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P-glycoprotein is not expressed in a majority of colorectal carcinomas and is not regulated by mutant p53 *in vivo*

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Summary Overexpression of the *MDR1* product, P-glycoprotein (Pgp), has been shown to be one of the mechanisms underlying the development of multidrug resistance (MDR). Recently, one mutant p53 has been shown to stimulate the *MDR1* gene promoter *in vitro*, whereas wild-type p53 repressed this activity. We measured Pgp and p53 expression by immunoblotting in 34 colorectal tumours, and performed mutation analyses on the p53-positive tumours to confirm the presence of mutant p53 protein. Tumour DNA indices (DIs) were also measured using flow cytometry. Pgp was detected in 44% (15/34) of the tumours and in 100% (13/13) of the normal mucosas ($P = 0.0005$), with highest levels of expression seen in normal mucosa, suggesting that initial drug resistance in colorectal tumours is not caused by Pgp. Highly DNA aneuploid tumours demonstrated the lowest levels of Pgp expression relative to moderately aneuploid and diploid colorectal tumours. p53 protein was detected in 53% (18/34) of the tumours, and 12 of 14 p53-positive tumours had p53 gene mutations. p53-negative tumours had approximately twice the level of Pgp expression of p53-positive tumours. Pgp expression was not associated with either p53 expression ($P = 0.73$) or incidence of p53 gene mutation ($P = 0.70$), suggesting that mutant p53 does not induce Pgp overexpression in colorectal carcinomas.

Keywords: P-glycoprotein; mutant p53; tumour DNA ploidy; colorectal tumours

A majority of colorectal carcinomas and other solid tumours are resistant to cytostatic drugs, which is a significant problem in their treatment (de Vries and Pinedo, 1991). The cause of such resistance may be determined not only by specific cellular mechanisms such as the multidrug resistance (MDR) phenotype associated with overexpression of the *MDR1* gene, but also by characteristics of the tumour population, such as the proportion of quiescent cells and adequacy of blood supply (Judson, 1992).

The *MDR1* gene product, a 170 kDa protein known as P-glycoprotein (Pgp), appears to be a bifunctional transmembrane protein that functions both as an energy-dependent drug efflux pump of broad specificity (e.g. for anthracyclines and other natural hydrophobic compounds) and as a chloride channel (Gill *et al.*, 1992). Several studies have shown that MDR can be conferred upon drug-sensitive cells by transfer of genes encoding Pgp (Gros *et al.*, 1986; Ueda *et al.*, 1987). *MDR1* RNA and Pgp are found at substantial levels in normal colon, small intestine, kidney, liver and adrenal gland (Fojo *et al.*, 1987), suggesting a normal transporter role for Pgp in these tissues. Pgp has also been found in untreated human cancers derived from normal tissues that express Pgp, such as carcinomas of the colon, liver, kidney, pancreas and adrenal gland (Goldstein *et al.*, 1989). However, it has also been shown that expression of low constitutive levels of *MDR1* mRNA/Pgp may not necessarily result in the functional expression of the MDR phenotype (Hamada *et al.*, 1987; Chambers *et al.*, 1990; Kramer *et al.*, 1993).

Chin *et al.* (1992) have recently shown that one mutant human p53 protein has a specific stimulatory effect upon the *MDR1* gene promoter in 3T3 cells and that wild-type p53 exerts specific repression. Expression of p53 protein in colorectal carcinomas is associated with a high degree of tumour DNA aneuploidy (Carder *et al.*, 1993; De Angelis *et al.*, 1993) and a high incidence of p53 gene mutation (De Angelis *et al.*, 1993). It could be speculated that highly aneuploid tumours with mutant p53 express excess Pgp as a result of the stimulatory effect of p53 on the *MDR1* gene. In this study we have measured Pgp expression and p53 expression in 34 colorectal tumours in order to determine whether Pgp expression is likely to confer initial drug resistance and whether mutant p53 stimulates Pgp expression *in vivo*.

Materials and methods

Clinical material

Thirty-four surgically removed and previously untreated colorectal carcinomas and 13 normal colonic mucosas were collected and studied. All tumours were classified according to Dukes' stage (Dukes, 1932) and histological grade (Jass *et al.*, 1986) (Table I, includes tumour site). Normal mucosa samples were selected from macroscopically normal areas of surgical specimens. Fresh tumour (T) and normal mucosa (N) samples were frozen without buffer at −80°C immediately after surgery. During tissue sectioning and preparation procedures for DNA ploidy analysis and immunoblotting, the tumours and normal mucosas were kept on dry ice until used.

DNA indices (DIs) were determined for the tumour set using laser flow cytometry as described previously (De Angelis *et al.*, 1993), and are presented in Table I. The DI characterises a tumour as either DNA aneuploid (DI > 1.00) or diploid (DI = 1.00) (Hiddemann *et al.*, 1984; Dressler and Bartow, 1989). Highly DNA aneuploid tumours were defined as those with a DI ≥ 1.30, and moderately DNA aneuploid (hyperdiploid) tumours as those with a DI > 1.00 and < 1.30. The DI has been used as a prognostic parameter (with modest impact, Bauer *et al.*, 1993) in addition to Dukes' stage and histological grade classifications for the prediction of prognosis of patients with colorectal tumours.

Immunoblotting

Slices (approximately 0.5–1.0 mm thick) of tumour and normal mucosa were cut with scalpels and boiled for 3–4 min in sodium dodecyl sulphate (SDS) sample buffer (Laemmli,

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Table I Tumour site, Dukes' stage, histological grade and DNA index (DI) for 34 colorectal tumours

Tumour	Tumour site	Dukes' stage	Histological grade	DNA index (DI)
90-1	Left colon	D	PD	1.21
90-4	Right colon	C	MD	1.00
90-7	Right colon	D	PD	1.10
90-8	Rectum	C	MD	1.00
90-9	Left colon	B	MD	1.22
90-10	Right colon	B	MD	1.00
90-19	Rectum	B	MD	1.77
90-20	Right colon	B	MD	1.26
92-1	Left colon	C	MD	1.70
92-2	Left colon	D	MD	1.37
92-4	Right colon	B	MD	1.00
92-5	Left colon	B	MD	1.49
92-6	Right colon	D	PD	1.31
92-8	Left colon	D	HD	1.60
92-9	Right colon	B	MD	1.15
92-10	Right colon	B	MD	1.00
94-1	Left colon	B	MD	1.56
94-3	Rectum	B	MD	1.00
94-6	Left colon	D	PD	1.71
94-7	Rectum	A	MD	1.76
94-8	Left colon	C	MD	1.94
94-9	Left colon	B	MD	1.78
94-10	Rectum	D	MD	1.97
94-13	Rectum	B	MD	1.59
94-14	Rectum	B	MD	1.00
94-15	Rectum	C	MD	1.58
94-16	Left colon	C	PD	1.39
94-17	Rectum	B	MD	1.00
94-18	Left colon	C	MD	1.69
94-19	Right colon	D	PD	1.00
94-21	Right colon	D	MD	1.74
94-23	Left colon	B	MD	1.20
94-24	Rectum	D	MD	1.70
94-25	Rectum	B	MD	1.00

PD, poorly differentiated; MD, moderately differentiated; HD, highly differentiated.

1970) containing protease inhibitors. Representative slices were also cut from the same areas of the tumours as used for immunoblotting, processed for routine histology, and examined by one of us (OPFC) in order to estimate the percentages of tumour and normal mucosal cells in each section. The mean percentage of tumour cells for all sections examined ($n = 31$) was 90.4% ± 12.6%.

The proteins were first separated by electrophoresis in 8.0% SDS-polyacrylamide gels (Laemmli, 1970). Immunoblotting was done as described previously (De Angelis *et al.*, 1993) using the anti-Pgp monoclonal antibody C219 (Centocor, PA, USA), the anti-p53 monoclonal antibodies PAb 1801 and PAb 421, and the anti-p83 monoclonal antibody 34C1 (courtesy of T Stokke). The amount of p83 (nucleoplasmic protein expressed at equivalent levels in proliferating and non-proliferating cells; T Stokke and S Funderud, unpublished) was determined on the same blots in order to control for gel loading, cell concentration and protein degradation. A biotin–streptavidin alkaline phosphatase staining procedure was used to detect the primary antibodies (Amersham, UK). The amounts of the different proteins were evaluated by densitometry using ImageQuant densitometry software (Molecular Dynamics, USA) to analyse stored gel images generated with an Agfa photo scanner (Agfa, Germany). Levels of protein expression are reported relative to p83 expression.

Mutation analysis within the p53 gene

DNA from 28 colorectal tumour samples was subjected to mutation analysis using polymerase chain reaction (PCR) followed by constant denaturant gel electrophoresis (CDGE). We screened for mutations in the conserved domains corresponding to the following codons: codons 128–153 and 155–185 of exon 5, codons 237–253 of exon 7 and codons 265–301 of exon 8. Twenty tumours were also analysed for mutations in codon region 189–215 of exon 6. The optimal conditions for PCR and CDGE have been described elsewhere (Borresen *et al.*, 1991; Smith-Sorensen *et al.*, 1993).

DNA sequencing

Tumour samples shown to be mutated by CDGE were submitted to direct sequencing in order to determine exactly which codon was affected. A new PCR product was made using primers outside the ones used for CDGE. The PCR products were sequenced directly with standard dideoxy-sequencing reactions using Dynabeads M280–streptavidin (Dynal AS, Oslo, Norway) as solid support (Hultman *et al.*, 1989). It has previously been shown that CDGE is a more sensitive method of detecting mutants than this type of sequencing (Borresen *et al.*, 1991). Therefore not all mutants found by CDGE were confirmed by sequencing.

Statistical analysis

Fisher's two-tailed 2 × 2 contingency test was used to check for significance of correlations (P-values) between any two parameters. P-values < 0.05 were considered to denote a statistically significant difference.

Results

Pgp was detected in 44% (15/34) of the tumours and in 100% (13/13) of the normal mucosas examined (Figure 1; Table II) ($P = 0.0005$), with higher levels of expression seen in normal mucosa generally (mean expression 0.260 ± 0.22) as assessed by densitometry, in contrast to a mean expression of 0.061 ± 0.12 for all of the tumours. Table III demonstrates the lower levels of Pgp expression in a subset of the tumour group compared with the levels seen in the corresponding normal mucosas; it was observed in two cases (92-5 and 92-9)

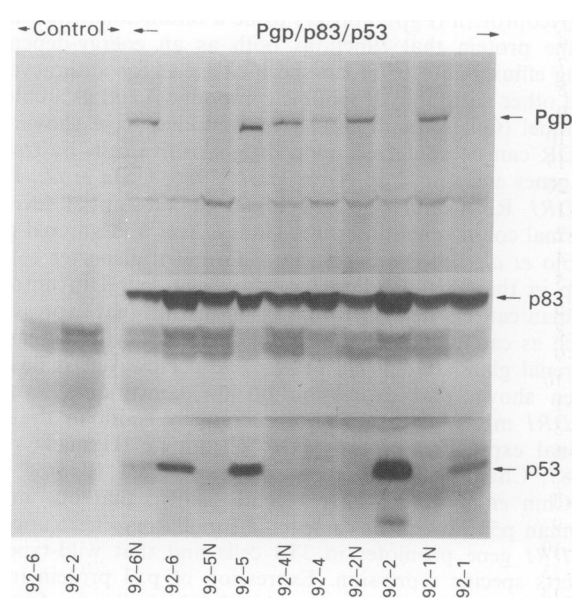

Figure 1 Immunoblot of colorectal tumours 92-1, 92-2, 92-4 to 92-6, and corresponding normal mucosas 92-1N, 92-2N, 92-4N to 92-6N, stained for Pgp, p83 and p53. The blot to the left is the control blot (without primary antibodies) for non-specific staining. The amounts of these proteins were quantified by densitometry and the results presented as fractional values relative to p83 (Tables II and III). Normal mucosa 92-5N expressed low levels of Pgp as demonstrated by the weakly stained Pgp band. The strong p83 bands for both the tumour and normal mucosal samples indicate lack of protein degradation and that relatively equal amounts of protein were loaded per lane.

that the level of Pgp expression in the tumours was higher than in the corresponding normal mucosas.

There was significant association neither between Pgp expression and Dukes' stage (A plus B *vs* C plus D; $P = 0.51$) nor between Pgp expression and tumour site in the colon ($P = 1.00$, right colon *vs* left colon/rectum). Additionally, there was not a significant association between Pgp expression and histological grade ($P = 1.00$, poorly differentiated *vs* moderately and highly differentiated tumours).

Fifty per cent (12/24) of the aneuploid tumours were positive for Pgp, compared with 40% (4/10) of the diploid tumours ($P = 0.71$). The densitometry results demonstrated a trend which showed that the highly aneuploid tumours expressed the lowest amounts of Pgp (mean = 0.034 ± 0.08), the moderately aneuploid tumours more than double that seen in the highly aneuploid tumours (mean = 0.086 ± 0.09), the diploid tumours approximately the same amounts as those seen in the moderately aneuploid tumours (mean = 0.078 ± 0.19) and the normal mucosas the highest amounts of Pgp (mean = 0.260 ± 0.22). However, this trend was not significant since the standard deviations for each sample group were high and overlapped with the other groups, and because each of the sample groups tended to be small in size.

p53 was detected in 53% (18/34) of the tumours (Figure 1; Table II), whereas none of the normal mucosas expressed p53. Twelve of 14 p53-positive tumours examined for mutations had p53 gene mutations.

Pgp expression was significantly associated neither with p53 expression ($P = 0.73$) nor with incidence of p53 gene mutations ($P = 0.70$). The level of Pgp expression in the p53-positive tumours was determined to be 0.043 ± 0.087, approximately half the level of expression seen for the p53-negative tumours (0.081 ± 0.156, although this difference was not significant because of the high and overlapping standard deviations for each group.

Discussion

Pgp was expressed in 44% of the colorectal tumours and in all of the normal mucosas examined in this study, with the highest levels of expression seen in normal mucosa. The positive tumours generally expressed less Pgp than normal mucosa. Our results are in agreement with those of a previous study (Fujii *et al.*, 1993), which demonstrated that 44% of colon carcinomas were positive for Pgp by immunofluorescence analyses using both flow cytometry and immunohistochemistry in sections, as well as with previous studies demonstrating Pgp expression generally in normal colonic mucosa (Fojo *et al.*, 1987) and colonic tumours (Goldstein *et al.*, 1989). Peters *et al.* (1992) found positive Pgp expression in all but one tumour; the tumours had significantly higher levels of Pgp than the normal mucosas.

Table III Pgp is not expressed in many tumours relative to the corresponding normal mucosas

Tumour	*Fraction Pgp expressed in normal mucosa*	*Fraction Pgp expressed in tumour*
90-1	0.576	0.124
90-7	0.074	0.0
90-8	0.059	0.037
90-10	0.733	0.0
92-1	0.269	0.0
92-2	0.207	0.0
92-4	0.298	0.128
92-5	0.091	0.324
92-6	0.344	0.0
92-8	0.131	0.112
92-9	0.076	0.211
Mean ± s.d.	0.260 ± 0.22	0.085 ± 0.11

Table II Pgp and p53 status for 34 colorectal tumours

Tumour	*Fraction Pgp expressed*	*Pgp status*	*Fraction p53 expressed*	*p53 status*	*p53 Gene mutations* *Exon codon*	*Mutation*	*Amino acid*
90-1	0.124	+	0.0	−			
90-4	0.610	++	0.0	−			
90-7	0.0	−	0.0	−	No mutation revealed		
90-8	0.037	+	0.0	−	No mutation revealed		
90-9	0.013	+	0.0	−	No mutation revealed		
90-10	0.0	−	0.0	−	No mutation revealed		
90-19	0.191	+	0.171	+			
90-20	0.025	+	0.0	−	No mutation revealed		
92-1	0.0	−	0.232	+	Exon 5: codon 175	CGC to CAC	Arg to His
92-2	0.0	−	0.724	++	Exon 8: codon 275	TGT to TTT	Cys to Phe
92-4	0.128	+	0.0	−	No mutation revealed		
92-5	0.324	++	0.860	++	Exon 8: unspecified codon		
92-6	0.0	−	0.457	++	Exon 8: codon 282	CGG to TGG	Arg to Trp
92-8	0.112	+	0.910	++	Exon 8: codon 273	CGT to CAT	Arg to His
92-9	0.211	++	0.0	−	No mutation revealed		
92-10	0.0	−	0.029	−			
94-1	0.0	−	0.0	−	No mutation revealed		
94-3	0.0	−	0.0	−	No mutation revealed		
94-6	0.012	+	0.162	+	Exon 5: unspecified codon		
94-7	0.0	−	0.206	+			
94-8	0.056	+	0.127	+	Exon 7: codon 245	GGC to AGC	Gly to Ser
94-9	0.015	+	0.216	+	Exon 5: unspecified codon		
94-10	0.062	+	0.240	+			
94-13	0.0	−	0.270	+	Exon 5: codon 130	CTC to CAC	Leu to His
94-14	0.0	−	0.113	+	Exon 5: codon 176	TGC to TAC	Cys to Tyr
94-15	0.0	−	0.0	−	Exon 6: codon 196	CGA to TGA	Arg to STOP
94-16	0.0	−	0.042	+			
94-17	0.0	−	0.062	+	No mutation revealed		
94-18	0.0	−	0.116	+	Exon 5: unspecified codon		
94-19	0.0	−	0.195	+	No mutation revealed		
94-21	0.0	−	0.269	++	Exon 6: unspecified codon		
94-23	0.142	++	0.0	−	No mutation revealed		
94-24	0.0	−	0.0	−	Exon 5: unspecified codon		
94-25	0.0	−	0.0	−	No mutation revealed		

In the columns 'Pgp status' and 'p53 status', the amounts of each protein were visually evaluated from the immunoblots and described as absent (−), present in low amounts (+) and present in high amounts (++).

This discrepancy could be explained by their lack of gel loading/concentration standards when quantifying Pgp expression (p83 in our study) and different methods of isolation of cells from tumour and mucosal samples.

More than half of the colorectal tumours examined in this study were negative for Pgp expression, possibly reflecting their clonal origin from a cell which did not originally express Pgp (Noonan *et al.*, 1990). This Pgp-negative cell might be a crypt epithelial cell, since these have been shown to be negative for Pgp expression, whereas the surface epithelial cells lining the lumen (apical brush border cells) are Pgp-positive (Cordon-Cardo *et al.*, 1990). This reflects a differentiation-dependent pattern of expression as normal mucosal cells move up the crypt toward the surface. We estimated the percentage of normal mucosal cells in the tumour sections which might influence the number of Pgp-positive tumours scored. We are confident that the immunoblotting results reflect the biological characteristics of tumour cells, since very few non-tumour epithelial cells were seen in the sections examined (<10%) from the same areas of the tumours used for immunoblotting. Several tumours in our study which contained some normal cells were not scored as positive, thus contamination is not a significant problem in this study. It may mean, however, that heterogeneous tumours with a low number of Pgp-positive cells may be scored as Pgp-negative. Heterogeneity of Pgp staining and tumour sampling are other factors which may influence the number of positive tumours scored. However, these factors do not influence our main conclusion, which is that colorectal tumours either do not express Pgp at all (lack of differentiated phenotype) or do so to a much lesser extent than normal mucosa.

There was no correlation of Pgp expression with Dukes' stage, histological grade, or tumour site, confirming the results of Pirker *et al.* (1993). We also found no significant correlation between Pgp expression and DNA aneuploidy, consistent with the results of Sinicrope *et al.* (1994). However, these results are not in agreement with those of Danova *et al.* (1992), who demonstrated a correlation between DNA aneuploidy and higher levels of Pgp expression relative to diploid tumours and normal tissue.

Chin *et al.* (1992) found that one mutant human p53 protein (point mutation at codon 175, Arg to His substitution) had a specific stimulatory effect upon the *MDR1* gene promoter in 3T3 cells, suggesting that colon tumours which express mutant p53 should overexpress Pgp (relative to the levels of Pgp expressed in normal mucosa). However, we found no association between mutant p53 and Pgp expression. Where there was Pgp expression in the p53-positive tumours, it was still lower than that seen in normal mucosa. Additionally, tumour 92-1 in our tumour set expressed the particular codon 175 mutant p53 as studied by Chin *et al.* (1992), but did not express Pgp. This independence of Pgp and mutant p53 expression is supported by recent studies of B-cell chronic lymphocytic leukaemia (El Rouby *et al.*, 1993), acute myelogenous leukaemia (Zhao *et al.*, 1992) and myelodysplastic syndromes (Preudhomme *et al.*, 1993), which demonstrated that *MDR1* gene overexpression is independent of p53 gene mutations/mutant p53 protein.

It was not possible in this study directly to correlate Pgp expression in colon carcinomas with response to cytostatics and overall survival, since only one of the patients received chemotherapy for his tumour. However, the lack of Pgp expression in many colon tumours suggests that resistance mechanisms other than MDR are responsible for the initial resistance to cytostatic drugs often seen in colon tumours. Non-MDR mechanisms which have been shown to be involved in mediating resistance to cytostatic drugs (other than anthracyclines) in colon tumours are the activity of glutathione *S*-transferase (Waxman, 1990; Clapper *et al.*, 1991; De Waziers *et al.*, 1991; Moorghen *et al.*, 1991; Peters *et al.*, 1992) and the alteration of DNA-associated topoisomerase II which is involved in post-replication repair (Redmond *et al.*, 1991).

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