Urol Res (1991) 19:99–103

Urological Research


# Cross-resistance patterns related to glutathione metabolism in primary human renal cell carcinoma

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Accepted: September 1, 1990

**Summary.** In 59 cases of primary human renal cell carcinoma (RCC), cross-resistance and collateral susceptibility patterns were determined in an MTT microculture assay. Concomitantly, the glutathione (GSH) content and the enzymatic activity of γ-glutamyl transpeptidase (GGT) were measured as distinct resistance characteristics. Resistance or chemoresponse towards Vinca alkaloids and anthracyclines were found to be highly coincident, suggesting that the classical multidrug resistance mechanism is active in human RCC. Strong resistance to platinum complexes combined with relative sensitivity to bleomycin was significantly associated with elevated glutathione levels, providing evidence for another pathway instigating chemoresistance. In contrast, despite substantial enzymatic activity, GGT effects revealed no correlation to the chemoresistance pattern. This result implies that it is the GSH-linked binding and reduction potential rather than the GGT-associated transportation capacity that has an impact on the expression of chemoresistance in human RCC.

**Key words:** Primary renal cell carcinoma – MTT staining assay – Chemoresistance – Glutathione metabolism

Cross resistance to a variety of chemotherapeutic agents with different cytotoxic actions, termed "multidrug resistance" (MDR) [4], is a frequent feature of solid human tumors [6]. Several mechanisms conferring drug resistance have been identified [17]. Apart from and independently of the so-called "multidrug transporter" [7], which produces MDR by rapid extrusion of antitumorous compounds from the cell, the glutathione metabolism, which owing to its effective reduction potential [12] is involved in the detoxification of antineoplastic substances, seems to contribute to this phenomenon [18].

Clinically, RCCs display a characteristically high degree of intrinsic drug resistance, including those of the MDR phenotype [24]. Normal renal cells already possess a higher GSH content than most other organs, resulting in an increased rate of turnover of GSH and probably GSH-linked cellular intermediates via translocation to GGT and subsequent transportation [8]. In previous studies, referring to a small number of human RCCs, we reported some evidence for the association of high GSH metabolism to doxorubicin resistance in an [$^3$H] uridine uptake assay [14].

The MTT test is a novel method of evaluating metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to purple colored formazan crystals [19]. The crystals are thought to be generated by the mitochondrial enzyme succinate dehydrogenase [22] and can be dissolved and quantified by measuring the absorbance of the resultant solution indicating the number of live cells. By using 96-well microculture plates and a multiwell spectrophotometer this assay can be semiautomated to process a large number of samples and to provide a rapid objective survey for drug [1] and, less widely studied, radiation responsiveness [21] in tumor cell lines. Recently, modifications have been introduced to investigate chemosensitivity in fresh human leukemia cells [20, 27] and this laboratory has described various factors interfering with the optimal application of the test to primary human RCC [13].

The present study determined the cross resistance pattern of 59 primary human RCCs to an array of structurally diverse drugs currently in clinical use and related the results to the enzymatically measured content of GSH and GGT as distinct resistance characteristics.

## Materials and methods

### *Cell cultures*

Representative samples from separate parts of 59 human RCCs were surgically obtained and processed under sterile conditions within 1 h after operation. The tumor segments were carefully freed from fat, connective tissue, and necrotic areas. H&E-stained smears were used as a matter of routine in order to exclude the major admixture of nontumorous material. Single cell suspensions were mechanically

**Table 1.** Degree of chemoresistance in human RCCs as determined by MTT microculture assay

| Drug | Max. conc. | *n* | Highly resistant | Less resistant |
|---|---|---|---|---|
| VBL | 1 μg/ml | 59 | 46 | 13 |
| CIP | 3 μg/ml | 58 | 51 | 7 |
| CAP | 7.5 μg/ml | 34 | 29 | 5 |
| BLE | 1 μg/ml | 40 | 37 | 3 |
| ADM | 3 μg/ml | 35 | 30 | 5 |
| EPI | 3 μg/ml | 35 | 30 | 5 |
| 5FU | 10 μg/ml | 25 | 25 | – |
| DAU | 3 μg/ml | 15 | 15 | – |
| MTX | 1 μg/ml | 12 | 12 | – |
| MMC | 1 μg/ml | 10 | 10 | – |

VBL, vinblastine; CIP, cisplatin; CAP, carboplatin; BLE, bleomycin; ADM, doxorubicin; EPI, epirubicin; 5FU, 5-fluorouracil; DAU, daunomycin; MTX, methotrexate; MMC, mitomycin C

**Table 2.** Cross-resistance patterns in human RCCs with a high degree of intrinsic resistance (numbers taken from Table 1)

| | VBL ($n=46$) | CIP ($n=51$) | CAP ($n=29$) | ADM ($n=30$) | EPI ($n=30$) | BLE ($n=37$) |
|---|---|---|---|---|---|---|
| VBL ($n=46$) | | 45 | 28 | 27 | 27 | 35 |
| CIP ($n=51$) | 45 | | 29 | 26 | 26 | 37 |
| CAP ($n=29$) | 28 | 29 | | 25 | 25 | 29 |
| ADM ($n=30$) | 27 | 26 | 25 | | 30 | 28 |
| EPI ($n=30$) | 27 | 26 | 25 | 30 | | 28 |
| BLE ($n=37$) | 35 | 37 | 29 | 28 | 28 | |

**Table 3.** Collateral susceptibility patterns in human RCCs with a low degree of intrinsic resistance (number taken from Table 1)

| | VBL ($n=13$) | CIP ($n=7$) | CAP ($n=5$) | ADM ($n=5$) | EPI ($n=5$) | BLE ($n=3$) |
|---|---|---|---|---|---|---|
| VBL ($n=13$) | | 4 | 1 | 5 | 5 | 2 |
| CIP ($n=7$) | 1 | | 5 | 3 | 3 | – |
| CAP ($n=5$) | 1 | 5 | | 3 | 3 | – |
| ADM ($n=5$) | 5 | 3 | 3 | | 5 | – |
| EPI ($n=5$) | 5 | 3 | 3 | 5 | | – |
| BLE ($n=3$) | 2 | – | – | – | – | |

prepared, the cells filtered through textile multilayer gauze (pore size 50–100 μm; SSGF) and purified by Ficoll centrifugation (density 1.07, 400 × g, 10 min, 4 °C; Seromed) after addition of 5 mg% DNase (Boehringer Mannheim). Cell viability and content to tumor cells were examined by the trypan dye exclusion test (final concentration 0.16%; Flow) and by H&E-stained smears, respectively. In specimens taken exclusively from well-defined small RCCs (maximum diameter 5 cm), the primary cell suspensions contained approximately 80% viable cells; more than 90% were classified cytologically as tumor cells. Cell suspensions were plated in monolayer flasks (Flow) containing culture medium (RPM1640, supplemented with 20% synthetic NU serum; Flow) and maintained until an exponential growing phase was reached.

### *MTT microculture assay*

Next, $2 \times 10^4$ tumor cells per well were then distributed by multichannel pipettes into 96-well, flat-bottomed microculture plates (Flow) and the MTT test was carried out as reported earlier [13]. Drug concentrations were selected to ensure highest reproducibility in vitro and were also applicable in vivo in transgenic mice carrying the human MDR-1 gene [16]. To reduce the incubation time conveniently to 16 h, maximum concentrations exceeded the peak levels traced in clinical trials and were serially diluted to the therapeutic range. In cases where a drug exposure time of 72 h was preferred (e.g., for vinblastine, bleomycin), 50 μl culture medium was added per well every 24 h to replace evaporated volume during the incubation period. Four replicate wells were used to determine each point. The values were expressed as percentage of the untreated trial groups in order to examine the effects of chemotherapeutic agents.

### *Glutathione metabolism*

Tumor specimens were first mechanically disintegrated (Potter Ellwejn) and then homogenized by ultrasound (Ultraturrax). The protein content was determined from aliquots of the solution using Folin-phenol reagent [11]. The extinction was measured at 578 nm and bovine serum albumin served as a standard. The homogeneous tissue solution was centrifuged at 5,000 × g for 20 min and GGT activity analyzed from aliquots of the supernatant using a certified test kit (Monotest; Boehringer Mannheim) as indicated by the manufacturer. After precipitation of the proteins by perchloric acid (Merck), the total GSH content was examined enzymatically in accordance with Tietze's method [22]. Experiments were run in triplicate; hemolyzed vials were rejected. Values were expressed as n*M*/mg protein (GSH) or U/mg protein (GGT). 1 U is defined as a conversion of 1 n*M* substrate/min.

All reagents were of p.a. grade and were, if not otherwise stated, purchased from Boehringer Mannheim.

Statistical analysis included the Student's *t* test, using the Bonferroni-Holm correction for curves [9].

## Results

With the help of the MTT staining procedure, human RCCs were divided into highly resistant and less resistant carcinomas according to the number of surviving tumor cells in the presence of a variety of anticancer agents (Table 1). A difference was taken to be significant when the *p*-value for the drug concentration point representing a 100-fold dilution of the maximum concentration applied was still less than 0.05, and the 10-fold dilution killed 50% or more of cells [13, 15]. Under these conditions a significant division was detected as from 0.01 μg/ml

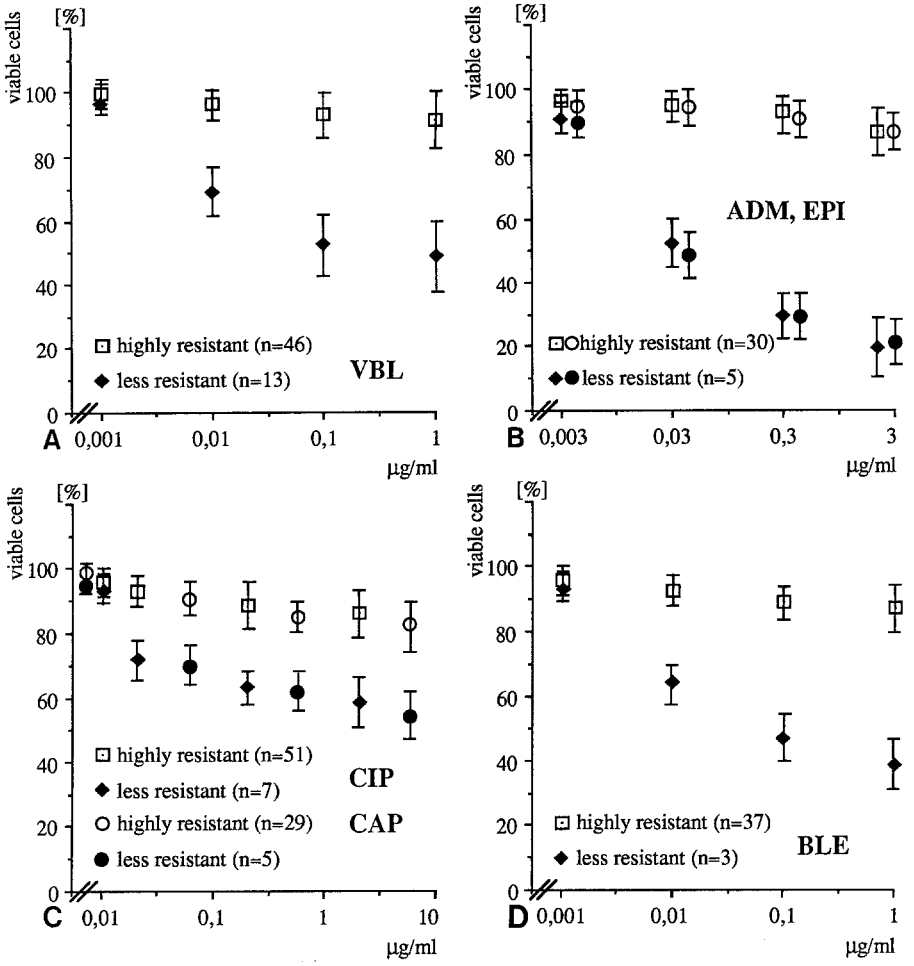


**Fig. 1A–D.** Chemoresistance patterns in human RCC against VBL, ADM/EPI, CIP/CAP, and BLE (data from Table 1; mean at each point ± SD)

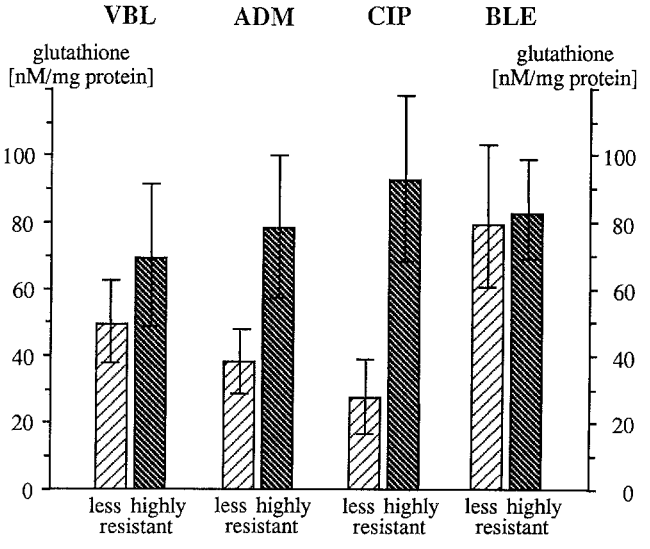


**Fig. 2.** GSH content in homogenized human RCCs related to the chemoresistance patterns (chemoresistance data from Table 1)

vinblastine (VBL) ($p < 0.01$) or 0.1 µg/ml doxorubicin (ADM)/epirubicin (EPI) ($p < 0.05$). Despite apparent differences in the quantity of live cells, the level of significance was not reached in the case of cisplatin (CIP)/carboplatin (CAP) or of bleomycin (BLE) due to the low number of chemoresponders (Fig. 1). Quantitatively, VBL influenced 13 of 59 RCCs (22%) whereas qualitatively ADM/EPI reduced the viable cell fraction most dramatically (Fig. 1).

A complete cross-resistance/collateral sensitivity pattern could only be noted in chemically related drugs like CIP/CAP or ADM/EPI where high and low chemoresistance was found to be coincident (Tables 2, 3]. Low ADM/EPI-resistance was always associated with low VBL resistance (Table 3), but in three tumors refractory to ADM/EPI a response to VBL could be ascertained (Table 2). Additionally, two tumors displayed low chemoresistance to BLE and none to CIP/CAP. Fluorouracil, daunorubicin, methotrexate, and mitomycin C could not be shown to be effective in human RCC (Table 1). Resistance or susceptibility to CIP/CAP obviously occurs independently of VBL activity (Tables 2, 3), whereas all three tumors with low BLE resistance were highly refractory to CIP/CAP (Table 3).

Using the enzymatic method, the average GSH content in homogenized RCC tissue amounted to 81 ± 16 n*M*/mg protein. On distributing the individual values obtained in accordance with the degree of chemoresistance as shown in Fig. 1, high ADM or CIP resistance was associated with a concentration of 80 ± 21 or 92 ± 34 n*M*/mg protein, low resistance with 41 ± 10 or 29 ± 11 n*M*/mg protein (Fig. 2).

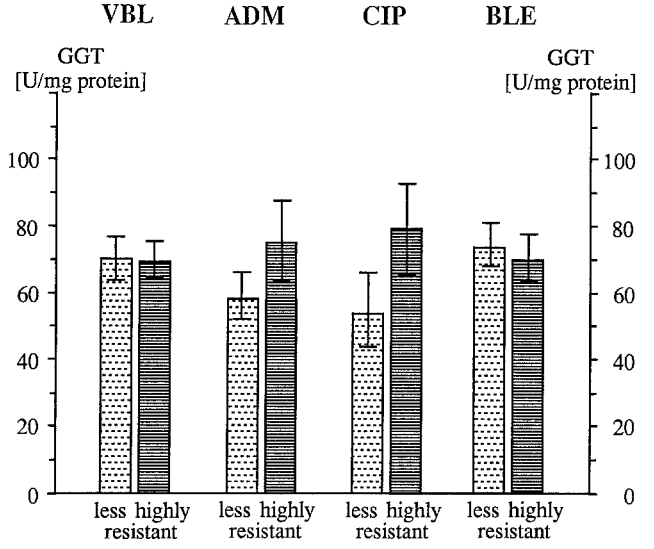


**Fig. 3.** GGT activity in homogenized human RCCs related to the chemoresistance pattern (chemoresistance data from Table 1; 1 U defined as conversion of 1 n*M* substrate/min)

GGT activity in human RCCs reached $69 \pm 12$ U/mg protein. The stratification of the data measured to the level of chemoresistance is depicted in Fig. 3. No difference with reference to in vitro resistance could be demonstrated.

## Discussion

On assessing the chemoresponse of a large number of human RCCs in a novel MTT assay, a high degree of chemoresistance to almost every substance tested could be confirmed (Fig. 1). We are aware of the fact that a definite decision for "high" or "low" resistance on the basis of the number of surviving tumor cells remains somehow arbitrary, as the test conditions may influence the test result; this is a persistent problem in all in vitro chemosensitivity testing systems [26]. However, the procedures described here are aimed at approximating the in vivo situation by using primary cell cultures rather than established cell lines and by adjusting exposure times and drug concentrations ([13]; Materials and methods). This strategy has been successfully applied in human leukemia samples [20, 27].

Furthermore, indirect support may be deduced from clinical studies in which VBL was proved to be the most effective single chemotherapeutic agent in human RCC, resulting in a maximum of 20% remissions [24]. This may correspond to our data, which showed 13 of 59 RCCs to have low VBL resistance (Fig. 1). For none of the other drugs was a similar effect demonstrated (Table 1).

On focussing on the cross-resistance pattern, consistency between anthracyclines like ADM or EPI and Vinca alkaloids like VBL became evident (Tables 2, 3). This finding may reflect the so-called "classical" MDR phenotype of cell lines [4, 17] mediated by the multidrug transporter [7]. In fact, when measuring MDR 1-gene mRNA in 42 RCCs, a clear correlation to ADM resistance was detected [10]. Moreover, our laboratory has reported earlier that the MDR 1-gene product P-170 glycoprotein could be immunohistochemically traced in 72% of 32 highly VBL-resistant tumors [15]. This compiled evidence suggests that MDR plays a crucial part in the expression of chemoresistance in human RCC.

GSH metabolism as a determinant of therapeutic efficacy has, in general, been associated with alkylating agents, anthracyclines, and platinum complexes, since their antineoplastic actions depend partially on oxidative cell damage and may therefore be impeded by GSH-linked reduction potential of the cell [3]. In contrast to the former, GSH is thought to be a prerequisite for BLE antitumor effects, in order to form the active species upon entry into the cell [2, 5]. This phenomenon has been illustrated in three human ovarian tumor cell lines exhibiting collateral susceptibility to BLE whilst being refractory to melphalan, ADM, and CIP [25]. All three RCCs with a low degree of resistance against BLE were noted to be highly resistant against CIP, CAP, ADM, and EPI (Table 2, 3). In addition, an elevated GSH content was coincident exclusively in highly CIP- or ADM-resistant cases (Fig. 2). Hence, it may be assumed that GSH content contributes to the level of chemoresistance against certain drugs in human RCC. However, the fact that GGT activity, although substantial enzymatic effects were measurable in homogenized RCC tissue, could not be related to the degree of chemoresistance (Fig. 3) suggests that the functional role of the GSH metabolism in chemoresistant RCC may be limited to the GSH-linked binding and reduction potential rather than to the GGT-associated transportation capacity.

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