Papers

Immunohistochemically detectable p53 and mdm-2 oncoprotein expression in colorectal carcinoma: prognostic significance

D Öfner, H Maier, B Riedmann, P Holzberger, M Nogler, M Tötsch, A Bankfalvi, G Winde, W Böcker, K W Schmid

Abstract

Aims—To investigate the correlation between the expression of the p53 and mdm-2 oncoproteins and to assess their prognostic value in colorectal cancer.

Methods—Using a polyclonal (CM1) and a monoclonal antibody directed against p53 and mdm-2, respectively, these oncoproteins were stained immunohistochemically in 109 colorectal adenocarcinomas.

Results—p53 was detected in less than 10% of tumour cells in 11 of 109 adenocarcinomas, in 10–50% of tumour cells, in 17 of 109 adenocarcinomas, and in more than 50% of tumour cells in 32 of 109 adenocarcinomas. Expression of mdm-2 was detected in 22 of 109 (20%) cases investigated, of which 19 showed concomitant p53 expression. In most cases mdm-2 immunoreactivity was strongly associated with a small proportion of p53 positive tumour cells. Both p53 and mdm-2 expression lacked statistical significance when correlated with common staging and grading parameters.

Conclusions—Detection of p53 and mdm-2 oncoprotein expression, detected using immunohistochemistry, is of no prognostic value in colorectal cancer. However, the close correlation between mdm-2 immunoreactivity and the proportion of p53 positive cells provides further evidence that the mdm-2 gene product interacts with p53 protein.

(J Clin Pathol: Mol Pathol 1995;48:M12-M16)

Keywords: Immunohistochemistry, p53, mdm-2, colorectal cancer.

Somatic mutations of the tumour suppressor gene p53 have attracted much attention as the most common single lesions in human tumours^{1–3} including colorectal cancer.^{4–8} p53 gene mutations are found in 50–70% of colorectal cancers. The mutant p53 protein has a different conformation to wild-type p53, is more stable and can be detected by immunohistochemistry. In colorectal,^{6,9} bladder,¹⁰

and lung cancer¹¹ good agreement was found between the frequency of gene mutations and immunohistochemically detectable p53 protein. However, the value of detecting p53 overexpression for the determination of prognosis in colorectal adenocarcinoma is still a matter of debate.^{12–16}

Recently, a cellular protein, the murine double-minute-2 (mdm-2) gene product, was shown to complex with wild-type and mutant p53 protein bringing about functional inactivation of the p53 gene.^{17–19} Amplification of this proto-oncogene was detected in sarcomas not carrying p53 mutations¹⁹ but has not been detected in colorectal carcinoma as yet.

In the present study an immunohistochemical analysis of 109 colorectal adenocarcinomas was performed using a polyclonal p53 antibody (CM1) and a monoclonal mdm-2 antibody. The study design was based on recent data suggesting that wild-type p53 protein may be detectable by immunohistochemistry in the presence of the mdm-2 gene product. The immunohistochemical results were compared with the clinical outcome of patients to evaluate the possible prognostic value of detecting one or both oncoproteins.

Methods

Tumour tissue from 109 consecutive cases of colorectal adenocarcinoma (43 rectal carcinomas and 45 carcinomas of the left and 21 of the right colon; 56 men, 53 women; mean age 67.8 years, range 35–90 years) was studied. All patients had undergone surgery between 1984 and 1986 at the Department of Surgery I, Innsbruck University Hospital, Austria, either with curative (n=92) or palliative intent (n=17). Patients who died within 30 days of surgery, with adjuvant chemotherapy and radiotherapy or both, or members of families with familial adenomatosis coli or hereditary non-polyposis colorectal cancer were excluded from this study.

Tumour tissue was routinely processed, fixed in formalin, embedded in paraffin wax and classified according to Dukes' classification,²⁰ with an added D stage for those patients with

Gerhard Domagk
Institute of Pathology,
University of Münster,
Germany

D Öfner
A Bankfalvi
W Böcker
K W Schmid

Department of
Surgery
G Winde

Department of
Surgery I, University
Hospital, Innsbruck,
Austria
D Öfner
B Riedmann
P Holzberger
M Nogler

Department of
Pathology
M Tötsch

Correspondence to:
Dr K W Schmid, Gerhard
Domagk Institute of
Pathology, University of
Münster/Westfalia,
Domagkstrasse 17, D-48149
Münster, Germany.

Accepted for publication
3 November 1994

Figure 1 Survival curve (Kaplan-Meier) for all 109 patients investigated in the study.

distant metastases,²¹ the TNM staging system,²² and the World Health Organisation (WHO) grading system.²³ Lymphocytic infiltration at the advancing edge of the tumour was determined according to the criteria outlined by Jass *et al.*²⁴ A detailed description of the staging and grading results is presented in table 1. Mean follow up was 79 months (fig 1).

For immunohistochemical staining, sections were cut at 2 µm and mounted on poly-L-lysine coated glass slides. All sections were dewaxed and rehydrated through a series of graded ethanols. Before staining, sections were pretreated with the wet autoclave method for antigen retrieval for 10 minutes, as described previously.²⁵ Incubations with the primary antibodies (polyclonal p53 antibody (CM1; Medac, USA) and monoclonal mdm-2 anti-

body (Oncogene Science, USA)), the enzyme colour reaction, light haematoxylin counterstaining, and section mounting were carried out as described elsewhere.²⁵

Semiquantitative evaluation of p53 and mdm-2 immunoreactivity was performed by one of the authors (KWS). Immunoreactivity was scored as follows: no immunoreactivity (–); positive staining in less than 10% of tumour cells (+); positive staining in 10–50% of tumour cells with a mainly focal distribution (++); positive staining in more than 50% p53 positive tumour cells (+++).

All data were analysed using the SYSTAT statistical package²⁶ including the SURVIVAL supplementary module.²⁷ Patients were followed according to the oncological follow up scheme of the Department of Surgery I: patients underwent clinical and laboratory examinations every three months within the first three years, every six months four and five years after surgery, and once a year thereafter. Colonoscopy or barium enema and chest x ray were performed twice a year in the first three years and once a year until five years after surgery. The data concerning the date and cause of death were confirmed by the Österreichisches Statistisches Zentralamt, an Institute of the Austrian government. Cumulative patient survival was estimated using the Kaplan-Meier method²⁸; the log rank test was used to compare survival curves (Mantel-Haenszel).²⁹ Descriptive statistics for continuous measures are given as the mean (SD); for discrete data, frequency counts and percentages are tabulated and groups were compared using χ^2 analysis with Yates' correction where appropriate.

Table 1 Frequency of prognostic parameters investigated in 109 colorectal adenocarcinomas

Parameter	Number	Percent
Tumour type		
intestinal	87	80
mucinous	17	16
signet ring cell	5	4
Histological grading		
well	15	13
moderate	69	64
poor	25	23
Lymphocytic infiltration		
mild	43	39
moderate	49	45
significant	17	16
Dukes' stage		
A	11	10
B	48	44
C	32	30
D	17	16
pT stage		
pT1	12	11
pT2	34	31
pT3	51	47
pT4	11	10
pN stage		
pN0	56	51
pN1	13	12
pN2	20	18
pN3	7	6
pNx	13	12
Tumour site		
right hemicolon	21	19
left hemicolon	45	41
rectum	43	40
Age		
≤ 65 years	38	35
> 65 years	71	65
Sex		
male	56	51
female	53	49

Table 2 Frequencies of p53 scores according to Dukes' Stage

p53	Dukes' A	Dukes' B	Dukes' C	Dukes' D
Negative	6	25	10	8
+	1	4	5	1
++	2	9	4	2
+++	2	11	13	6

p53 scores: + = <10%; ++ = 10–50%; +++ = >50%.

Table 3 Frequencies of p53 scores according to tumour site

p53	Proximal colon	Distal colon	Rectum
Negative	12	20	17
+	2	7	2
++	1	8	8
+++	6	10	16

p53 scores: + = <10%; ++ = 10–50%; +++ = >50%.

Table 4 p53 immunoreactivity with regard to mdm-2 scores

p53	mdm-2		
	Negative	+	++
Negative (n = 49)	46	3	0
+	1	8	2
++ (n = 17)	10	1	6
+++ (n = 32)	30	1	1

p53 scores: + = <10%; ++ = 10–50%; +++ = >50%.
mdm-2 scores: + = <10%; ++ = >10%.

Table 5 Correlation between p53, mdm-2 scores and the various prognostic parameters investigated

Parameter	p53			mdm-2		
	χ^2	DF	p	χ^2	DF	p
M Stage	0.6	1	NS	0.9	2	NS
pN stage* (PN0 vs pN1-3)	1.5	1	NS	2.0	2	NS
Dukes' classification	1.2	3	NS	4.0	6	NS
pT stage	3.0	3	NS	3.9	6	NS
Tumour site	3.9	2	NS	4.0	4	NS
Lymphocytic infiltration	0.9	2	NS	7.7	4	NS
Tumour type	1.3	2	NS	3.3	4	NS
Histological tumour grade	4.8	2	NS	2.1	4	NS
Age (≤ 65 v >65 years)	0.02	1	NS	0.5	2	NS
Sex	1.9	1	NS	2.5	2	NS

* pNx cases (n=13; four with Dukes' A and nine with Dukes' D cancer) excluded.

Results

Positive p53 stained nuclei were detected in 60 of 109 (55%) colorectal carcinoma cases. Immunohistochemical staining results with respect to the Dukes' classification are summarised in table 2. Pronounced p53 immunoreactivity (+++) was found in advanced tumour stages (in two of 11 (18%) with Dukes' A; in 11 of 49 (22%) with Dukes' B; in 13 of 32 (41%) with Dukes' C; and in six of 17 (35%) with Dukes' D cancer) and was accompanied by a slight decrease in the number of totally negative cases. The frequency of p53 negative cases was higher in left sided compared with right sided tumours. None of these comparisons were statistically significant.

Expression mdm-2 was detected using immunohistochemistry in 22 of the 109 (20%) of cases investigated. The p53 and mdm-2 scores are presented in table 4. In general, mdm-2 and p53 immunoreactivities were detected in serial sections from the same tumour areas. In three p53 negative cases mdm-2 was detected in less than 10% of tumour cells. Only one of 11 cases with less than 10% p53 positive cells lacked mdm-2 immunoreactivity. Of the 49 cases with p53 overexpression in more than 10% of tumour cells (++/+ + +), nine showed mdm-2 staining. This association was highly statistically significant (total χ^2 69.3, DF=6, $p=0.0001$). Comparisons of p53 and mdm-2 scores with respect to various staging and grading parameters are presented in table 5.

Results of univariate survival analysis of all parameters investigated are given in table 6. Kaplan-Meier survival curves with regard to all p53 scores investigated (Mantel-Haenszel: χ^2 3.4, DF=3, NS) are shown in fig 2. The survival curve of mdm-2 positive cases was

similar to the survival curve of cases with positive p53 immunoreactivity (Mantel-Haenszel: χ^2 = 3.7, DF=2, NS) (fig 3).

Figure 2 Kaplan-Meier estimations according to immunohistochemically demonstrable p53 expression: — negative; --- <10%; -.-.- >10% to 50%; >50% positive tumour cells for p53 protein (Mantel-Haenszel: χ^2 = 4.3, DF=3, NS). Comparison between p53 negative and p53 positive tumours showed no statistical significance (Mantel-Haenszel: χ^2 = 3.4, DF=1, NS).

Figure 3 Kaplan-Meier estimations according to immunohistochemical demonstrable p53 and associated mdm-2 staining patterns: — negative for both p53 and mdm-2; --- positive for mdm-2 protein; -.-.- positive for p53 but negative for mdm-2 expression (Mantel-Haenszel: χ^2 = 3.7, DF=2 (NS)).

Table 6 Prognostic factors examined in 109 colorectal carcinomas: a univariate approach to cancer specific mortality

	Univariate χ^2 for the log-rank test	DF	p
Dukes' stage	88.1	3	0.0001
pT stage	17.7	3	0.0001
pN stage*	25.7	3	0.0001
Lymphocytic infiltration	8.6	2	0.01
Histological tumour grade	7.4	2	0.02
p53 scores (-/+ v +/+ + +)	1.5	1	NS
mdm-2 scores (negative v +/+ +)	0.1	1	NS
Tumour type	4.0	2	NS
Tumour site	2.2	2	NS
Age (≤ 65 v >65 years)	0.1	2	NS
Sex	0.001	1	NS

* pNx cases (n=13; four with Dukes' A and nine with Dukes' D cancer) excluded.

Discussion

The extended palette of adjuvant chemotherapy and/or radiotherapy schedules in combination with biological response modifiers requires determination of groups of patients with (morphological) prognostic features which are independent of common staging and grading classifications. Recent advances in the understanding of tumorigenesis of colorectal cancer suggest that p53 tumour suppressor gene dysfunction may play a central role. Therefore, promising results were expected when p53 abnormalities were evaluated on both the molecular and immunohistochemical level.³⁰ Yet only one report dealing with p53 mutations in a large series of sporadic colorectal cancers demonstrated a statistical correlation between p53 mutation and patient survival.⁸ On the other hand, because of the clearly demonstrated association between increased p53 protein stability and mutation,^{6,9,31} several immunohistological investigations have been performed previously. In these studies, however, strict correlations between survival and p53 overexpression were not observed.^{13–16}

The results of the present study reveal a highly statistically significant ($p=0.0001$) correlation between immunohistochemically detectable mdm-2 expression and low (+) or moderate (++) p53 overexpression (table 4). Only three of 109 cases (2.8%; two with Dukes' B and one with Dukes' D cancer) showed positive staining for mdm-2 with a concomitant lack of p53 expression. By contrast, two samples (1.8%; one with Dukes' A and one with Dukes' B cancer) classified as strongly (+++) overexpressing p53 also showed mdm-2 positive immunoreactivity. Moreover, the survival curve of mdm-2 positive cases (irrespective of p53 expression) more closely resembled that of p53 positive than p53 negative patients (fig 2).

Concerning p53 frequency distribution and correlations with common prognostic parameters, the findings of the present study confirm those of other investigators.^{13–16} Although not statistically significant, a preponderance of left sided colon cancers with p53 positive immunoreactivity was observed. Furthermore, immunohistochemically detectable p53 was more frequently observed in advanced cancer stages and was associated with poor clinical outcome. All statistical comparisons, including survival analysis, lacked significance. These observations strongly suggest that p53 overexpression in less than 10% of nuclei probably occurs through complexing with the mdm-2 protein. We did not observe a correlation between immunohistochemically detectable p53 expression and clinical outcome.

Several molecular studies suggest that tumorigenesis in colorectal cancer occurs in a stepwise manner through a series of genetic alterations.^{32,33} This study demonstrates that a recently described oncoprotein, the mdm-2 gene product, is expressed in colorectal cancer. Its association with cases showing a small proportion of cells with immunohistochemically detectable p53 expression suggests that the mdm-2 gene may be involved in the auto-

regulation of p53 gene function. These multiple interactions may be responsible for the lack or only marginal value of proto-oncogenes as independent prognostic parameters. Thus, the Dukes' classification, which is easy to reproduce and relates well to survival, still remains the "gold standard" with regard to prognosis in colorectal cancer. Immunohistochemical or genetic, or both, investigations of proto-oncogenes, however, may be useful in helping to understand the biological mechanism(s) underlying this simple and reliable "gold standard".

The authors would like to thank Ms U Neubert, Ms A Muhmann and Ms B Kunk for technical and Mrs H Gerdes-Funnekötter for photographic assistance. Thanks are due to ORat Dr HP Friedl for prompt and helpful advice.

- Curtis CH, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993;329:1318–27.
- Levine AJ, Perry ME, Chang A, Silver A, Dittmer D, Wu M, et al. The 1993 Walter Hubert Lecture: the role of p53 tumour-suppressor gene in tumorigenesis. *Br J Cancer* 1994;69:409–16.
- Soussi T, Legros Y, Lubin R, Ory K, Schlichtholz B. Multifactorial analysis of p53 alteration in human cancer: a review. *Int J Cancer* 1994;57:1–9.
- Baker SJ, Fearon ER, Nigro J, Hamilton S, Preisinger AC, Jessup JM, et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinoma. *Science* 1989;244:217–21.
- Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson JKV, et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* 1990;50:7717–22.
- Rodriges NR, Rowan A, Smith MEF, Kerr IB, Bodmer WF, Gannon JV, et al. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990;87:7555–9.
- Shirasawa S, Urabe K, Yanagawa Y, Toshihara K, Iwama T, Sasazuki T. p53 gene mutations in colorectal tumors from patients with familial polyposis coli. *Cancer Res* 1991;51:2874–8.
- Hamelin R, Laurent-Puig P, Olschwang S, Jeco N, Asselain B, Remvikos Y, et al. Association of p53 mutations with short survival in colorectal cancer. *Gastroenterology* 1994;106:42–8.
- Baas IO, Mulder J-WR, Offerhaus GJA, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5–12.
- Cordon-Cardo C, Dalbagni G, Saez GT, Oliva MR, Zhang Z-F, Rosai J, et al. p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. *Int J Cancer* 1994;56:347–53.
- Bodner SM, Minna JD, Jensen SM, Damico D, Carbone D, Mitsudomi T, et al. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. *Oncogene* 1992;7:743–9.
- Purdie CA, O'Grady J, Piris J, Wyllie AH, Bird CC. p53 expression in colorectal tumors. *Am J Pathol* 1991;138:807–13.
- Scott N, Sagar P, Stewart J, Balir GE, Dixon MF, Quirke P. p53 in colorectal cancer: clinicopathological correlation and prognostic significance. *Br J Cancer* 1991;63:317–19.
- Starzynska T, Bromley M, Ghosh A, Stern PL. Prognostic significance of p53 overexpression in gastric and colorectal cancer. *Br J Cancer* 1992;66:558–62.
- Sun XF, Carstensen JM, Stal O, Zhang H, Nilsson E, Sjodahl R, et al. Prognostic significance of p53 expression in relation to DNA ploidy in colorectal adenocarcinoma. *Virchows Arch A Pathol Anat Histopathol* 1993;423:443–8.
- Yamaguchi A, Nakagawa G, Kurosaka Y, Nishimura G, Yonemura Y, Miyazaki I. p53 immunoreaction in endoscopic biopsy specimens of colorectal cancer, and its prognostic significance. *Br J Cancer* 1993;68:399–402.
- Barak Y, Oren M. Enhanced binding of a 95-kDa protein to p53 in cells undergoing p53-mediated growth arrest. *EMBO J* 1992;11:2115–21.
- Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992;69:1237–45.
- Oliner JD, Kinzler KW, Meltzer PS, Georges DL, Vogelstein B. Amplification of a gene encoding p53 associated protein in human sarcomas. *Nature* 1992;358:80–3.
- Dukes CE, Bussey HJR. The spread of rectal cancer and its effect on prognosis. *Br J Cancer* 1958;12:309–20.
- Lineweaver W. Staging colon cancer. *Contemp Surg* 1984;25:19.
- Spiessl B, Behrs OH, Hermanek P, Hutter RVP, Scheibe O, Sobin LH, et al. *TNM atlas: illustrated guide to the TNM/pTNM classification of malignant tumors*. New York, Berlin, Heidelberg: Springer-Verlag, 1992.
- Morson BC, Sobin LH. *International histological classification of tumours*. Geneva: World Health Organisation, 1976.
- Jass JR, Atkins WS, Cuzick J, Bussey JH, Morson BC,

- Northover JM, et al. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. *Histopathology* 1986;10:437-59.
- 25 Bankfalvi A, Navabi H, Bier B, Böcker W, Jasani B, Schmid KW. Wet autoclave pretreatment for antigen retrieval in diagnostic immunochemistry. *J Pathol* 1994;174:223-8.
 - 26 Wilkinson L. *SYSTAT; The system of statistics*. Evanston, Illinois: Systat Inc., 1989.
 - 27 Steinberg D, Colla P. *Survival: a supplementary to module for SYSTAT*. Evanston, Illinois: Systat Inc., 1988.
 - 28 Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457-81.
 - 29 Kalbfleisch JD, Prentice RL. *The statistical analysis of failure time data*. New York: John Wiley and Sons, 1980.
 - 30 Jensen RA, Page DL. p53: the promising story continues to unfold. *Mod Pathol* 1993;25:455-6.
 - 31 Iggo R, Gatter K, Bartek J, Lane DP, Harris A. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 1990; 335:657-9.
 - 32 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
 - 33 Aaltonen LA, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin J-P, et al. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993;260:812-16.