MDR1/P-glycoprotein and MRP-1 Drug Efflux Pumps in Pancreatic Carcinoma

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Abstract. *Background: Pancreatic cancer is one of the most challenging solid organ malignancies. This is due to its aggressiveness, frequent late presentation as advanced disease and chemoresistance. A better understanding of the molecular basis of its drug resistance is needed. Materials and Methods: In this study, the first of its kind, the expression of both MDR1 P-gp and MRP-1 protein in pancreatic tumour specimens was examined by immunohistochemistry. Expression of these drug efflux pumps was examined using semi-quantitative immunohistochemistry according to the percentage of cells within the tumour, demonstrating another staining intencity. Results: Overall, 93.3% of pancreatic carcinomas expressed MDR1 P-gp, approximately 31% co-expressed MRP-1 with MDR1 P-gp, while 6.7% expressed neither of these proteins. Conclusion: Our results show that drug efflux pumps, in particular that of MDR1 P-gp, are frequently expressed in pancreatic cancer. While a causative role for these efflux pumps in pancreatic cancer chemoresistance cannot necessarily be concluded, the information presented here should be considered when selecting chemotherapy/drug efflux pump inhibitors for future therapies.*

Pancreatic cancer, the 4-5th leading cause of cancer-related death in the Western world, is a devastating disease with poor prognosis (1). More than 232,000 cases of this cancer are diagnosed each year (2), with cigarette smokers 2-3 times more likely to develop this disease than non-smokers (3). The majority of pancreatic cancers develop in people older than 50 years, the risk increases with advancing years (4), and 57% of new cases occur in females (5). The most common form of pancreatic cancer is adenocarcinoma of the ductal epithelium and the majority of patients present with advanced disease (4). This is reflected in the 5-year survival rate estimation of <5%, with an average survival after diagnosis of 3 to 6 months, and an overall long-term survival rate of only 0.4% (2, 6). Only a minority of the presenting patients are suitable for resection; the majority of tumours are not resectable due to metastasis and invasion of major vessels posterior to the pancreas (4). The resectable minority (2.6-9%) demonstrate 5-year survival rates of 10-18% with morbidity and low mortality (2).

Pancreatic cancer is a serious challenge to oncology due to its rising incidence and poor survival results (7). At present, surgery offers the only chance of cure to the pancreatic cancer patient; however, morbidity remains high at 30-40%, complications are common, and even after complete resection (where possible) most tumours recur, since local and systemic disease is still likely to be present even after the best resection (1, 8). Adjuvant chemotherapy, but not adjuvant radiotherapy, has been proposed to result in a slight survival benefit. Meta-analysis of five randomised trials including adjuvant chemotherapy and chemoradiation following resection of ductal adenocarcinomas showed a 19-month survival with chemotherapy – compared to 13.5 months without this treatment – and an associated 25% significant reduction in risk of death. This was not found to be the case with chemoradiation; indeed, it has been found that patients receiving chemoradiation may even do worse than those not (9). Some studies have reported gemcitabine-based therapies to be the best possible option currently available for pancreatic cancer patients (10-12), while other studies have shown that, although post-operative chemotherapy significantly improves survival, there is no difference between the regimes of adjuvant chemotherapy used (when comparing intra-arterial 5-fluorouracil twice

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weekly; systemic gemcitabine, by intravenous injection, bi-weekly; or intra-arterial 5-fluorouracil and systemic gemcitabine bi-weekly (13)). Similarly, trials including 5-fluorouracil, leucovorin, epirubicin and carboplatin, with or without gemcitabine, have shown encouraging results with only mild toxicity (14). No standard second-line treatment has been identified for patients who are refractory to first-line treatment.

The very limited use of chemotherapy for pancreatic cancer patients is associated with the inherent chemoresistant nature of this devastating disease. While the ATP-dependent membrane-bound drug efflux pumps, including MDR1 P-glycoprotein (MDR1 P-gp), and MRP-1 may be mediators of clinically relevant chemo-resistance/multiple drug resistance (MDR), studies of the prevalence of MDR1 P-gp and MRP-1 in pancreatic tumours have been limited and conflicting. To determine the potential involvement of these drug efflux pumps in the clinical setting, we report the first study of both MDR1 P-gp and MRP-1 protein expression in pancreatic tumours.

Materials and Methods

Patients. The patient group studied comprised of 45 consenting patients with primary tumours of the pancreas. All patients were treated at St. Vincent's University Hospital (SVUH), Dublin, between 1999 and 2003 and approval to conduct this study was granted by SVUH Ethics Committee. Pathological material was examined on each case by SK. Formalin-fixed paraffin-embedded material was available for all patients. Representative 4-μm sections of tissue blocks were cut using a microtome, mounted onto poly-l-lysine coated slides and dried overnight at 37˚C. Slides were stored at room temperature until required.

Immunohistochemistry. All immunohistochemical studies on formalin-fixed paraffin wax-embedded tissue sections were performed following the method of Hsu *et al.* (15), using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (Vectastain Elite ABC, Vector Laboratories, UK) plus an appropriate secondary antibody. Sections (4-μm thick) were dewaxed in xylene (2x5 minutes) rehydrated in grading alcohols 100%, 90% and 70% (2x3 minutes) and placed in Tris-buffered saline (TBS/0.1% Tween-20). Endogenous peroxidase activity was quenched by placing tissue sections in 3% (v/v) H_2O_2/distilled water for 5-7 minutes at room temperature. All slides were blocked for non-specific staining with 20% normal rabbit serum (X-902, Dako, Glostrup, Denmark)/TBS for 20 minutes at room temperature. Primary antibodies were applied to each specimen optimally diluted in TBS/0.1% Tween-20 (anti-MDR-1, clone 6/1C (National Institute for Cellular Biotechnology (16): ascites diluted 1:40; anti-MRP-1 monoclonal antibody (AlexisALX-801-007-C125: neat/undiluted)). Primary antibodies were incubated overnight at 4˚C. Specimens were then washed (3x5 minutes) with TBS/0.1% Tween-20, followed by a 30-minute incubation with biotinylated secondary antibody (rabbit anti-mouse IgG (1/300 dilution in TBS/0.1% Tween-20) (Dako, E345) or rabbit anti-rat (1/500 dilution in TBS/0.1% Tween-20). Finally, following another 3x5 minute wash step, Vectastain Elite ABC reagent (HRP conjugated) (Vector Laboratories, UK) was applied for 25 minutes at room temperature. The peroxidase substrate, 3',3-daminobenzidine tetrahydrochloride (DAB) containing 0.02% H_2O_2 (Vector Laboratories) was added for 10 minutes at room temperature. All slides were washed (3x5 minutes) TBS/0.1% Tween-20. Tissue sections were then lightly counterstained with haematoxylin (Vector Laboratories).

Following this, slides were dehydrated in graded alcohols 70%, 90% and 100% (2x3 minutes). Specimens were then cleared in xylene and mounted in DPX (BDH, UK). Negative control specimens in which primary antibody were replaced by 1X TBS/0.1% Tween-20 were included in all experiments. Positive controls (normal kidney and lung tissue) using the same experimental conditions were included in all experiments.

Immunohistochemical scoring. MRP-1 and MDR1 P-gp immunohistochemical staining was evaluated semi-quantitatively, according to the percentage of cells showing specific immunoreactivity and the intensity of this immunoreactivity. Scoring involved evaluation of at least 5 fields of view per slide, by two independent observers. In the case of MRP-1 protein and MDR1 P-gp, membrane and cytoplasmic staining was scored as positive or negative. A semi-quantitative measurement was used in which overall positivity of the tumour was assessed and a score of 1+ was given where up to 25% of cells showed MRP-1/ MDR1 P-gp positive staining; a score of 2+ was given where ≥25 but <50% of cells showed MRP-1/ MDR1 P-gp positive staining; a score of 3+, where ≥50% but <75% of cells showed positive staining and a score of 4+, where ≥75% of cells showed positive staining. For assessment of both MRP-1 protein and MDR1 P-gp, the intensity of immunoreactivity was scored as 1 (weak), 2 (moderate), or 3 (strong).

Results

Patient characteristics. This study involved analysis of tumours from 45 pancreatic cancer patients. For those for whom relevant information was available (*i.e.* 41 of the cases), ages ranged between 32 and 79 years at the time of diagnosis (median age = 62 years). Of the 42 cases where information on tumour type was available, 34 were adenocarcinoma, 4 were endocrine/neuro-endocrine, 3 were cholangiocarcinoma and one was a retro-pancreatic malignant gastrointestinal stromal tumour. Seven tumours were known to be poorly-differentiated; twenty-five were- moderately differentiated; and six were well-differentiated (data was unavailable for 7).

MDR1 P-gp expression. MDR1 P-gp specific staining was observed in 93.3% of the pancreatic tumours analysed. A representative MDR1 P-gp positive tumour is shown in Figure 1A. Specific staining was localised to the cell membrane/cytoplasm. Approximately 7% (3/45) of tumours did not express MDR1 P-gp protein. As indicated in Table I, the breakdown of the distribution of MDR1 P-gp in positive tumours was as follows: approximately 12% (5/42) scored 1, 9.5% (4/42) scored 2, 24% (10/42) scored 3, while approximately 55% (23/42) scored 4. Weak staining was

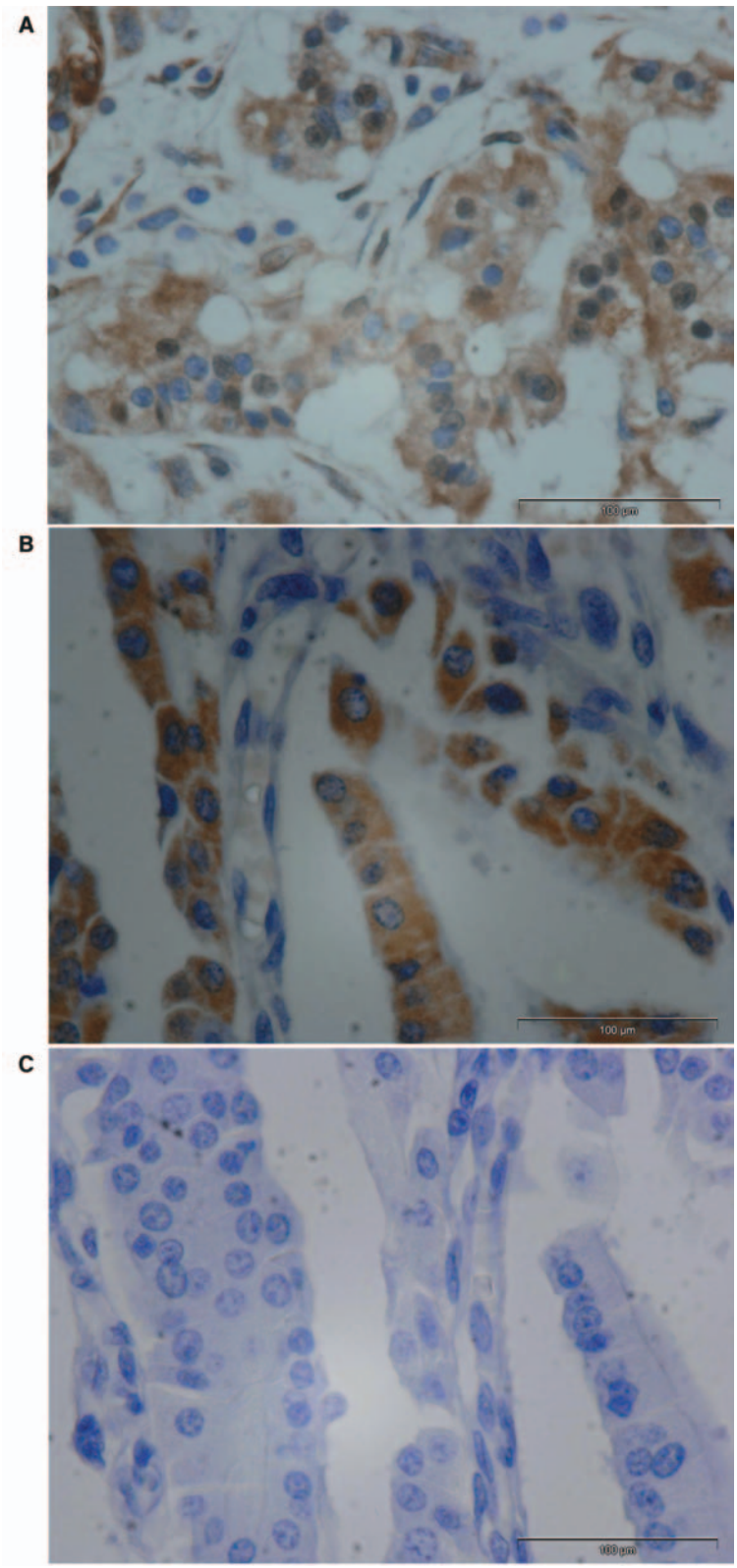

Figure 1. *Pancreatic adenocarcinoma from a patient with a moderately-differentiated tumour showing intense (score 4+3, both cases) (A) MDR1 P-gp and (B) MRP-1 positive staining in tumour cells, stained using peroxidase substrate, 3',3-daminobenzidine tetrahydrochloride (DAB) substrate. (C) Negative control where primary antibody was not included (original magnification 40x; scale bar=50 µm).*

Table I. *MDR1 P-gp and MRP-1 protein expression in pancreatic tumours.*

	No. of cases	Staining Intensity		
		Strong	Moderate	Weak
MDR1 P-gp				
4+ (≥75%)	23	13	9	1
3+ (≥50%-<75%)	10	3	6	1
2+ (≥25-<50%)	4	0	4	0
1+ (1-<25%)	5	0	3	2
0	3	0	0	0
MRP-1				
4+ (≥75%)	2	1	1	0
3+ (≥50%-<75%)	8	2	3	3
2+ (≥25-<50%)	2	1	0	1
1+ (1-<25%)	2	1	0	1
0	31	0	0	0

Table II. *Comparison of MDR1 P-gp and MRP-1 co-expression in all pancreatic carcinomas studied.*

Drug efflux pump	% Cases
$MRP1^+/MDR1Pgp^+$	31
$MRP1^-/MDR1Pgp^+$	62.3
$MRP1^+/MDR1Pgp^-$	0
$MRP1^-/MDR1Pgp^-$	6.7

Table III. *Comparison of MDR1 P-gp and MRP-1 co-expression in adenocarcinomas only.*

Drug efflux pump in adenocarcinomas (34 cases)	% Cases
$MRP1^+/MDR1Pgp^+$	29.4
$MRP1^-/MDR1Pgp^+$	64.7
$MRP1^+/MDR1Pgp^-$	0
$MRP1^-/MDR1Pgp^-$	5.9

observed in 9.5% (4/42) cases, moderate staining in 52.5%, and 38% stained strongly.

MRP-1 expression. MRP-1 specific staining was observed in 31% (14/45) of tumours analysed. Figure 1B illustrates a typical example; Figure 1C is a typical negative control. Sixty-nine percent (31/45) of tumours did not show MRP-1 protein expression. The breakdown of distribution of MRP-1 staining in positive tumours was as follows: 14.3% (2/14) cases scored 1 and scored 2 respectively, 57% (8/14) scored 3 and 14.3% (2/14) scored 4. Weak staining occurred in approximately 43% (6/14) cases, with moderate staining in 28.6%. The remaining 28.6% (4/14) stained strongly (Table I).

MDR1 P-gp and MRP-1 co-expression. As summarised in Table II, the majority (93.3%) of pancreatic tumours expressed drug efflux pumps analysed in this study; only 6.7% expressed neither MDR1 P-gp nor MRP-1 protein. Most of the pancreatic tumours (62.3%) expressed MDR1 P-gp protein, without detectable MRP-1, while a further 31% co-expressed both of these efflux pumps. No cases were identified where MRP-1 was detectable independent of MDR1 P-gp expression. Table III indicates that this trend was reflective of the adenocarcinoma sub-population (which was the majority of cases included). The analysis of the other pancreatic tumour types indicated that most (3/4 cases) endocrine/neuro-endocrine cases expressed MDR1 P-gp, but not MRP1, while the remaining specimen expressed both efflux pumps. Three cholangiocarcinoma specimens were analysed; one expressed both proteins, one expressed MDR1 P-gp alone, and the third expressed neither efflux pump. The retro-pancreatic malignant GIST specimen had an MDR1 $P\text{-gp}^+/MRP1^-$ phenotype.

Discussion

Pancreatic cancer is one of the most challenging solid organ malignancies due to its aggressive nature, frequent late presentation with advanced disease, and chemoresistant biology. While palliative chemotherapy treatment is reported to be superior to best supportive care (12), its effectiveness is compromised and very limited due to the yet undefined resistant nature of this cancer.

The drug efflux pumps, MDR1 P-gp and MRP-1, have been associated with chemoresistance (multiple drug resistance/MDR) in a number of cancer types; however, their correlation with resistance in pancreatic cancer remains to be elucidated. To date, the main focus of research in this area has been based on pancreatic cell line models. While these studies suggest a role for MDR1 P-gp and MRP-1 in pancreatic carcinoma chemoresistance, results from such analyses are far from definitive. Experimental research using pancreatic cell line models have indicated that both MDR1 P-gp and MRP-1 may have a role to play in intrinsic drug resistance in pancreatic cancer, although the results from these studies are somewhat conflicting with the apparent relative importance of these efflux pumps differing from study to study and from cell line to cell line. Western blotting and cellular accumulation (of rhodamine 123, substrate for MDR1 P-gp; [^{3}H] vincristine, substrate for MRP-1) functional assays, assessing PANC-1, BxPC-3, AsPC-1, and Capan-1 cell lines, indicate that pancreatic cells express MRP-1 but very little, if any, MDR1 P-gp. This suggests that intrinsic MDR in

pancreatic cancer may be due in part to MRP-1, but not MDR1 P-gp (17). This observation was supported by reported expression of MRP-1 in 97% (18) and 87% (19) of pancreatic tumour cell lines. Conversely, analysis of SUIT-2 and its taxotere-selected populations suggest that both intrinsic and acquired drug resistance in pancreatic carcinoma cells may, at least partly, be mediated by MDR1 P-gp rather than MRP-1 (20-21). Using a hammerhead ribozyme to target *mdr1*, Holm *et al.* (22) reported induced sensitivity in the daunorubicin-resistant human pancreatic cell line, EPP85-181RDB, while Nieth *et al.* (23) found that siRNA directed against *mdr1* reduced chemoresistance to daunorubicin (to 89%) in this cell line. In agreement with this, in their analysis of MDR1 P-gp mRNA and protein in SW1990 and Capan-1 cell lines, Zhao *et al.* (24) reported expression levels to correlate with rhodamine extrusion.

A potential involvement of MDR1 P-gp and MRP-1 in the clinical setting is further confused by the very limited number of studies reported to date and the fact that, in these few studies, MDR1 P-gp or MRP-1 alone was analysed; co-analysis of expression of these efflux pumps in pancreatic tumours has not previously been reported. Results from a study of 103 cases of pancreatic tumours indicated a high level expression of MDR1 P-gp in ~73% of cases (25). In a related study, Zalatnai (7) reported that MDR1 P-gp expression was not detected in human pancreatic tumour xenografts, while treatment of mice with the cell cycle regulator mimosine induced expression in 30-60% of the carcinoma cells

Analysis of MRP-1 in 36 pancreatic ductal adeno-carcinomas and 6 normal pancreas specimens showed expression of mRNA and protein to be detected in both tissue types. However, results from immunofluorescence staining indicated that MRP-1 protein was apparently expressed only by fibroblasts, rather than acinar cells or pancreatic carcinoma cells – which lacked MRP-1 staining (6).

Here, in the first study of its kind, we report both MDR1 P-gp and MRP-1 to be expressed by the tumour cells of pancreatic carcinomas. The majority (~93%) of these carcinomas expressed MDR1 P-gp, with ~31% of cases co-expressing MRP-1. No pancreatic tumours were identified where MRP-1 was expressed independently of MDR1 P-gp, and only 6.7% of cases were found to lack expression of both drug efflux pumps.

In the case of MDR1 P-gp, the majority (52%) of tumours that expressed this protein had detectable levels present in >75% of cells within the tumour mass and in most cases (more than 90%) cases, staining was moderate or strong in intensity. While MRP-1 was detected in a substantial number of these tumours (31%), the percentage of stained cells and the staining intensity was generally lower, when compared to that detected for MDR1 P-gp.

Conclusion

The results from this study indicate that the expression of drug efflux pumps (MDR1 P-gp and/or MRP-1) is common in pancreatic tumours and so potentially could contribute, at least in part, to the chemoresistant biology of this cancer. Although these findings do not prove a causal role, the expression of these efflux pumps – and, in particular, the predominant expression of MDR1 P-gp – suggests that they may be important contributors to this resistance. While inhibition of MDR1 P-gp alone has not been shown to improve chemotherapy efficacy in controlled clinical trials, these findings suggest that the inhibition of multiple drug efflux pumps might be necessary if clinically relevant MDR reversal is to be achieved.

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