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Involvement of ABC Transporters in Chemosensitivity of Human Renal Cell Carcinoma, and Regulation of MRP2 Expression by Conjugated Bilirubin

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Abstract. *In this study, the involvement of ATP-binding cassette (ABC) transporters in in vitro chemosensitivity of surgically removed human renal cell carcinomas was investigated. The relative expression levels of transporter mRNAs in the renal tumors from 13 patients were similar to those in the surrounding normal kidney tissues. Five renal cell carcinomas cultured successfully in vitro for 14 days showed significantly decreased expression of multi-drug resistance-associated proteins 2 and 6 (MRP2 and MRP6) mRNAs. In vitro chemosensitivity testing of the same specimens using the collagen-gel matrix assay indicated that some anticancer drugs were effective, especially cisplatin, which is an MRP2 substrate. MRP2 mRNA expression in renal carcinoma was significantly increased when cells were cultured in the presence of conjugated bilirubin. In an established renal proximal tubule epithelial cell line (RPTEC), conjugated bilirubin increased MRP2 expression at the mRNA and protein levels, and decreased the cisplatin sensitivity of the cells. These results indicate that MRP2 expression in renal cell carcinoma may be regulated by conjugated bilirubin in the body and decreased during in vitro culture. Thus, the effectiveness of anticancer drugs selected on the basis of in vitro chemosensitivity testing of clinical cancers may be overestimated.*

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Several *in vitro* chemosensitivity assays have been developed to assess the sensitivity of human tumor tissues to various anticancer drugs. An organ culture system using the collagen-gel matrix (CGM) assay has been developed as a useful method to determine *in vivo*-like responses of tumor tissues to anticancer drugs (1, 2). Oyama *et al.* (3) and Furukawa *et al.* (4) have established a rapid and convenient *in vitro* chemosensitivity assay by modification of the CGM assay. The reliability and utility of the *in vitro* chemosensitivity assay have been evaluated for several human tumor specimens, and a positive correlation between sensitivity and clinical response has been reported (3-10). However, this method does not completely reproduce the *in vivo* environment for cancer cell growth and unexpected results, such as low or little response of cancer to selected drugs, have been obtained.

The CGM assay has been used on surgical specimens of renal cell carcinoma to estimate the activity of anticancer drugs against recurrent cancer, although renal cell carcinomas are generally resistant to many anticancer drugs and are suitable for surgical operation. On the other hand, it has been reported that a rat ascites hepatoma cell line, AH66, was highly cisplatin-resistant *in vivo*, but not *in vitro* (11), and the *in vivo* resistance was dependent upon induction of an enhanced expression of the multi-drug resistance-associated protein 2 (MRP2) by ascites fluid and its component, conjugated bilirubin (12, 13). Thus, clinical *in vivo* resistance to anticancer drugs, which is not detectable by *in vitro* sensitivity tests, may occur *via* environmental modulation of tumor characteristics, such as the expression of transporters.

ATP-binding cassette (ABC) transporters, including MRP2, are multi-domain integral membrane proteins that

utilize the energy of ATP hydrolysis to translocate substances across cellular membranes. ATP transporters play central roles in many biomedical phenomena, including the drug resistance of cancers (14, 15). Therefore, some of the clinical *in vivo* resistance may be explained by modulation of the expression of ABC transporters by environmental factors that differ *in vitro* and *in vivo*, as in the case of *in vivo* resistance of AH66 cells. In this study, the modification of the expression levels of the ABC transporter mRNAs in human renal cell carcinomas by treatment with conjugated bilirubin *in vitro*, as observed in rat ascites hepatoma (11-13), was assessed.

Materials and Methods

Materials. The anticancer drugs used were cisplatin, doxorubicin, 5-fluorouracil and etoposide, all of which were purchased commercially. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and conjugated bilirubin were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Collagenase type 1 and dimethyl sulfoxide (DMSO) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Alpha modification of Eagle's minimal medium (α-MEM) and 1% non essential amino acids for MEM Eagle were from ICN Biomedicals, Inc. (Irvine, CA, USA). MCDB 131 medium, M-MLV reverse transcriptase and *Taq* DNA polymerase were from Invitrogen Corp. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Bio-Whittaker (Walkersville, MD, USA). ISOGEN was from Nippon Gene (Osaka, Japan). Canalicular multispecific organic anion transporter (cMOAT/MRP2) monoclonal antibody was from Alexis Biochemicals (Lausen, Switzerland). Kanamycin sulphate was purchased from EMD Biosciences (Calbiochem; San Diego, CA, USA).

Cancer specimens and primary culture. Cancers and surrounding normal tissue samples were obtained by surgical removal from 13 patients, who gave their informed consent for the present study before the procedure, at the Department of Urology, Kanazawa University Hospital, Japan. For primary culture, the tissues were minced in α-MEM and filtered through a 250-μm nylon filter. The tissues were pelleted by centrifugation (10 min, 2,000 rpm), washed and resuspended in the medium. The tissues were subsequently seeded into collagen-coated 10-cm plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) and cultured in α-MEM supplemented with 10% heat-inactivated FBS, 1% non essential amino acids for MEM Eagle and 50 μg/ml kanamycin, in an atmosphere of 5% CO_2 at 37˚C.

Cell culture. Human renal proximal tubular epithelial cells (RPTECs), obtained from Bio-Whittaker, were cultured in MCDB 131 medium supplemented with 10% FBS and 0.5 mg/ml hydrocortisone, 10 μg/ml human epidermal growth factor, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 6.51 μg/ml triiodothyronine, gentamicin sulfate and amphotericin-B. Cells were passaged using standard trypsinization procedures and cultured in an atmosphere of 5% CO_2 at 37˚C.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated with an RNA isolation solution, ISOGEN. Synthesis of cDNA from the isolated mRNA was carried out using M-MLV reverse transcriptase. The reverse transcription (RT) mixture was amplified by PCR using *Taq* DNA polymerase in the presence of 0.2 μM sense and antisense primers (Sigma Genosys Ltd., Haverhill, Suffolk, UK). Primers used for human *MRP 1* were 5'-TGG GAC TGG AAT GTC ACG-3' and 5'-AGG AAT ATG CCC CGA CTT-3' (260 bp); *MRP 2*, 5'-TGT CTT CAC CAT CAT CGT CATT-3' and 5'-TCC TGC CCA CCA CAC CAA TCTT-3' (644 bp); *MRP 3*, 5'-TTC TGG TGG TTC ACA AAG AT-3' and 5'-TAG TGT TGT AAG ATC AGC GA-3' (452 bp); *MRP 4*, 5'-CAC AGA AGC CTT CTT TAA CA-3' and 5'-CAC GAT ATT CCT ATC TCC AT-3' (487 bp); *MRP 5*, 5'-CAA GAA GAG CTG AAT GAA GT-3' and 5'-GTT GGT CCC AGA ATA ATT AC-3' (494 bp); *MRP 6*, 5'-TTC CTG GAG TTT ATT GGT GA-3' and 5'-GAA CTT GAT GGT CTT CGA GT-3' (507 bp); *MDR 1*, 5'-CTA ATA AGA AAA AGA TCA ACT-3' and 5'-GGC TAG AAA CAA TAG TGA AAA CAA-3' (300 bp); *β-actin*, 5'-TTC TAC AAT GAG CTG CGT GTG GC-3' and 5'-CTC (A/G)TA GCT CTT CTC CAG GGA GGA-3' (456 bp). Each cycle consisted of 45 sec at 94˚C, 60 sec at 58˚C and 120 sec at 72˚C for *MRP1*, *MRP3*, *MRP4*, *MRP5*, *MRP6* and *β-actin*, 60 sec at 94˚C, 60 sec at 56˚C and 60 sec at 72˚C for *MRP 2*, and 45 sec at 94˚C, 60 sec at 55˚C and 120 sec at 72˚C for *MDR1*, with 33 cycles for *MRP1*, *MRP3*, *MRP4*, *MRP5*, *MRP6*, 35 cycles for *MRP2* and *MDR1*, and 23 cycles for *β-actin*.

In vitro chemosensitivity assay using the CGM assay (3). A 5-mm cube of specialized collagen-gel matrix derived from pig skin (Spongostan, Johnson & Johnson Medical Ltd., Gargrave, Skipton, UK) was placed in each well of a 24-well tissue culture plate (Falcon) and then hydrated adequately with α-MEM supplemented with 10% heat-inactivated FBS. Cancer specimens obtained from patients were cut into fragments (5 mg) and placed on the matrix. The medium was replaced with medium containing anticancer drugs and incubated for 7 days at 37˚C in a humidified CO_2 incubator. The drugs were used at the following concentrations (cisplatin, 24.9 μg/ml; doxorubicin, 6.0 μg/ml; 5-fluorouracil, 0.6 mg/ml; etoposide, 0.34 mg/ml), which are 10 times the therapeutic peak plasma concentrations estimated by Scheithauer *et al.* (16) To evaluate the viability of the cancer fragments, 25 μl aliquots of PBS solutions of MTT (2 mg/ml) and collagenase type 1 (1%) were added to each well, and the plate was incubated for 4 h at 37˚C. The plate was then centrifuged at 2,500 rpm for 5 min, and the supernatants were discarded. The resulting MTT-formazan crystals were dissolved in 500 μl of DMSO. A portion of the MTT-formazan solution (150 μl) was transferred to a 96-well flat-bottomed microplate, and the absorbance at 540 nm was measured with a microplate reader, Multiskan BICHROMATIC (Labsystems Japan, Tokyo, Japan). The effect of each drug was expressed as an inhibition rate (IR%) calculated by applying the following formula; (1–T/C) x 100, where T is the mean absorbance of the drug-treated group and C is that of the control group.

Flow cytometry. Renal proximal tubule epithelial cells (RPTECs), treated or not treated with conjugated bilirubin for 72 h, were washed with ice-cold PBS and fixed in 70% ethanol for 1 h at –20˚C. After having been washed, the cells were reacted with human MRP2 monoclonal antibody and then stained with the FITC-conjugated mouse IgG second antibody. The MRP2 expression level was determined in terms of fluorescence intensity

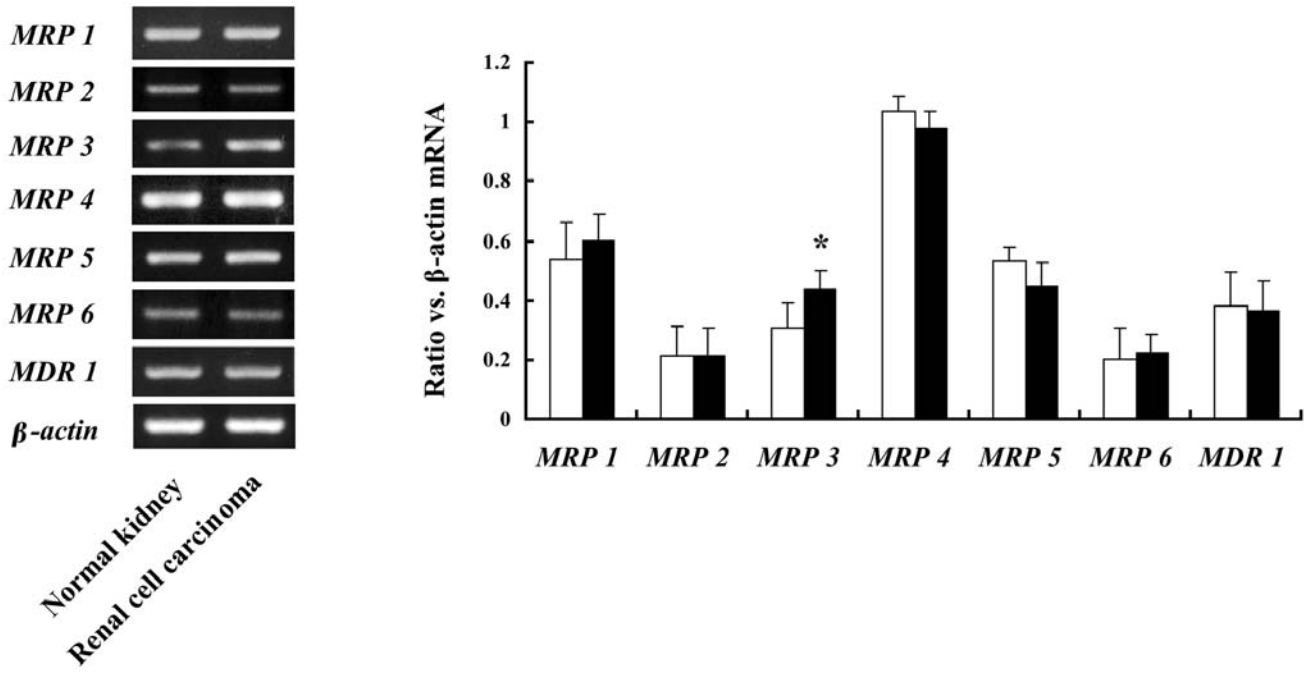

Figure 1. *Relative expression levels of ABC transporter mRNAs in renal cell carcinomas and normal kidney tissues. Total RNAs were extracted from renal cell carcinomas (shaded column) and surrounding normal kidney tissues (unshaded column) from 13 patients. ABC transporter expression levels were detected by RT-PCR (left), and normalized by β-actin level (right). Each value is the mean±SE (n=13). *Significantly different from the normal kidney tissues at p<0.05.*

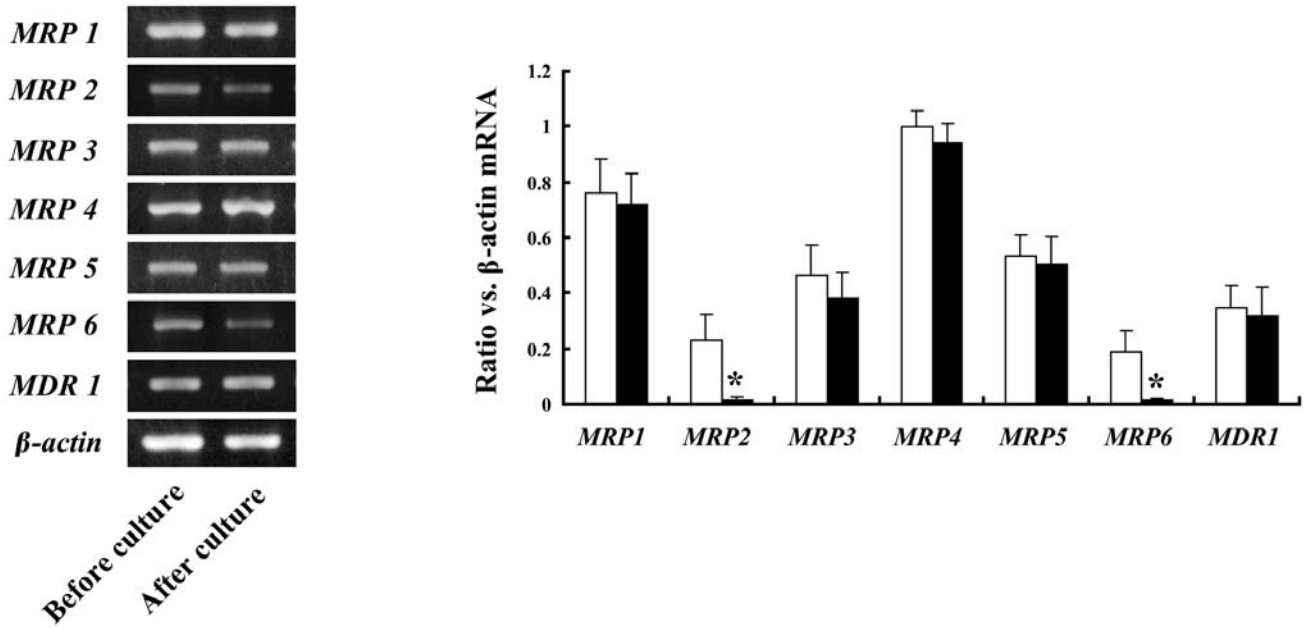

Figure 2. *Relative expression levels of ABC transporter mRNAs in renal cell carcinomas before and after culture in vitro. Total RNAs were extracted from renal cell carcinomas just removed from patients (unshaded column) and after culture in vitro in α-MEM containing 10% FBS for 14 days (shaded column). ABC transporter expression levels were evaluated by RT-PCR (left), and normalized by β-actin level (right). Each value is the mean±SE (n=5). *Significantly different from the renal tumors just removed from patients at p<0.05.*

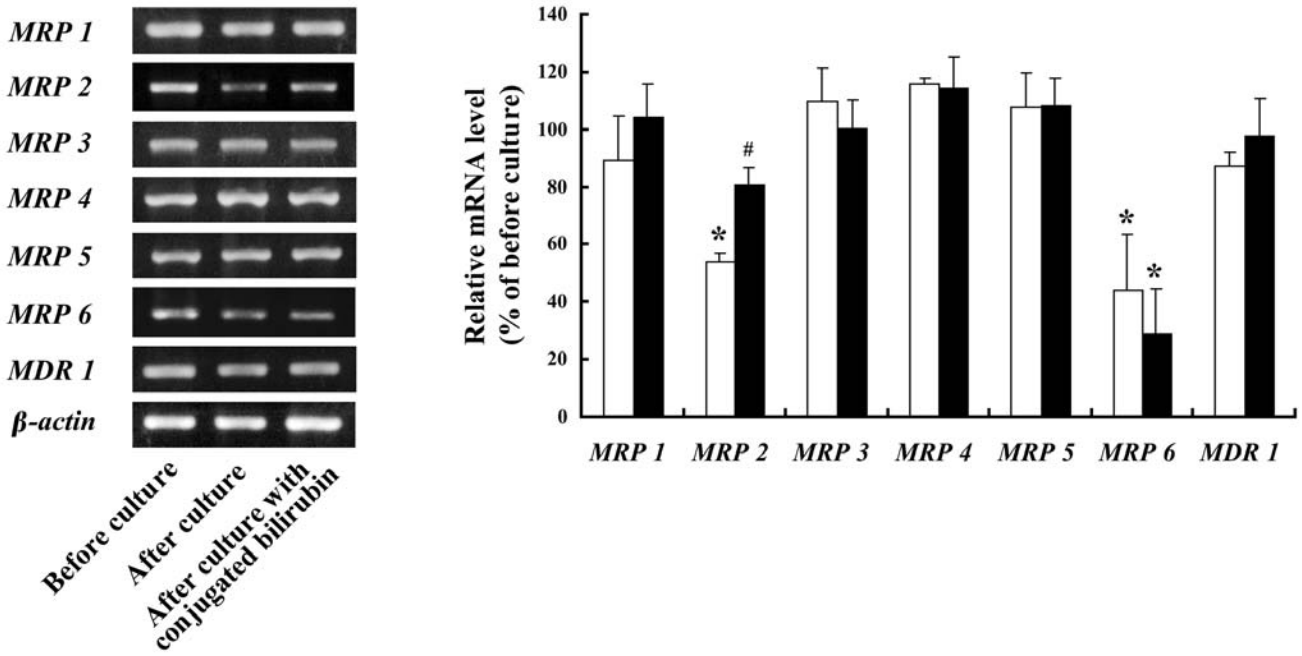

Figure 3. *Effect of conjugated bilirubin on ABC transporter mRNA expression in renal cell carcinomas. Renal cell carcinomas removed from patients were cultured in α-MEM containing 10% FBS for 14 days, and further cultured in the medium without (unshaded column) or with 20 μg/ml conjugated bilirubin (shaded column) for 72 h. Total RNA was extracted and ABC transporter expression levels were evaluated by RT-PCR (left). They were normalized by β-actin level and are shown as a percentage of the expression level of the renal cell carcinoma before culture (right). Each value is the mean±SE (n=3). *Significantly different from the carcinoma before culture at p<0.05. #Significantly different from the carcinoma cultured without conjugated bilirubin at p<0.05.*

by flow cytometry (FACS Calibur; Becton Dickinson) and analyzed by Cell Quest (Becton Dickinson).

MTT assay. RPTECs ($5x10^4$) were seeded in each well of collagen-coated 96-well micrometer plates (Falcon) and allowed to attach overnight. The cells were then treated or not with conjugated bilirubin in the presence of the indicated concentration of an anticancer drug for 72 h. Subsequently, 25 μL of MTT (2 mg/ml) in PBS was added to each well, followed by incubation for 4 h at 37˚C. MTT-formazan crystals were dissolved in DMSO. Absorbance was determined with a microplate reader, Multiskan BICHROMATIC (Labsystems Japan), at 540 nm.

Results

Expression of ABC transporter mRNAs in renal cell carcinomas and the surrounding normal tissues. The expression levels of ABC transporter mRNAs in 13 surgically removed renal cell carcinomas and their surrounding normal kidney tissues are illustrated in Figure 1. The relative level of *MRP4* mRNA was high and those of *MRP2* and *MRP6* mRNAs were low in both the renal cell carcinomas and the normal tissues. The expression level of *MRP3* mRNA in the renal cell carcinomas was slightly but significantly higher than that of the normal tissues.

Table I. *In vitro chemosensitivity of renal cell carcinomas to anticancer drugs.*

	Inhibition rate (%)			
Pt. No.	CDDP	DXR	5-FU	VP-16
No.1	**62.9**	28.7	**69.4**	**40.1**
No.2	8.8	-29.4	-24.2	-1.3
No.3	**47.2**	23.7	**31.3**	**42.8**
No.4	**43.9**	**31.9**	27.6	**N.T.**
No.5	**40.9**	17.1	23.9	**38.3**

Bold: > IR 30%

CDDP; cisplatin, DXR; doxorubicin, 5-FU; fluorouracil, VP-16; etoposide
N.T.; not tested

Changes in ABC transporter mRNA expression in renal cell carcinomas cultured in vitro. Five renal cancer cell specimens were succesfully cultured *in vitro* for 14 days. As shown in Figure 2, the expression of *MRP2* and *MRP6* mRNAs was significantly lowered after culturing *in vitro* for 14 days.

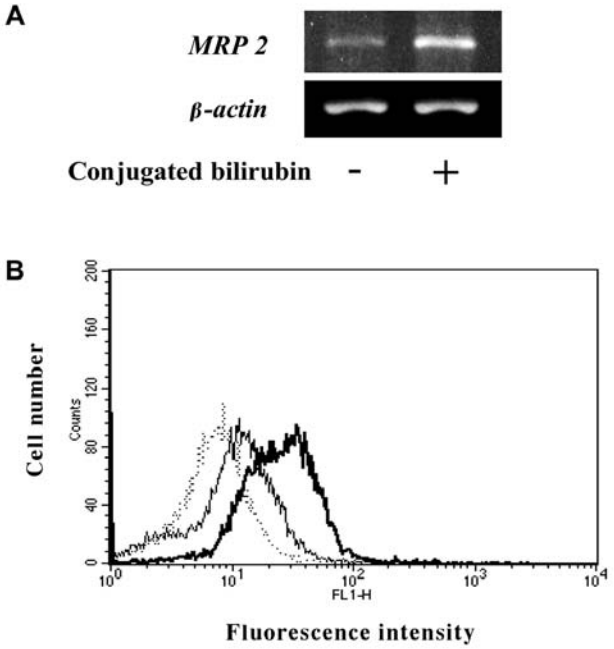

Figure 4. *Effect of conjugated bilirubin on MRP2 expression in RPTECs. A, MRP2 mRNA expression level in RPTECs treated without (–) and with (+) 20 μg/ml conjugated bilirubin for 72 h as evaluated by RT-PCR. B, RPTECs were cultured in α-MEM containing 10% FBS in the absence (thin line) or presence (bold line) of 20 μg/ml conjugated bilirubin for 72 h. Background reactivity (dotted line) of the cells was obtained by staining with FITC-conjugated second antibody alone.*

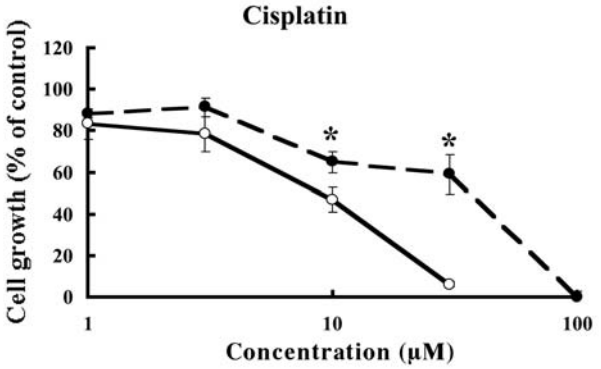

Figure 5. *Influence of bilirubin treatment on the growth-inhibitory effects of cisplatin. RPTECs were treated with various concentrations of cisplatin in the absence (open circle with solid line) or presence (closed circle with dotted line) of 20 μg/ml conjugated bilirubin for 72 h. Each value is the mean±SD (n=3). *Significantly different from the value in the absence of conjugated bilirubin at p<0.05.*

In vitro chemosensitivity. The results of the *in vitro* chemosensitivity test with the CGM assay on the same specimens as in Figure 2 are provided in Table I. Although renal cancers are generally resistant to various anticancer drugs, the results indicate that cisplatin (CDDP) may be effective against 4 out of the 5 cancer specimens in this study.

Effect of conjugated bilirubin on ABC transporter mRNA expression in renal cell carcinomas. The relative changes in ABC transporter mRNA expression in primary cultured carcinoma cells after *in vitro* culturing in the presence or absence of 20 μg/ml conjugated bilirubin for 72 h are depicted in Figure 3. *MRP2* and *MRP6* mRNA expression levels in primary cultured cancer cells were significantly lower than those in surgical specimens, as shown in Figure 2, and the treatment with conjugated bilirubin (20 μg/ml) significantly increased the expression of *MRP2* mRNA, but not of *MRP6* or other mRNAs (Figure 3).

Effect of conjugated bilirubin on MRP2 expression and cisplatin sensitivity in RPTECs. When human renal proximal tubular epithelial cells (RPTECs) were cultured with conjugated bilirubin (20 μg/ml) for 72 h, *MRP2* mRNA expression increased (Figure 4A). Increased MRP2 protein expression was also observed by flow cytometric analysis (Figure 4B). Cisplatin sensitivity of RPTECs treated with conjugated bilirubin is illustrated in Figure 5, where it can be seen that conjugated bilirubin significantly decreased the cisplatin sensitivity of the cells. The IC_{50} values (μM, mean±SD) of cisplatin alone and cisplatin with conjugated bilirubin were 9.5±1.2 and 39.4±9.9, respectively ($p<0.05$).

Discussion

Multiple drug resistance, both intrinsic and acquired, has a grave impact on the outcome of cancer treatment. It is well established that ABC transporters, such as P-glycoprotein and the MRP family, contribute to drug resistance (14, 15, 17, 18). Some investigators have reported that the expression of ABC transporters, such as MDR1, MRP1 and MRP2, in tumors and cancers tends to be higher than that in the corresponding normal tissues (19-21). In this study, all transporter mRNAs examined were expressed in renal cell carcinomas and the surrounding normal tissues to similar extents (Figure 1). This supports the idea that renal cell carcinoma is intrinsically resistant to many anticancer drugs, which are ABC transporter substrates.

On the other hand, in *in vitro* chemosensitivity testing with the CGM assay, 4 out of 5 specimens were found to be sensitive to cisplatin (Table I). It has previously been reported that MRP2 expression in rat hepatoma AH66 cells harvested from tumor-bearing rats was markedly decreased after *in vitro* culture (12, 13, 17). In this study, *MRP2* mRNA expression as well as that of *MRP6* mRNA in renal cell carcinomas significantly decreased during *in vitro* primary culture (Figure 2). The results of the *in vitro* chemosensitivity

tests indicated that renal cell carcinomas were sensitive to some drugs, especially cisplatin. Hirano *et al.* (9) have also reported that renal cell carcinomas had high sensitivity to cisplatin *in vitro*. Cisplatin is known to be an MRP2 substrate, which is excreted from the cells by this transporter (11-13, 22, 23). Therefore, it is suggested that the decreased MRP2 expression after *in vitro* culture might lead to an increase in the intracellular cisplatin concentration, resulting in an apparent increase of drug sensitivity. Regarding MRP6, its physiological function is not yet known, so it is unclear whether the decrease in its expression influences drug sensitivity. These results indicate that changes in the expression of transporters, including MRP2, may occur not only in hepatoma and renal carcinoma, but also in tumors and cancers in general. Care may be needed in assessing the results of *in vitro* chemosensitivity tests for selecting anticancer drugs.

Another major aim of this study was to identify the regulator or enhancer of MRP2 expression in renal cell carcinoma *in vivo*. Previously, it has been reported that conjugated bilirubin increased MRP2 expression and induced *in vivo* cisplatin resistance of AH66 cells (13). The effect of conjugated bilirubin on ABC transporter mRNA expression in surgically removed human renal cell carcinomas and in an established RPTEC cell line was examimed. The plasma concentration of conjugated bilirubin in healthy humans is generally less than 4 μg/ml. In this study, 20 μg/ml of conjugated bilirubin were employed, because the conjugated bilirubin concentration (20.0±4.1 μg/ml) in the kidney was found to be more than 10 times the plasma level (1.8±1.1 mg/ml) in normal rats (unpublished data). Treatment with conjugated bilirubin specifically increased the expression of *MRP2* mRNA in human renal cell carcinomas (Figure 3), as well as in AH66 cells (12, 13). Moreover, in RPTEC, conjugated bilirubin increased *MRP2* mRNA and protein levels (Figure 4) and lowered the sensitivity to cisplatin (Figure 5). However, expression of other ABC transporter mRNAs was not changed by conjugated bilirubin (data not shown). The MRP2 regulation mechanism and regulator may be different from those of other ABC transporters.

MRP2 and *MRP6* mRNA expression was significantly reduced in *in vitro* cancers of human renal cell carcinomas, while *MRP2* mRNA expression levels were restored in the presence of conjugated bilirubin, an endogenous substance. Conjugated bilirubin also increased MRP2 protein expression and decreased the cisplatin sensitivity. These findings suggest that the expression of transporters, which influence anticancer drug sensitivity, is modulated by differences between *in vitro* and *in vivo* environments. Therefore, the discovery of other endogenous transporter expression regulators and their addition to the assay system would enhance the effectiveness of *in vitro* chemosensitivity testing in the selection of optimum anticancer drugs for individual patients.

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References

1 Freeman AE and Hoffman RM: *In vivo*-like growth of human tumors *in vitro*. Proc Natl Acad Sci USA *83*: 2694-2698, 1986.

2 Vescio RA, Redfern CH, Nelson TJ, Ugoretz S, Stern PH and Hoffman RM: *In vivo*-like drug responses of human tumors growing in three-dimensional gel-supported primary culture. Proc Natl Acad Sci USA *84*: 5029-5033, 1987.

3 Ohyama S, Tanaka M, Yonemura Y, Kinoshita K, Miyazaki I and Sasaki T: *In vitro* chemosensitivity test of human gastric carcinomas using collagen gel matrix. Jpn J Cancer Res *82*: 607-612, 1991.

4 Furukawa T, Kubota T and Hoffman RM: Clinical applications of the histoculture drug response assay: Clin Cancer Res *1*: 305-311, 1995.

5 Ariyoshi Y, Shimahara M and Tanigawa N: Study on chemosensitivity of oral squamous cell carcinomas by histoculture drug response assay. Oral Oncol *39*: 701-707, 2003.

6 Singh B, Li R, Xu L, Poluri A, Patel S, Shaha AR, Pfister D, Sherman E, Goberdhan A, Hoffman RM and Shah J: Prediction of survival in patients with head and neck cancer using the histoculture drug response assay. Head Neck *24*: 437-442, 2002.

7 Morioka H, Yabe H, Morii T, Yamada R, Kato S, Yuasa S and Yano T: *In vitro* chemosensitivity of human soft tissue sarcoma. Anticancer Res *21*: 4147-4151, 2001.

8 Furukawa T, Kubota T, Tanino H, Oura S, Yuasa S, Murate H, Morita K, Kozakai K, Yano T and Hoffman RM: Chemosensitivity of breast cancer lymph node metastasis compared to the primary tumor from individual patients tested in the histoculture drug response assay. Anticancer Res *20*: 3657-3658, 2000.

9 Hirano Y, Ushiyama T, Suzuki K and Fujita K: Clinical application of an *in vitro* chemosensitivity test, the Histoculture Drug Response Assay, to urological cancers: wide distribution of inhibition rates in bladder cancer and renal cell cancer. Urol Res *27*: 483-488, 1999.

10 Uchibayashi T, Egawa M, Hisazumi H, Hasegawa T, Tanaka T and Sasaki T: *In vitro* chemosensitivity test for human genitourinary tumors using collagen gel matrix. Eur Urol *24*: 267-270, 1993.

11 Minamino T, Nomura M, Tamai M, Moritani S, Ohshima T and Miyamoto K: *In vivo* cisplatin resistance of rat ascites hepatoma AH66. Cancer Lett *108*: 153-156, 1996.

12 Minamino T, Tamai M, Itoh Y, Tatsumi Y, Nomura M, Yokogawa K, Suzuki H, Sugiyama Y, Ohshima T and Miyamoto K: *In vivo* cisplatin resistance depending upon canalicular multispecific organic anion transporter (cMOAT). Jpn J Cancer Res *90*: 1171-1178, 1999.

13 Tamai M, Ito Y, Mirura H, Tsukada H, Yokogawa K, Nomura M, Moritani S and Miyamoto K: Conjugated bilirubin induces multidrug resistance-associated protein 2 mRNA expression and *in vivo* cisplatin resistance in rat hepatoma AH66 cells. Anticancer Res *23*: 4781-4787, 2003.
14 Kruh GD and Belinsky MG: The MRP family of drug efflux pumps. Oncogene *22*: 7537-7552, 2003.
15 Leonard GD, Fojo T and Bates SE: The role of ABC transporters in clinical practice. Oncologist *8*: 411-424, 2003.
16 Scheithauer W, Clark GM, Salmon SE, Dorda W, Shoemaker RH and Von Hoff DD: Model for estimation of clinically achievable plasma concentrations for investigational anticancer drugs in man. Cancer Treat Rep *70*: 1379-1382, 1986.
17 Itoh Y, Tamai M, Yokogawa K, Nomura M, Moritani S, Suzuki H, Sugiyama Y and Miyamoto K: Involvement of multidrug resistance-associated protein 2 in *in vivo* cisplatin resistance of rat hepatoma AH66 cells. Anticancer Res *22*: 1649-1653, 2002.
18 Gottesman MM: Mechanisms of cancer drug resistance. Annu Rev Med *53*: 615-627, 2002.
19 Cho YS and Kim MJ: Expression of multidrug resistance-related genes in oral squamous cell carcinomas. Oral Oncol *37*: 652-659, 2001.
20 Soini Y, Jarvinen K, Kaarteenaho-Wiik R and Kinnula V: The expression of P-glycoprotein and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) in human malignant mesothelioma. Ann Oncol *12*: 1239-1245, 2001.
21 Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM and Pastan I: Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA *84*: 265-269, 1987.
22 Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M and Kuwano M: A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res *57*: 5475-5479, 1997.
23 Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D: Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol *55*: 929-937, 1999.

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