Expression of multidrug resistance-associated protein 2 is involved in chemotherapy resistance in human pancreatic cancer

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Abstract. Despite the recent introduction of the new anticancer agents gemcitabine (GEM) and TS-1, as well as combination regimens such as GEM plus cisplatin (CDDP), pancreatic cancer treatment remains relatively ineffective. Both intrinsic and acquired resistance to chemotherapy are major roadblocks to the successful treatment of pancreatic cancer patients. The aims of this study were to examine the expression of multidrug resistance-associated proteins (MRPs) MRP1, MRP2 and MRP3 and to evaluate the correlation between MRP2 expression and CDDP resistance in human pancreatic cancer. Five human pancreatic cancer cell lines and several surgically resected pancreatic cancer tissues were subjected to reverse-transcriptase (RT)-PCR, real-time PCR and immunohistochemical analysis. While MRP1 and MRP2 mRNA was expressed in all cell lines, MRP3 mRNA was only detected in two cell lines. In resected pancreatic cancer tissues, only MRP2 mRNA was expressed and it was overexpressed compared with normal pancreatic tissues. MRP2 protein expression was observed in 77.5% (31/40) of cancer tissues, primarily in the cytoplasm of cancer cells, but was not observed in normal pancreatic tissue. Two CDDP-resistant pancreatic cancer cell line SUIT-2 variants, SUIT-2-CD3 and SUIT-2-CD4, were established by continuously administering 10 nM CDDP to SUIT-2 cell lines for 3 and 4 months, respectively. Incubation of these cells with CDDP in the presence of anti-MRP2 antibody or the MRP2 inhibitor MK-571 in a growth inhibition assay demonstrated that the CDDP-resistant variants were more resistant to CDDP than the parent cell line and this resistance was diminished by either anti-MRP2 antibody or MK-571. Moreover, RT-PCR and real-time PCR revealed that while induction of MRP2 mRNA expression was increased in CDDP-resistant compared with parent cells, MRP1 and MRP3 expression remained unchanged. These observations suggest that MRP2 may correlate to intrinsic and acquired resistance for CDDP in human pancreatic cancer.

Introduction

Pancreatic cancer is currently the fifth leading cause of cancer death in Japan (1) and the incidence of pancreatic cancer-related death is steadily increasing. At the time of diagnosis, many patients have locally advanced or metastatic disease. Consequently, chemotherapy remains the only treatment strategy for patients with non-resectable disease (2,3). In recent years, gemcitabine (GEM) and TS-1 (tegafur, gimestat and otastast potassium) have become standard therapies for patients with advanced pancreatic cancer; however, these new treatments have not led to improved prognoses. The efficacy of combination chemotherapy, such as GEM plus cisplatin [cis-diamminedichloroplatinum (CDDP)], has also been disappointing (4-6). Overall, the response of pancreatic cancer to chemotherapy is generally quite limited, due in part to resistance to chemotherapeutic agents (2,7).

Acquired drug resistance in cancer cells is a major roadblock to effective chemotherapy. In general, multidrug resistance is caused by P-glycoprotein and multidrug resistance proteins (MRPs), which belong to the ATP-binding cassette transporter family (8-11). The human MRP family currently has nine members, of which at least three (MRP1, MRP2 and MRP3) are known to be correlated with chemoresistance (9,12-14). The best characterized are MRP1 and MRP2. MRP1 is a 190-kDa glycoprotein that mediates the extra- and intracellular transport of many glutathione S-conjugates (8,15-17). Its preferred substrates are organic anions, e.g., drugs conjugated to glutathione, sulphate, or glucuronate (9). MRP2, or human canalicular multispecific organic anion transporter (cMOAT), was first identified from the canalicular membrane of hepatocytes and is also involved in the transport of organic anions across the plasma membrane. In addition, MRP2 has been found to be overexpressed in a number of CDDP-resistant human cancer cell lines, including hepatocytes.
cellular carcinoma, hepatoblastoma, bladder cancer, prostate cancer, colon cancer, ovarian cancer, adrenocortical cancer, and melanoma cells (18-21). In vitro data have also suggested that MRP2 may play an important role in multidrug resistance during chemotherapy (19-24). Finally, MRP3 has been shown to transport methotrexate (MTX) and etoposide, but not glutathione (25).

Liu et al have reported that docetaxel-related drug resistance in pancreatic cancer cell lines is primarily mediated by P-glycoprotein and has no relationship to MRPs or lung resistance protein (26,27). In contrast, Miller et al reported that intrinsic drug resistance in pancreatic duct cancer may be due in part to the presence of MRPs (28). Overall, few studies have investigated the expression of MRPs in pancreatic cancer and there have been no studies about MRP2 expression and CDDP resistance in this disease. The aims of the present study were to examine the expression profile of MRPs and to evaluate the correlation between MRP2 expression and CDDP resistance in human pancreatic cancer.

Materials and methods

Cell culture and tumor tissues. Four human pancreatic cancer cell lines, PANC-1, MIAPaCa-2, Capan-2 and BxPC3, were purchased from the American Type Culture Collection (Rockville, MD). The pancreatic cancer cell line SUIT-2 was purchased from the Health Science Research Resources Bank (Osaka, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY), pH 7.4, containing 100 U/ml penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO) and 10% fetal bovine serum (FBS; Iwaki, Tokyo, Japan) and were grown in 25-cm² plastic flasks (Becton-Dickinson, Franklin Lakes, NJ) in 5% CO₂ at 37°C. We examined 40 pancreatic cancer tissue specimens that were surgically resected between 1995 and 2006 at the Hiroshima University Hospital. Five specimens were used for reverse transcriptase (RT)-PCR and six were used for real-time PCR, after informed consent was obtained from patients.

RT-PCR. RT-PCR analysis was performed, as described previously (29). Briefly, total RNA was extracted from five human pancreatic cancer cell lines and five surgically resected pancreatic cancer tissues using a FastRNA® kit, Green (Bio 101, Inc., Vista, CA), according to the manufacturer’s instructions. A sample containing 200 ng total RNA in a 20-μl mixture was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan) at 42°C for 30 min in the presence of 25 pmol oligo (dT) 20 primer. Subsequently, 20 μl reverse-transcribed mixture was subjected to PCR in a 100-μl reaction mixture in the presence of 1 U Kod Plus™ (Toyobo) and 20 pmol of each primer. The following cycling conditions were used: 40 cycles consisting of denaturation at 94°C for 30 sec, annealing at 56°C (MRP1), 50°C (MRP2), 54°C (MRP3), or 60°C [glyceraldehyde-3-phosphate dehydrogenase (G3PDH; control)] for 30 sec and extension at 72°C for 60 sec. A 10-μl sample of each PCR product was separated in a 1.5% agarose gel, stained with SYBR-Green (Molecular Probes, Eugene, OR) and visualized using ultraviolet light. The following primer sequences were used: MRP1, sense 5'-AGT GAC CTC TGG TCC TTA AAC AAG G-3' and antisense 5'-GAG GTA GAG AGC AAG GAT GAC TTG C-3'; MRP2, sense 5'-AGG ATG ACA TCA GAA ATA GAG ACC-3' and antisense 5'-CTA CTC CAT CAA TGA TAA TCT GAC C-3'; MRP3, sense 5'-GAT ACG CTC GCC ACA ACG T-3' and antisense 5'-CAG TTT GTC GTC GTC GTC GTC G-3'; and G3PDH, sense 5'-TCC ACC ACC CTG TTAG CTG TA-3' and antisense 5'-ACC ACA GTC CAT GCC AC-3'.

Immunohistochemical staining. Consecutive 4-μm sections were cut from each specimen block. Immunohistochemical staining was performed using a Vectastain® Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). Sections were subjected to immunohistochemical analysis of MRP2 expression using a mouse monoclonal antibody (M2IIJ-6; Sanbio b.v., Uden, The Netherlands) at a 1:50 dilution. Tissues in which >10% of cells were stained were considered to be positive.

Establishment of CDDP-resistant SUIT-2 variants. Two CDDP-resistant variants of the SUIT-2 pancreatic cancer cell line, SUIT-2-CD3 and SUIT-2-CD4, were cultivated by continuous exposure to 10 nM CDDP for 3 and 4 months, respectively. Briefly, SUIT-2 cells were grown as monolayers in culture medium at 37°C in a 5% CO₂ humidified atmosphere and exposed to 10 nM CDDP for 3 or 4 months, with fresh culture medium and drug added every 3 days. Cells were then maintained in a CDDP-free culture medium for at least 3 weeks before analysis.

Growth inhibition assay. We used a cell proliferation ELISA system™ (Amersham Pharmacia Biotech, Piscataway, NJ) (30) to measure cell growth inhibition, according to the manufacturer’s instructions. Briefly, SUIT-2, SUIT-2-CD3, and SUIT-2-CD4 cells (5x10⁴) were plated in 96-well plates and treated with various concentrations of CDDP (0-16 μM) and anti-MRP2 antibody (0-25 ng/ml) or MK-571 (0-20 μM) (Biomol International, LP, USA), which is reported to be an MRP2 inhibitor (31,32), in DMEM supplemented with 0.1% FBS. After 72 h, 5-bromo-2’-deoxyuridine (BrdU) was added to a final concentration of 10 μM and the reaction mixture was incubated for 2 h at 37°C. Sequentially, cells were fixed with ethanol, blocked with blocking solution and incubated with anti-BrdU for 90 min. Finally, cells were reacted with 3,3’,5,5’-tetramethylbenzidine (TMB) substrate and the absorbance at 450 nm of supernatant aliquots from each well was measured using an automatic plate analyzer (ETY-3A; Toyo Sokki, Tokyo, Japan). Each experiment was performed two times in triplicate.

Preparation of MRP2 and G3PDH cDNA for standard curves. Select MRP2 and G3PDH PCR products were gel-purified, cloned by ligation into the pGEM®-T easy vector (Promega, Madison, WI) and transformed into JM 109 competent bacteria (Toyobo). Plasmid DNA was extracted by the Automatic DNA Isolation System™ (Kurabo, Osaka, Japan). Sequencing reactions were analyzed in an automatic sequencing system (ABI 310; Applied Biosystems, Foster City, CA) using the BigDye terminator cycle sequencing reaction kit (Applied...
Biosystems). The derived MRP2 and G3PDH gene sequences were confirmed by comparison with corresponding germline genes using Genbank (EMBL). Isolated cDNA fragments were used as templates for external standards for use in real-time PCR.

Real-time PCR. Total RNA was extracted and reverse-transcribed as described above. PCR reactions were monitored in real-time with the LightCycler® system (Roche Molecular Systems, Inc., Pleasanton, CA) and LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Meylan, France), which binds preferentially to double-stranded DNA. The 20-μl reaction mixtures contained LightCycler FastStart DNA Master SYBR-Green I, 3 mM MgCl₂, primers (each at 0.5 μM) and 2 μl template. The amplification program with MRP2 and G3PDH primers included an initial denaturation step at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 sec, annealing at 50°C (MRP2) or 60°C (G3PDH) for 5 sec and extension at 72°C for 10 sec. The temperature transition rate was 20°C/sec. After amplification, a melting curve was acquired by heating the product at 20°C/sec to 95°C, cooling it at 20°C/sec to 65°C, maintaining it at 65°C for 15 sec and then slowly heating at 0.1°C/sec to 95°C. With this technique, reactions are characterized using the time during cycling when amplification of the PCR products is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the template, the earlier a significant increase in fluorescence is observed. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline. For each sample, the amounts of the MRP2 and G3PDH mRNA were determined from a standard curve. Quantities of MRP2 and G3PDH mRNA were determined by comparison to a DNA external standard. Product specificity was determined by melting curve analysis, as described in the LightCycler handbook and by visualization of PCR products on agarose gels and direct DNA sequencing. Results are expressed as ratios of MRP2 mRNA to G3PDH mRNA.

Statistical analysis. Statistical analysis was performed using Scheffé’s F test. Results are presented as the average ± SD of triplicate samples from at least two independent experiments. A probability level of p<0.05 was considered statistically significant.

Results

MRP1, MRP2 and MRP3 mRNA expression in pancreatic cancer cell lines and resected pancreatic cancer tissue (RT-PCR). First, MRP1, MRP2 and MRP3 mRNA expression in pancreatic cancer cell lines and surgical tissues was examined by RT-PCR analysis. As shown in Fig. 1A, MRP1 and MRP2 mRNA was detected in all cancer cell lines. In contrast, MRP3 mRNA was only expressed in MIAPaCa-2 and SUIT-2 cells. MRP2 mRNA was detected in all five resected pancreatic cancer tissue specimens examined; however, neither MRP1 nor MRP3 mRNA was detected in these specimens (Fig. 1B).

Semiquantitative analysis of MRP2 mRNA expression in resected pancreatic cancer tissue (real-time PCR). Since MRP2 mRNA was detected in all cancer cell lines and resected cancer tissues, we next performed semiquantitative analysis of MRP2 mRNA expression using cancerous and normal pancreatic tissues. Initially, to determine whether the LightCycler assay system was reliable for semiquantification of MRP2 mRNA, serial concentrations of the derived MRP2 gene were amplified and fluorescence intensities were measured. MRP2 was linearly amplified by the LightCycler, depending on the template concentration. The run profile and standard curve of MRP2 are shown in Fig. 2A and B. Subsequently, we performed real-time PCR on six pancreatic
cancer tissues and matched normal pancreatic tissue. We found that MRP2 mRNA expression was 1.2- to 30-fold higher in cancer lesions than in matched normal pancreatic tissue (Fig. 2C).

Expression of MRP2 protein in resected pancreatic cancer tissue (immunohistochemistry). We used immunohistochemistry to evaluate MRP2 protein expression in 40 pancreatic cancer tissues obtained by surgical resection. Immunohistochemical staining revealed that MRP2 protein was expressed in 31 out of 40 specimens (77.5%), with immunoreactive signals observed primarily in the cytoplasm of cancer cells, as well as in the cell membrane (Fig. 3). In contrast, no evidence of MRP2 protein was observed in surrounding normal pancreatic tissues, including pancreatic

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Figure 2. Semiquantitative analysis of MRP2 mRNA expression. Using the LightCycler system, serial concentrations of derived DNA fragments of MRP2 (5x10^{-1} - 5x10^{3} ng/ml) were amplified and the fluorescence intensities were measured, as described in Materials and methods. (A) Run profile of fluorescence vs. PCR cycle (MRP2 external standard). (B) Standard curve for MRP2 mRNA. MRP2 fragments were linearly amplified by the LightCycler depending on the template concentration. (C) Total RNA from pancreatic cancer tissues and normal pancreas was extracted and reverse-transcribed. MRP2 mRNA was overexpressed in human pancreatic cancer tissues compared to matched normal pancreas.
ducts, acini and islets of Langerhans. These results demonstrate that MRP2 protein is highly and preferentially expressed in pancreatic cancer lesions.

**Growth inhibition assay.** We next used a cell proliferation ELISA system to determine whether SUIT-2-CD3 and SUIT-2-CD4 cells are resistant to CDDP. Treatment of SUIT-2 cells with 1 μM CDDP induced a cell growth inhibition rate of 50% (Fig. 4). However, the cell growth inhibition rates in SUIT-2-CD3 and SUIT-2-CD4 cells were reduced to 10 and 8%, respectively, following CDDP treatment. The 50% inhibitory concentrations of CDDP against SUIT-2, SUIT-2-CD3 and SUIT-2-CD4 cells were 1.2, 1.9 and 2.0 μM, respectively. These results indicate that continuous administration of CDDP to SUIT-2 cells over 3-4 months results in CDDP resistance.

**Induction of MRP2 mRNA expression by CDDP.** We studied whether CDDP resistance in pancreatic cancer cells involves changes in MRP2 expression. RT-PCR analysis demonstrated that MRP2, but not MRP1 or MRP3, is overexpressed in CDDP-resistant SUIT-2 cells (Fig. 5). A semiquantitative analysis using the LightCycler system was then used to confirm that induction of MRP2 mRNA expression is significantly increased by 1.5- and 2.5-fold in SUIT-2-CD3 and SUIT-2-CD4 cells, respectively, compared to parent SUIT-2 cells (Fig. 6).

**Reduction of resistance to CDDP.** SUIT-2 and SUIT-2-CD3 cells were treated with various concentrations of CDDP, as well as anti-MRP2 antibody or MK-571 and cell proliferation was examined. SUIT-2 cell proliferation was not affected by 0-25 ng/ml anti-MRP2 antibody (Fig. 7A) or by 0-20 μM MK-571 (Fig. 7B). However, CDDP resistance in SUIT-2-CD3 cells was diminished in the presence of anti-MRP2 antibody (2.5 ng/ml) or MK-571 (5.0 μM) (Fig. 7C), suggesting that MRP2 is involved in CDDP resistance in human pancreatic cancer.

**Discussion**
The resistance of cancer cells to anticancer agents continues to be a major problem in the treatment of non-resectable solid tumors. Particularly in advanced pancreatic cancer
results of Wittwer et al, who reported that real-time PCR using the LightCycler system was an effective method for measuring mRNA expression levels in tissue samples (33). We performed this semiquantitative analysis for MRP2 mRNA in cancer and normal surrounding pancreatic tissues. MRP2 mRNA expression was 1.2- to 30-fold higher in cancer lesions than in surrounding normal pancreatic tissue. In addition, immunohistochemical analysis demonstrated that MRP2 protein is present in cancer lesions, but not in normal pancreas. The tissues used in this study were obtained from chemotherapy-naïve patients, suggesting that MRP2 expression in pancreatic cancer is not related to drug exposure. Narasaki et al reported that MRP2 is expressed in various human cancer cells, including drug-resistant cells (34).

Together with this study, our results suggest that MRP2 expression in pancreatic cancer may be a source of intrinsic drug resistance.

In many cancers, including pancreatic cancer, long-term chemotherapy decreases the response of cancer cells to anticancer agents. Acquired drug resistance plays an important role in this process. In the present study, we investigated acquired drug resistance using a cell inhibition assay and real-time PCR in the SUIT-2 cell line as well as CDDP-resistant SUIT-2 variants. Hong et al reported that CDDP-resistant lung cancer cell lines could be cultivated by continuous exposure to gradually increased CDDP doses (35). We modified this method to establish the CDDP-resistant SUIT-2-CD3 and SUIT-2-CD4 cell lines by means of continuous exposure to 10 nM CDDP for 3 and 4 months, respectively. A cell inhibition assay confirmed that SUIT-2-CD3 and SUIT-2-CD4 cells were more resistant to CDDP than was the parent SUIT-2 cell line. RT-PCR analysis revealed overexpression of MRP2 mRNA in SUIT-2-CD3 cells compared to parent cells, while real-time PCR demonstrated a 1.5-fold greater induction of MRP2 mRNA. Moreover, the CDDP resistance of SUIT-2-CD3 cells was diminished by treatment with anti-MRP2 antibody and the MRP2 inhibitor MK-571. The involvement of MRP2 in chemotherapy resistance has been shown in several previous studies. For example, Evers et al demonstrated that overexpression of MRP2 mediates vinblastine transport in polarized cells (24). Further, overexpression of MRP2 in transfected cells results in resistance to MTX (36), CDDP,
etoposide, doxorubicin and epirubicin (37). Kuwano et al also showed that MRP2 expression is involved in drug resistance in head and neck carcinoma, hepatocellular carcinoma and colorectal carcinoma (18,23,38). Together with these studies, our results suggest that MRP2 is associated with acquired resistance to CDDP, as well as with intrinsic resistance. Although SUIT-2-CD4 cells demonstrated a 1.7-fold greater induction of MRP2 expression compared to SUIT-2-CD3 cells, the degree of CDDP resistance in the two cell lines was similar. This finding indicates that acquired CDDP resistance involves other genes in addition to MRP2.

In summary, we have demonstrated that MRP2 is overexpressed in pancreatic cancer patients who have not undergone chemotherapy, as well as in CDDP-resistant pancreatic cancer cell lines. These results suggest that MRP2 is associated with intrinsic and acquired CDDP resistance in human pancreatic cancer. Moreover, MRP2 expression may be considered a novel chemoresistance marker in patients with pancreatic cancer who are being treated with CDDP-based chemotherapy.

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