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P-Glycoprotein Is Positively Correlated with p53 Protein Accumulation in Human Colorectal Cancers

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To explore the relationship between mutant p53 and Pgp expression, we have examined the levels of both proteins in human colorectal adenocarcinomas. Serial frozen sections of 40 surgical samples were stained with an anti-Pgp (MRK16) and two different anti-p53 protein antibodies (Abs), PAb421 and PAb1801. Nineteen (47.5%) of 40 samples examined were positive for Pgp, and 18 (45%) of 40 were positive for p53. The samples that stained positively with PAb421 also stained positively with PAb1801. Pgp expression was detected in 13 (76.5%) of 17 samples that were positive for p53 using PAb421 and in 15 (83.3%) of 18 samples that were positive for p53 using PAb1801. Thus, we found that p53 and Pgp were co-expressed in a significant number of samples ($P<0.002$). There was no relationship between Pgp or p53 protein accumulation and histologic grade or stage. The present results demonstrate that Pgp expression is closely associated with p53 protein accumulation in human colorectal cancers. These data provide evidence to support the idea that mutant p53 activates the *MDR1* gene *in vivo*.

Key words: P-glycoprotein — p53 — Colon cancer — Multidrug resistance — *MDR1* gene

The 170,000 dalton human P-glycoprotein (Pgp), encoded by the *MDR1* gene, is an energy-dependent drug efflux pump that confers multidrug resistance (MDR) in cancer cells.[1)] Although the distribution of Pgp/*MDR1* in normal human tissues has been examined in some detail, the normal physiologic function of this protein remains largely unknown.[1)] Tumors originating from tissues that normally express Pgp/*MDR1* often express high levels of Pgp.[1)] Evidence to support this finding derives from the observation that normal colonic mucosa[2,3)] and colorectal cancers[1,4)] both frequently express Pgp/*MDR1*.

p53 is a nuclear tumor-suppressor protein that is frequently mutated in a wide variety of human cancers, and much evidence indicates that mutant p53 plays a role in the process of tumorigenesis.[5)] Moreover, several recent studies suggest that the p53 tumor-suppressor protein plays a role in determining the sensitivity or resistance of cancer cells to chemotherapeutic agents.[6–9)] *In vitro* *MDR1* gene expression is activated following exposure to multiple agents including anticancer drugs, heat shock, and heavy metals.[1)] Experiments using different mutant *p53* expression vectors co-transfected together with *MDR1* gene promoter reporter constructs indicate that mutant p53 proteins can trans-activate *MDR1* gene expression in cultured cells.[10–13)] However, attempts to corroborate the association between mutant *p53* and *MDR1* gene expression in clinically relevant studies have yielded mixed results.[14–19)] Several studies have reported an association between mutant *p53* expression and tumorigenesis or tumor progression in human cancers including colorectal cancer.[5,20)] Pgp-positive colon cancer cells were found to have an increased potential for dissemination.[21)] When considered together, these findings raise the possibility of an association between Pgp and mutant p53 expression in human colorectal cancers. Since mutant p53 protein has a prolonged half-life when compared with the wild-type protein, it accumulates in tumor cells.[22)] This feature permits its detection with antibodies directed against p53. In the present study we have investigated the possible relationship between Pgp and p53 protein expression in colorectal adenocarcinomas using immunohistochemical techniques.

MATERIALS AND METHODS

Clinical samples Forty colorectal adenocarcinoma tissue samples were obtained following surgery at the Nagasaki University Hospital. None of the cancer patients had received chemotherapy prior to surgery. The samples, taken from the center of tumors, were frozen immediately after removal and soaked in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN). The embedded samples were then frozen in liquid nitrogen and stored at −70°C until processing. An aliquot of each fresh sample was fixed in 10% formalin and routinely processed for he-

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matoxylin-eosin staining. Differentiation of histologically confirmed cancer was graded, and the samples were classified into well, moderately, and poorly differentiated adenocarcinomas.

Immunohistochemistry for Pgp and p53 protein Serial 6 μm thick cryostat sections were mounted on glass slides coated with poly-L-lysine. The samples were air-dried for 30 min, fixed for 20 min in cold acetone, and stored at $-70^{\circ}C$ until staining. To block endogenous peroxidase activity, the slides were incubated with 0.3% H_2O_2/methanol for 10 min prior to staining. They were rinsed three times in 0.01 *M* phosphate-buffered saline, pH 7.2 (PBS) followed by incubation for 15 min with a blocking solution of 1% normal rabbit serum in PBS. The primary antibodies were applied overnight at 4°C. Two different antibodies, mouse monoclonal antibody PAb421 (Ab-1; Oncogene Science, Manhassett, NY) and PAb1801 (Ab-2; Oncogene Science) recognizing different epitopes of p53 protein were used at a final concentration of 5 μg/ml to detect p53 protein accumulation more accurately. A mouse monoclonal antibody (MRK16, KAMIYA Biomedical Co., Thousand Oaks, CA) was used at a concentration of 5 μg/ml to detect Pgp. After rinsing with PBS, the slides were incubated with a streptavidin-biotin complex peroxidase (Histofine SAB-PO Kit; Nichirei Inc., Tokyo) as described previously by Shi *et al.*[23] Peroxidase activity was detected by incubation with diaminobenzidine. The slides were then counterstained with hematoxylin. As controls for nonspecific staining, the same process was performed on sections using an antibody directed against an antigen that is not expressed in colon cells. Sections of normal human kidney and a colon adenocarcinoma with elevated p53 protein expression were used as positive controls for Pgp and p53 protein, respectively.

All slides were independently evaluated for Pgp and p53 expression by two different pathologists (Authors: A.

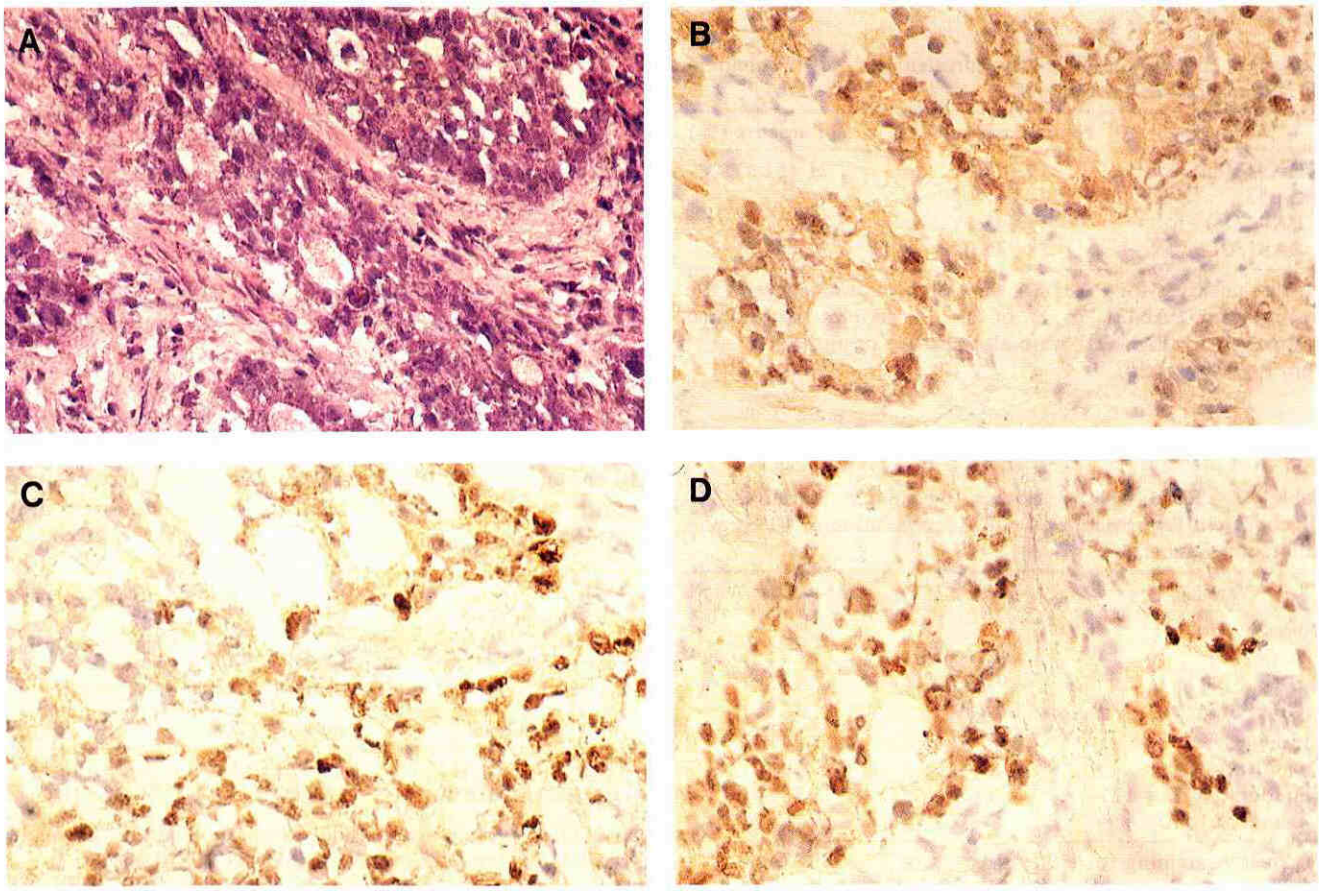

Fig. 1. Immunohistochemical staining for human P-glycoprotein expression using MRK16, and p53 protein with PAb421 and PAb1801 antibodies in a representative positive sample from one patient. A, Hematoxylin-eosin staining; B, MRK16; C, PAb421; D, PAb1801.

S. and N. I.). Staining for p53 was determined by comparing each sample with a slide of normal cells as negative controls. For Pgp, at least 1,000 tumor cells on each section were counted to determine the percentage of positive cells. Positive staining was defined if more than 25% of the tumor cells were stained.

The results were evaluated statistically by the χ^2 test, and a two-tailed $P<0.05$ was considered to indicate statistical significance.

RESULTS

Immunoreaction of colorectal cancer with MRK16, PAb 421 and PAb1801 Forty tissue sections obtained from colorectal cancer patients were stained for p53 and Pgp. A representative slide from one patient that was positive for both Pgp and p53 protein expression is presented in Fig. 1. Nineteen (47.5%) of the 40 tumor samples examined were found to express Pgp following incubation with the MRK16 antibody (Table I). If samples were scored as positive when only 5% of the population stained, the percentage of Pgp-positive samples would be increased to 65%. A strong correlation was observed between the results obtained with the two different Abs to p53: 42.5% of the samples reacted with both the PAb421 and PAb1801 antibodies (Table I), while only one sample stained positively with PAb1801 and negatively with PAb421.

No relationship could be demonstrated when the data for Pgp and p53 staining were compared with histologic grade or Dukes' stage of the colorectal carcinoma samples examined (Table II).

Relationship between Pgp and p53 protein accumulation Data demonstrating a relationship between Pgp and p53 protein accumulation are presented in Table III. Thirteen (76.5%) of 17 samples positive for p53 using PAb421 were also positive for Pgp using MRK16. In contrast, only six (26.1%) of 23 samples that failed to react with PAb421 were found to be positive for Pgp with MRK16. Thus, Pgp expression was significantly correlated with p53 expression in the samples examined with PAb421 ($P<0.002$). Similarly, a strong correlation between Pgp and p53 expression was observed when the samples were stained with PAb1801. Fifteen (83.3%) of 18 sections that stained positively for p53 with PAb1801 were also positive for Pgp ($P<0.0001$). When considered together these data demonstrate a close association of p53 protein accumulation with Pgp expression in colorectal cancers.

Table I. Expression of P-Glycoprotein and p53 Protein in 40 Human Colorectal Cancers

Antibody	No. of positive (%)	No. of negative (%)
MRK16 (anti-Pgp)	19 (47.5)	21 (52.5)
anti-p53		
PAb421	17 (42.5)	23 (57.5)
PAb1801	18 (45.0)[a]	22 (55.0)

a) The positives for PAb1801 were highly consistent with the positives for PAb421, as 17 of the 18 samples that stained positively with PAb1801 were also positive using PAb421.
Pgp, P-glycoprotein.

Table II. Relationship between Pathologic Variables, P-Glycoprotein Expression and p53 Accumulation in 40 Human Colorectal Cancers

Variable	Pgp-positive (%)	p53-positive (%)[a]
Dukes[b]		
A ($n=2$)	0 (0)	1 (50)
B ($n=21$)	9 (43)	8 (38)
C ($n=17$)	10 (59)	9 (53)
Grade[b]		
well ($n=18$)	8 (44)	8 (44)
moderately ($n=12$)	6 (50)	5 (42)
poorly ($n=10$)	5 (50)	5 (50)

a) Positive staining for either PAb421 or PAb1801 antibody.
b) There was no statistically significant difference between pathologic variables and positive staining for Pgp or p53 protein.
Pgp, P-glycoprotein.

DISCUSSION

The present study demonstrated that Pgp expression was closely associated with p53 protein accumulation in human colorectal adenocarcinomas as judged from immunohistochemical staining. These findings suggest that mutant p53 proteins may activate the *MDR1* gene of colorectal cancer cells *in vivo*. If mutation does function to enhance Pgp/*MDR1* expression *in vivo*, certain malignantly transformed colorectal cells may display a more drug-resistant phenotype.

Table III. Relationship between P-Glycoprotein Expression and p53 Protein Accumulation in 40 Colorectal Cancers

Antibody	+/+ (%)	+/− (%)
MRK16/PAb421	13/17 (76.5)[a]	6/23 (26.1)
MRK16/PAb1801	15/18 (83.3)[b]	4/22 (18.2)

a) P-Glycoprotein expression was significantly correlated with p53 staining detected with PAb421 ($P<0.002$).
b) P-Glycoprotein was significantly correlated with p53 staining detected with PAb1801 ($P<0.0001$).
MRK16, anti-human P-glycoprotein antibody; PAb421 and PAb1801, anti-p53 protein antibodies.

Several studies have reported a positive relationship between Pgp/*MDR1* and p53 expression in human solid tumors.[5, 15, 16] Previous studies have shown that immunohistochemistry is an important method for assessing *p53* gene mutations in colorectal neoplasms.[24] The frequency of p53-positive specimens in this study was similar to that reported in previous studies.[25, 26] Moreover, the specimens that stained positively for p53 with PAb421 consistently reacted with PAb1801. The number of samples that stained positively for Pgp/*MDR1* in this study was somewhat lower than the frequency reported in other studies.[21, 27]

There is a great deal of controversy in the literature regarding the effects of mutant p53 on the promoter of the *MDR1* gene. While most studies performed *in vitro* have found that mutant p53 activates the *MDR1* promoter,[9, 10–12] one study reported that wild-type p53 was capable of stimulating this promoter.[28] The discepancy between these results may be due to artefacts inherent with *in vitro* transfection studies or to differences in experimental materials, such as cell lines and expression vectors.

Discrepancies between Pgp/*MDR1* expression and mutant p53 have also been reported in clinical studies. A positive relationship between Pgp/*MDR1* and p53 was detected in breast carcinomas,[5] but not in gynecologic tumors, B-cell chronic lymphocytic leukemias or myelodysplastic syndromes.[13–16] One possibility to reconcile these differences may be that the conformation and function of mutant p53 are influenced by other cellular factors, depending upon the type of tumor involved. In support of this hypothesis, *p53* mutation profiles differ between hematologic and colorectal cancers.[5, 29] Aas *et al.* reported recently that specific *p53* mutations (codons 163–195 and 236–251) are associated with resistance to doxorubicin in human breast cancers.[8]

Wild-type p53 functions as a sequence-specific DNA binding protein that has transcriptional activity.[5, 30–32] However, the mechanism by which mutant p53 activates the promoter of the *MDR1* gene remains undetermined. Since the *MDR1* promoter does not contain a characteristic p53 DNA binding sequence,[28] it is not clear whether the protein binds directly to the DNA or acts via alternative protein:protein interactions.[12, 28] Additional *in vitro* studies will be required to understand the mechanism by which mutant p53 activates this gene.

Mutations in the *p53* gene and *Pgp/MDR1* overexpression have been reported as independent prognostic or tumor progression-favoring factors in some cancers.[5, 16, 21, 33] In this study, we found no association between p53 accumulation or Pgp expression and tumor progression. In a previous study involving a large number of colorectal cancers, no association was found between *MDR1* gene expression and any clinical variable examined.[27] One possible role for Pgp in tumor progression may relate to the vectorial transport of unidentified growth or angiogenic factors from tumor cells.[34] The functions of this protein in normal and cancer cells remain largely unknown, with the exception of its role as a drug efflux pump. Thus, further *in vitro* and *vivo* studies are needed to clarify the exact role of Pgp/*MDR1* in cancer cell biology.

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