

Influence of Chemotherapeutic Treatment on Expression of Human Multidrug Resistance Protein (ABCC, ABCB) Family Members in Pancreatic Cancer Cell Lines

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ABSTRACT

AIM: Chemotherapeutic treatment of pancreatic carcinomas is often impeded by intrinsic multidrug resistance (MDR). This MDR phenotype can be caused by transporters of the MDR P-glycoprotein (ABCB) or multidrug resistance related protein (MRP) family (ABCC). To elucidate the role of ABCB and ABCC family members in pancreatic carcinomas, we analyzed mRNA expression of MDR-1, MDR-3, MRP-1, MRP-3 and MRP-5, which have been shown to confer resistance to chemotherapeutic drugs.

METHODS: mRNA expression was quantified in 10 different human pancreatic carcinoma cell lines before and during continuous in vitro chemotherapy including gemcitabine (0.5 µg/mL), 5-fluorouracil (5-FU; 0.5 µg/mL), cisplatin (0.05 µg/mL) or the combination of 5-FU and cisplatin.

RESULTS: Quantitative RT-PCR demonstrated high base line expression levels of MRP-3>MRP-1>MRP-5 in the pancreatic cell lines Panc-1, KCI-MOH1, MIAPaCa-2, PK-1, PK-8, PK-9 and AsPC-1, whereas PaTu8902, HuP-T4 und FAMPAC revealed low base line mRNA transcript levels. 5-FU and cisplatin caused a significant elevation of MRP-3>MRP-1>MRP-5, while gemcitabine affected the mRNA expression less.

CONCLUSION: MRP-1, MRP-3 and MRP-5 are likely to be involved in the MDR phenotype. The majority of pancreatic carcinoma cells express high levels of MRPs even without prior chemotherapeutic treatment. Quantification of MRP expression may predict individual tumor responses and guide chemotherapeutic treatment.

INTRODUCTION

Pancreatic carcinoma is the fifth leading cause of human cancer death in Western countries, with an overall 5 year survival of less than 5% despite improved treatment regimens^[1,2]. At the time of diagnosis, many patients are not resectable due to advanced tumor stage or metastases. Consequently, chemotherapy is the remaining treatment strategy in these patients^[3,4]. Gemcitabine (GEM) has become standard therapy for patients with advanced pancreatic cancer in recent years^[5] but its efficacy is limited. Impact of combination chemotherapy, such as GEM plus cisplatin is still under debate^[6,7].

Primary and acquired resistance of tumor cells to antineoplastic drugs is a major cause of the limited efficiency of chemotherapy^[8] and consists of several processes as increased drug efflux and decreased accumulation of drugs in the cell^[9].

Major determinants of chemoresistance in tumor cells are efflux transporters of the ATP-binding cassette transporter family as the ABCB1 (multidrug resistance 1, MDR1) and the ABCC (multidrug resistance-associated protein, MRP) family^[10,11,12]. These transporters are capable of regulating the intracellular concentration and tissue distribution of drugs and their metabolites and therefore represent an important mechanism in drug resistance^[13].

Currently the human MDR family has eleven members while the MRP family consists of nine members. At least three members of the MRP family (MRP-1, MRP-2 and MRP-3) are known to be correlated with chemoresistance^[10].

Some studies show in pancreatic cancer tissue and cell lines a high rate of MDR1 expression^[14,15] while other studies demonstrate an association of MDR1 with sensitivity to GEM^[16].

The members of the MRP family show different substrate specificity, tissue distribution, and cellular localization^[10]. It is known that MRP-1, MRP-2, and MRP-3 transport lipophilic compounds conjugated with glutathione, glucuronate, or sulphate while MRP4 and MRP-5 transport nucleotide analogs and cyclic nucleotides. MRPs are as well involved exporting organic anions of xenobiotics and are important factors for resistance to drugs^[10].

In our study we analyzed the mRNA expression of MDR-1, MDR-3, MRP-1, MRP-3 and MRP-5 in 10 pancreatic carcinoma cell lines. We subsequently quantified the mRNA expression of MDR-1, MDR-3, MRP-1, MRP-3 and MRP-5 by quantitative real-time RT-PCR.

There is only limited data concerning the change in expression profile of human multidrug resistance proteins in pancreatic cancer after chemotherapeutic treatment. To elucidate the role of different chemotherapeutic agents on mRNA expression of these proteins, we quantified mRNA expression during continuous *in vitro* chemotherapy (14 days or 28 days) including GEM (0.5 µg/mL), 5-fluorouracil (5-FU) (0.5 µg/mL), cisplatin (0.05 µg/mL) or the combination of 5-fluorouracil and cisplatin.

In terms of response prediction quantification of MDR/MRP expression levels may be helpful to guide therapy decisions and to predict individual tumor responses towards chemotherapeutic treatment.

METHODS

Cell culture

Ten human pancreatic carcinoma cell lines (Table 1) were kindly provided by the tumor bank of the DKFZ (German Cancer Research Center, Heidelberg, Germany). All cell lines were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Mycoplasma screening was performed routinely using a standard PCR technique.

Table 1 Analyzed pancreatic cancer cell lines with corresponding references.

| Cell line | Reference |
|------------|--|
| HuP-T4 | Nishimura N <i>et al</i> ^[35] |
| KCI-MOH1 | Mohammad RM <i>et al</i> ^[36] |
| PaTu 8902 | Elsässer HP <i>et al</i> ^[37] |
| AsPC-1 | Tan MH <i>et al</i> ^[38] |
| FAMPAC | Eisold S <i>et al</i> ^[39] |
| Panc-1 | Lieber M <i>et al</i> ^[40] |
| MIA PaCa-2 | Yunis AA <i>et al</i> ^[41] |
| Pk-1 | Kobari M <i>et al</i> ^[42] |
| Pk-8 | Kobari M <i>et al</i> ^[42] |
| Pk-9 | Kobari M <i>et al</i> ^[42] |

Drugs and chemotherapeutic treatment

Drug concentrations used were determined by long-time *in vitro* treatment of the cell line FAMPAC with several concentrations of the drugs. The concentrations which gave best outgrowth of resistant clones were used subsequently to treat all cell lines used in this study. This treatment consisted of single and combined application of 5-Fluorouracil (GRY-Pharma, Kirchzarten, Germany), Cisplatin (Bristol-Myers Squibb, Munich, Germany) and GEM (Lilly, Gießen, Germany). For single application 3.84 µM of 5-FU and 1.67 µM of cisplatin was added to the medium for 28 days. Due to higher toxicity 1.90 µM GEM was added to the medium for only 14 days. The combined treatment with 3.84 µM of 5-FU plus 1.67 µM of cisplatin was administered for 28 days.

RNA isolation and Reverse Transcription

The High pure RNA isolation kit from Roche was used to achieve

high quality nucleic acid purification. After centrifugation of 1×10^6 cells at 1200 rpm the cells were lysed by using a buffer containing 4.5 M Guanidiniumchloride, 50 mM Tris-HCL supplemented with 30% Triton X-100. Thereafter the lysates were transferred to high pure filter tubes. After the following centrifugation at 10.000 rpm for 15 min, DNase I was added for 15 min. The lysates were washed twice with wash buffer I/II, resuspended and incubated in 70-100 µL of sterile water at -70°C. One µg of RNA was reverse transcribed using 0.8 µL of avian myeloblastosis virus reverse transcriptase and 2 µL of the oligo-(dt) primer (1st Strand cDNA-Synthesis Kit for RT-PCR, Roche), according to the kit's instructions. The final volume of 20 µL was stored at -20°C until PCR analysis.

Quantitative real time PCR

Specific primer sets for MDR-1, MDR-3, MRP-1, MRP-2, MRP-3 and MRP-5 for the LightCycler™ system were developed as summarized for MDR-1, MDR-3 and MDR-5 in table 2. Quantitative real time PCR was performed using SYBR Green. A working mix of 10 µL was adjusted, consisting of 6 µL of sterile water, 1 µL of each sense- and antisense primer, and 2 µL SYBR Green Mix. The copy number was calculated from a standard curve, obtained by plotting known concentrations of control plasmids at log dilutions to the PCR-cycle number at which the detected fluorescence intensity reached a fixed value. To correct differences in the content of total RNA, calculated copy numbers were normalized according to the average expression of two housekeeping gene, cyclophilin B and β-actin. Values were given as input adjusted copy number per microliter of cDNA.

Statistical analysis

The Student's *t*-test was used to test whether differences between two groups were significant. Data are expressed as means±SD, if not otherwise indicated. Analyses were performed using Graphpad Prism software, and *p*-values <0.05 were considered significant.

RESULTS

Overview of the RT-PCR results of the multidrug resistance protein family members in pancreatic cancer cells

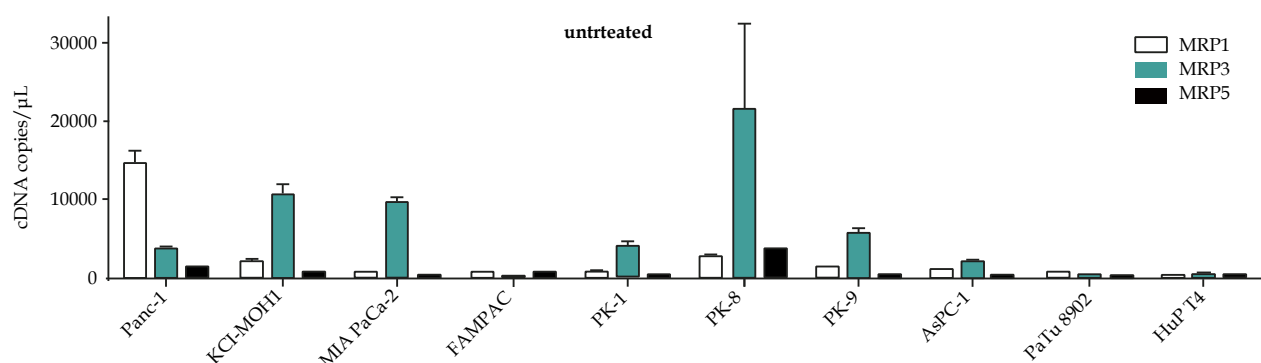
The first aim was to investigate the mRNA expression profile of the ABCB and ABCC family members, namely MDR-1, MDR-3, MRP-1, MRP-2, MRP-3 and MRP-5 in 10 different human pancreatic cancer cell lines. The expression analysis was performed using the LightCycler™ system. Indicated primer pairs were tested for their specificity and affinity against the respective cDNA template to ensure that this experimental setup can be used for detecting specific MDR or MRP mRNA and for quantifying the amount of the respective mRNA in a given sample.

An overview of the PCR results of all analyzed pancreatic cancer cells lines given as mean values (cDNA copies/µL) is shown in figure 1. RT-PCR results for MRP-1, MRP-3 and MRP-5 showed a high base line expression of transcripts (Figure 1), whereas mRNA of MRP-2, MDR1 and MDR3 could only be found in concentrations at the detection limit in all investigated pancreatic cancer cell lines (data not shown). Interestingly transcripts for MDR1 were below the detection limit in 5 of 10 pancreatic cancer cell lines and transcripts for MDR3 in 3 of 10 pancreatic cancer cell lines. The highest base line mRNA levels in untreated pancreatic cancer cell lines were detected for MRP-3 (range of 127-21685 copies/µL), followed by MRP-1 (range of 175-14736 copies/µL) and MRP-5 (range of 54-3697 copies/µL) (Figure 1).

Extent of base line expression was different in the examined pan-

Table 2 Primer positions and length of the amplified fragment used for quantitative RT-PCR.

| | Sense primer | Position | Antisense Primer | Position | Fragment length (bp) |
|------|-------------------------------|-----------------|-----------------------------|-----------------|----------------------|
| MRP1 | 5'-CTGACAAGCTAGACCATGAATGT-3' | bp 4244-bp 4269 | 5'-TCACACCAAGCCGGCGTCTTT-3' | bp 4596-bp 4576 | 353 |
| MRP3 | 5'-GGACCTGCGCATGAACCTG-3' | bp 4133-bp 4152 | 5'-AGGCAAGTCCAGCATCTCTGG-3' | bp 4582-bp 4562 | 450 |
| MRP5 | 5'-GCTGTTCAGTGGCACTGTCAG-3' | bp 3834-bp 3854 | 5'-TCAGCCCTTGACAGCGACCTT-3' | bp 4314-bp 4294 | 481 |

**Figure 1** Overview of base line mRNA expression of MRP-1, MRP-3 and MRP-5 in pancreatic cancer cells. Synthesized single stranded DNA's were purified using a high pure isolation kit and MRP-1, MRP-3 and MRP-5 mRNA levels were determined in relation to two housekeeping genes β -actin and cyclophilin-B (CBP) using the LightCycler System and the FastStart DNA Sybr Green I kit. mRNA expression level for each parameter is shown as mean SD (cDNA copies/ μ L).

creatic cell lines. Panc-1, KCI-MOH1, MIA PaCa-2, PK-1, PK-8, PK-9 and AsPC-1 showed high base line expression levels whereas PaTu 8902, HuP-T4 und FAMPAC revealed low base line levels of these mRNA transcripts (Figure 1).

Relationship of the mRNA expression levels of multidrug resistance protein family members to the action of different chemotherapeutic drugs

The next step of our study was to analyze the changes in mRNA expression of our defined multidrug resistance proteins due to the treatment with different chemotherapeutic drugs. As indicated above we found no significant elevation of mRNA expression of MRP-2, MDR1 and MDR3, therefore we limited our analysis on MRP-1, MRP-3 and MRP-5.

After chemotherapeutic treatment significantly elevated mRNA expression of MRP-1 could be detected in 8 of 10 cell lines. Continuous in vitro 5-FU treatment induced an elevated mRNA expression in 5 out of 10 cell lines, the combination of Cisplatin and 5 FU in 5 cell lines, Cisplatin alone in 5 cell lines and GEM in 3 out of 10 cell lines (Table 3).

Continuous chemotherapeutic treatment up regulated MRP-3

Table 3 Summary of significant elevation of mRNA expression levels of MRP-1, MRP-3 and MRP-5 in the analyzed cell lines classified to chemotherapeutic drugs. Results $p > 0.05$ are marked as ns.

| | Chemotherapeutic treatment | | | |
|------------|----------------------------|-----------|-----------------|----------------|
| | 5-FU | cisplatin | 5-FU/ cisplatin | GEM |
| Panc-1 | MRP-3 | MRP-3 | MRP-3 | MRP-3 MRP-5 |
| KCI-MOH1 | MRP-1 | MRP-1 | ns | MRP-1 |
| | MRP-3 | MRP-3 | | MRP-3 |
| MIA PaCa-2 | MRP-1 | MRP-1 | MRP-1 | MRP-1 |
| FAMPAC | MRP-1 | MRP-1 | MRP-1 | ns |
| | MRP-3 | MRP-3 | MRP-3 | |
| | MRP-5 | | | |
| PK-1 | MRP-5 | ns | MRP-5 | ns |
| PK-8 | ns | MRP-1 | ns | ns |
| PK-9 | MRP-1 | ns | MRP-1 | ns |
| AsPC-1 | MRP-1 | MRP-3 | MRP-1 | MRP-1 |
| | MRP-3 | | MRP-3 | MRP-3 |
| | MRP-5 | | | |
| PaTu 8902 | MRP-3 | MRP-3 | MRP-1 | MRP-3 |
| | | | MRP-3 | |
| HuP-T4 | MRP-3 | MRP-3 | MRP-3 | ns |

mRNA markedly higher when compared to MRP-1 and MRP-5 (range copies/ μ L). In 6 out of 10 cell lines a significant mRNA elevation of MRP-3 could be detected. Treatment with 5-FU alone induced a significant elevation in 6 cell lines, combination of 5-FU and cisplatin in 5 cell lines, cisplatin alone in 6 cell lines and GEM in 4 cell lines (Figure 2, Table 3).

At last our mRNA expression analysis showed that MRP-5 is least inducible compared to MRP-1, MRP-3. Continuous chemotherapeutic treatment induced a significant rise in MRP-5 expression levels in 4 out of 10 cell lines. Chemotherapeutic treatment with 5-FU alone induced significant expression of MRP-5 in 3 cell lines, combination of 5-FU and cisplatin in 1 cell line as well as GEM in 1 cell line. Continuous cisplatin treatment did not induce a significant elevation of MRP-5 expression in any of the ten cell lines (Figure 2, Table 3).

Different potency of chemotherapeutic drugs on induction of expression of multidrug resistance protein family members

To study the impact of different chemotherapeutic drugs on induction of mRNA expression of multidrug resistance proteins, pancreatic cancer cell lines were in vitro treated either with 5-FU, cisplatin, 5 FU/cisplatin or GEM.

Here, MRP-1, MRP 3 and MRP-5 expression was found to be triggered best by 5-FU with a total of 14 significant up regulations (MRP1, MRP3 and MRP5 taken together). Cisplatin treatment induced an up regulation of 10 mRNAs. The combination treatment of 5-FU and cisplatin led to a significant up regulation of 11 mRNAs. It was thus very similar to cisplatin alone. Treatment with GEM affected the expression of MRP mRNAs less than the other in vitro treatments with a total of 8 significant up regulations (Figure 2, Table 3).

DISCUSSION

Treatment of non-resectable solid tumors is significantly influenced by resistance of cancer cells to anticancer agents. Particularly treatment of advanced pancreatic cancer is dependent of response to chemotherapeutic treatment as many patients are inoperable at time of diagnosis.

Multidrug resistance proteins are an important defence mechanism of cancer cells against carcinostatic drugs^[10]. These proteins are efflux transporters of the ATP-binding cassette as the ABCB1

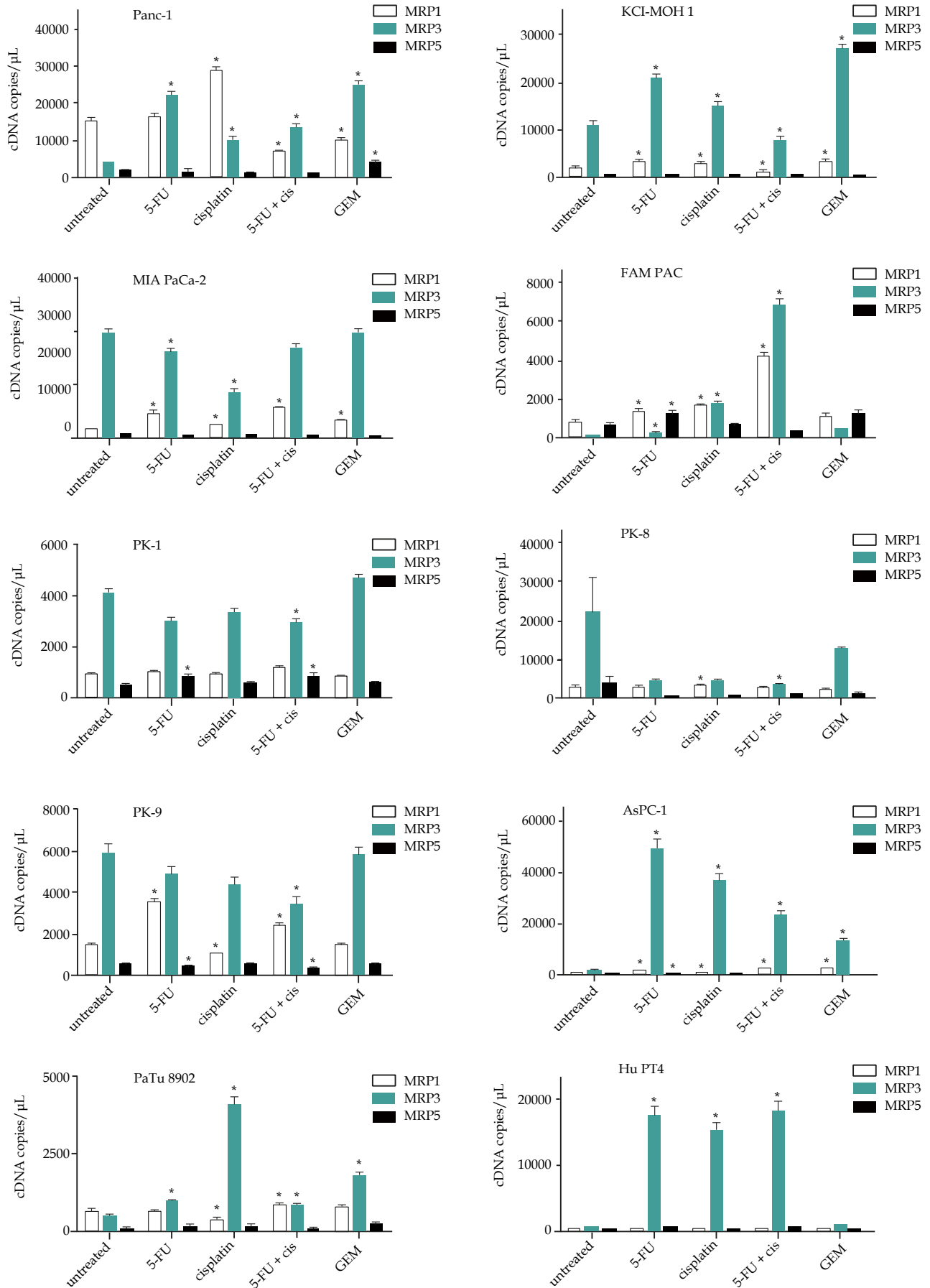


Figure 2 mRNA expression of MRP-1, MRP-3 and MRP-5 in pancreatic cells after *in vitro* treatment with 5-FU, cisplatin, 5-FU/ cisplatin or GEM. The Student's *t*-test was used to test whether differences in comparison to the untreated controls were significant. Data are expressed as means±SD, if not otherwise indicated. Analyses were performed using Graphpad Prism software, and *p*-values<0.05 were considered significant.

(multidrug resistance 1, MDR1) and the ABCC (multidrug resistance-associated protein, MRP) family^[10,11,12]. Quantification of expression levels of these proteins may be helpful to guide therapy decisions and to predict individual tumor responses towards chemotherapeutic treatment. There are several reports concerning the role of multidrug resistance proteins in pancreatic carcinoma^[9,17,18,19,15] but little is known about the change in expression levels of these proteins after chemotherapeutic treatment.

To further address this question we treated different pancreatic carcinoma cell lines with current chemotherapeutic drugs and analyzed mRNA expression levels of multidrug resistance proteins MDR-1, MDR-3, MRP-1, MRP-2, MRP-3 and MRP-5.

In the examined cell lines we found relevant expression of MRP-1, MRP-3 and MRP-5 whereas mRNA levels of MRP-2, MDR1 and MDR3 were found at the lowest level of detection. These findings are on par with observations showing expression of MRP1, MRP3, MRP4 and MRP5 on mRNA level in tissue samples of normal human pancreas and pancreatic carcinoma^[9,20]. Another aspect of this quantitative RT-PCR analysis is the observed different base line expression pattern. The cell lines KCI-MOH1, MIA PaCa-2, PK-1, PK-8, PK-9 and AsPC-1 showed high base line expression of MRP-1, MRP-3 and MRP-5 whereas PaTu 8902, HuP-T4 und FAMPAC revealed low base line levels of these mRNA transcripts. This *in vitro* observed heterogeneous cell line specific expression pattern of MRP's may to some extent reflect the individual response of human pancreatic tumors *in vivo* with respect to expression of relevant transporters involved in chemotherapeutic resistance.

More interesting than base line expression of these MRPs are changes in expression patterns after chemotherapeutic treatment as possible adaptive mechanism of the cell, diminishing the potential impact of the drug by accelerating it's elimination from intracellular compartments. Corresponding to examination of base line expression we found only relevant expression of MRP-1, MRP-3 and MRP-5 whereas mRNA levels of MRP-2, MDR1 and MDR3 were at the lowest level of detection even after chemotherapeutic treatment.

5-FU is a key drug in the treatment of a variety of tumors including colorectal, breast and pancreatic cancer. Several reports could show resistance to 5-FU due to MRP associated cellular efflux processes^[21,22]. In the present study we were able to show a significantly elevated expression of MRPs after 5FU treatment *in vitro* in different cell lines. In descending order MRP3, MRP1 and MRP5 were significantly expressed after 5-FU treatment but in a heterogeneous pattern in the analyzed cell lines. Haggmann *et al*^[23] observed elevated mRNA expression of these MRPs in the pancreatic carcinoma cell line Capan-1 and Hoffmann *et al*^[24] could show an elevated expression of MRP1 and MRP3 in an orthotopic Panc02 pancreatic carcinoma mouse model after 5-FU treatment. Recent reports demonstrated the involvement of MRP5 in conferring drug resistance to 5-FU especially in pancreatic cancer^[23,25]. Our findings underline these results and support a strong involvement of at least MRP5 in the resistance to 5-FU.

Cisplatin is a widely used drug in cancer therapy for different digestive tumors although therapy effectiveness is limited due to high intrinsic resistance of some targeted cells. In this study, we found significant elevated expression of MRP3 and to a lesser extent of MRP1 after cisplatin treatment. Involvement of MRPs in resistance to platinum based chemotherapeutic drugs in pancreatic carcinoma is rarely examined. However, there are several reports on elevated expression of MRPs in different cell lines resistant to platinum based chemotherapeutic drugs in other tumor entities including

melanoma, hepatocellular carcinoma, hepatoblastoma as well as bladder, prostate, colon, ovarian and adrenocortical cancer^[26,27,28,29]. In addition, high MRP1 expression seems to correlate with poorer outcome in cisplatin treated non-small-cell lung cancer^[30]. Noma *et al*^[31] demonstrated MRP2 and MRP1 elevation in samples of human pancreatic carcinoma whereas MRP3 was only expressed in cisplatin resistant pancreas cancer cell lines (SUIT-2, MIA PaCa2).

Another interesting aspect of the present study was the finding that combination treatment of 5-FU and cisplatin induced mRNA elevation comparable to single drug treatment.

Resistance mechanisms of tumor cells to GEM are not fully understood but there is increasing evidence of the involvement of efflux transporters of the ABCC family. GEM or its phosphorylated metabolites were reported to be a typical substrate for the MRP5 efflux pump^[32]. One study demonstrated a significant association between MRP5 expression levels and GEM in a non-small cell lung cancer cell line^[33]. Another study by Davidson *et al*^[32] demonstrated that MRP5 over expressing HEK293 cells were twice as resistant to GEM as vector control cells. A recent study by Haggmann *et al*^[34] demonstrated increased expression levels of MRP5 after GEM treatment in several pancreatic cancer cell lines.

The analysis showed that GEM, compared to 5-FU or cisplatin, was a markedly less potent trigger for MDR expression. In five (Panc-1, KCI-MOH1, MIA PaCa-2, AsPC-1, PaTu 8902) out of ten analyzed cell lines, a significant elevation of MDRs was observed, but only Panc-1 showed a significant elevation of MRP5, whereas PaTu 8902 and FAMPAC showed no significant albeit twofold elevated expression of this efflux transporter. The observed missing induction of MRPs expression after GEM treatment might contribute to the clinically observed better efficacy of GEM compared to 5-FU or cisplatin in chemotherapeutic treatment of pancreatic carcinoma.

In conclusion our results showed that (1) MRP1, MRP3 and MRP5 are likely to be involved in the multidrug resistance phenotype often seen in pancreatic carcinomas, whereas MDR-1, MDR-3 and MRP2 seem to be of minor importance; (2) the majority of pancreatic carcinoma cells express high levels of MRPs even without prior chemotherapeutic treatment; (3) the observed response heterogeneity of different pancreatic cancer cell lines might reflect *in vitro* the known heterogeneous response to chemotherapeutic treatment of pancreatic cancer *in vivo*; (4) chemotherapeutic treatment of pancreatic cancer cells with 5-FU, cisplatin or GEM can significantly change the expression levels of these relevant efflux pumps and finally; and (5) that 5-FU and cisplatin show a significant higher capability of inducing increased MDR expression when compared to GEM.

Thus, quantification of MDR expression before and while chemotherapeutic treatment might be an important tool to improve effectiveness of chemotherapeutic regimens and an important step towards a more individualized treatment in terms of guidance of therapy decisions and prediction of individual tumor responses. In addition, the development of specific inhibitors of MDR and MRP efflux pumps might enhance chemotherapeutic effectiveness.

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