in tumor cells to the cell line KB-8, P < 0.05; Fig. 2).

In the non-oncocytoma tumors, there was less MDR1 on average than in adjacent normal kidney cortex, but the difference did not achieve statistical significance (0.89 ± 0.10 versus 1.11 ± 0.12; P = 0.07). However, when the clear cell tumors were further divided into Fuhrman grade 3 (more undifferentiated, n = 12) and Fuhrman grade 2 (more differentiated, n = 8) tumors, the MDR1 level in Fuhrman grade 3 tumors was found to be lower than normal kidney cortex (0.74 ± 0.10 versus 1.11 ± 0.12, P < 0.05). The MDR1 level in Fuhrman grade 2 tumors was not significantly different from adjacent normal kidney (0.91 ± 0.19 versus 1.11 ± 0.12, P = 0.94). A comparison of MDR1 expression in tumors of Fuhrman grades 2 and 3 with adjacent normal kidney cortex is found in Fig. 3.

MDR1 levels in the non-oncocytomas were lower in early stage tumors [T1N0M0,T2N0M0 (21), n = 14] than in those that
Table 1  MDRI gene expression in RCC and adjacent normal kidney

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age (yr)</th>
<th>Symptoms on presentation</th>
<th>Stagea</th>
<th>Tumor grade</th>
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<th>Normal kidney MDRIb</th>
<th>Tumor MDRIb</th>
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<tr>
<td>M</td>
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<td>S</td>
<td>T2</td>
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*TNM Classification.
*Ratio of MDRI vs. KB8 cell line (see "Patients and Methods").
*F. female; M. male; S. symptomatic; A. asymptomatic at presentation; ONCO, oncocytoma; GRAN, granular; C2, clear cell grade 2/4; C3, clear cell grade 3/4.

had spread into perinephric tissue or metastasized tissue (n = 6; 0.77 ± 0.08 versus 1.24 ± 0.30 P < 0.05). At 18 months of follow-up all patients were still alive.

DISCUSSION

Using the highly sensitive technique of rt-PCR, we have found that MDRI levels are lower in poorly differentiated renal clear cell tumors than in normal kidney cortex with intermediate levels of MDRI in the more differentiated tumors. This supports the very similar findings of Kanamaru et al. (22) who measured MDRI RNA using the less sensitive slot blot technique and found that the mean MDRI RNA level was higher in well-differentiated RCCs than in those that were poorly differentiated. These findings suggest that MDRI gene expression in RCC is related to the increased expression in renal proximal tubule cells (22), and thus multidrug resistance is not a property of malignant transformation, but rather is constitutive in normal and malignant renal tubular cells and may be reduced as tumors become less differentiated. In some of the tumors in this study, MDRI levels are too low (Table 1) with ratios well below the KB8 cell line (ratios of 0.11, 0.05, and 0.1) and are unlikely to confer drug resistance. Some are borderline, with ratios around the KB8 cell line. Since the threshold of MDRI values that represents functional drug resistance (15) is unknown, these tumors could all be borderline drug sensitive or resistant. This supports our view that MDRI expression is constitutive and not likely the main drug resistance mechanism of RCC.

A surprising finding was lower MDRI levels in oncocytomas than in clear cell carcinomas, although the oncocytomas were better differentiated. Of note is the report by Rochlitz et al. (23) who found no P-gp in the single oncocytoma in their series. Our study had only three oncocytomas; however, confirmation of this finding in a larger series would support the hypothesis that the cell of origin of oncocytomas may be different from that of clear cell carcinomas (24), which are thought to arise from the proximal renal tubular cells which are the only renal cells rich in P-gp.

The significance of MDRI in RCC is unclear at present. Immunohistochemical staining for P-gp using the C219 antibody recognizes the protein products of MDRI and MDRI, while it is only the protein product of MDRI, P-gp, which is known to confer multidrug resistance in RCC (12, 25). Thus, cross-reactivity of the product of MDRI could potentially confound quantitative analysis of P-gp using immunohistochemical techniques. This does not appear to be a major problem, however, since studies using immunohistochemical staining have reported similar findings to our own (23, 26–28). In a study by Nishiyama et al. (28), P-gp levels measured by immunoblotting with the monoclonal antibody C219 were lower in RCC than in adjacent normal kidney cortex. Rochlitz et al. (23) found that the degree of staining with the C-219 antibody correlated with the histopathological subtypes of RCC, with the more differentiated tumors having a higher expression of P-gp.

In contrast to the findings of Rochlitz et al. (23), who could not demonstrate a correlation between P-gp content and disease stage, we found significantly higher MDRI levels in six tumors that had invaded through the renal capsule or metastasized than in noninvasive tumors. Kanamaru et al. (22) did not find higher MDRI RNA levels in primary RCC tumors from patients with metastatic disease compared to those without metastases. How-
ever, on average, in four pairs of tumors and metastases, there was a trend to higher MDRI in the metastases (22). A positive correlation between tumor stage and MDRI expression has been reported for a variety of tumors (5, 29, 30). The finding of lower levels of MDRI in more undifferentiated RCCs and yet significantly higher levels in more invasive tumors is unexpected. It is possible that alterations in proto-oncogenes or tumor suppressor genes associated with increased biological aggressiveness may lead to activation of MDRI. This relationship has been reported by Chin et al. (31) who found that mutant p53 overexpression activated the MDRI gene in murine NIH 3T3 cells. Schneider et al. (32) found a relationship between P-gp and HER-2/neu in aggressive inoperable mammary carcinomas, but could find no significant association with mutant p53 expression and MDRI activation. Increased expression of MDRI may be one of the genetic events that occurs when the malignant cell develops the potential to metastasize. In cancers such as RCC, which are derived from P-gp-rich tissues, expression of MDRI may perhaps be biphasic, i.e., decreasing as the cells dedifferentiate and increasing again as they develop the ability to metastasize.

Although the MDRI assay by rt-PCR and immunocytochemical assays for P-gp using C219 seem to correlate well with each other when studying patient populations, it is not at all clear which of these methods is optimal for determining the probability of response to P-gp inhibition in an individual patient. rt-PCR was a more sensitive method, and does not suffer from the possibility of MDRI cross-reactivity. Immunocytochemical methods, however, have the advantage of identifying that it is indeed the tumor cells which are staining, allowing assessment of tumor heterogeneity, and also confirm MDRI expression at the protein level as increased P-gp expression may occur without a concomitant increase in the level of mRNA (33).

Our work supports the findings of other authors that P-gp expression in RCC correlates with tumor differentiation with the less-differentiated tumors having lower levels of MDRI mRNA. Clinical drug resistance is universal for RCCs while the level of MDRI is variable, suggesting that the tumor MDRI level and drug resistance may be independent of each other. Attempts to reverse multidrug resistance in clinical studies in RCC or other tumors derived from P-gp-rich tissues have not yet resulted in chemoinsensitivity, and this has been the experience of our group and others (34–37). For tumors where multidrug resistance is first acquired as part of malignant transformation, such as pediatric, hematological, and breast malignancies or in response to exposure to cytotoxic agents, resistance modulation may have an important role, which should be tested.

REFERENCES
Expression of the multiple drug resistance gene in human renal cell carcinoma depends on tumor histology, grade, and stage.

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