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Original Paper

Expression of p53 and mdm2 mRNA and Protein in Colorectal Carcinomas

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The aim of our study was to investigate the expression of p53 and mdm2 mRNA and protein in colorectal adenocarcinoma. For the detection of mRNA, 60 fresh frozen human tumour samples and 12 samples of corresponding normal tissue were examined. After total RNA extraction, reverse transcription (RT) was performed followed by cDNA amplification with specific primers using RT–polymerase chain reaction (PCR). Immunohistochemical detection of protein was examined in 81 formalin-fixed and paraffin-embedded human tumour specimens as well as 15 samples of adjacent normal colorectal tissue. p53 mRNA was detected in 80% (48/60) of the tumours and in 67% (8/12) of normal tissue samples; 87% (52/60) of tumours had mdm2 mRNA in contrast to only 17% (2/12) of normal tissue specimens. Nuclear p53 protein expression was observed in 52% (42/81) of the tumour specimens and in none of the 15 normal specimens, whereas mdm2 protein was found in the nucleus (31%, 25/81) and also in the cytoplasm (86%, 70/81) of tumour samples. In normal tissue, mdm2 protein expression was only observed in the cytoplasm (13%, 2/15) and not in the nucleus. There was a significant correlation between coexpression of p53 and mdm2 protein and the occurrence of lymph node metastases ($P = 0.03$) as well as between p53 protein expression and the occurrence of distant metastases ($P = 0.007$). Additionally, significant associations were found between p53 mRNA and p53 protein, p53 mRNA and mdm2 mRNA or protein, and also between mdm2 mRNA and mdm2 protein expression, supporting the existence of a regulatory mechanism involving p53 and mdm2.

Key words: *TP53* tumour suppressor gene, *mdm2* oncogene, colorectal carcinomas, mRNA level, protein level, lymph node metastases, distant metastases
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INTRODUCTION

In 1990 Fearon and Vogelstein described a cascade of genetic changes leading to the development of colorectal cancer in which loss of the *TP53* tumour suppressor function plays a key role in the step from benign adenoma to colorectal cancer [1]. The physiological function of wild-type *TP53* is to 'guard the genome' [2] by transcriptional activation of genes leading to cell cycle arrest. Activated by DNA damage, p53 binds specifically to DNA sequences, resulting in the expression of factors (e.g. WAF-1) which lead to cell cycle arrest in the G1 phase so that DNA damage can be repaired or apoptosis induced [3, 4]. Mutant p53 lacks the capability of specific DNA binding, so its presence as well as the absence of wild-type p53 lead to tumour progression [2, 5]. In fact, many alterations have been detected which inhibit p53 function, such as spontaneous mutation of *TP53*, binding of the protein to viruses or interaction with other proteins such as mdm2.

The *mdm2* gene (murine double minute 2) was originally identified as being amplified and overexpressed in a tumorigenic derivative of mouse 3T3 cells. The amplified sequences were located on extrachromosomal double minute particles [6]. mdm2 has been described to be a cellular phosphoprotein with at least four splice variants having different molecular masses of 57, 59, 67 and 90 kDa [7].

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In normal cells, p53 stimulates the transcription of *mdm2* by binding to an internal specific *mdm2* promoter (P_2) which is located near the 3′ end of intron 1 [8, 9]. This leads to increased mdm2 mRNA and protein expression. mdm2 itself binds to p53 resulting in stabilisation of the complex and subsequent blockage of p53 function [10–12]. Thus, the activity of p53 and mdm2 are kept in balance by an autoregulatory feedback loop first described by Wu and colleagues [13]. In altered cells, high levels of mdm2, resulting from mutation or amplification of *mdm2*, lead to a total block of p53 function and so to increased cell proliferation.

Until now, this autofeedback loop has been mostly examined in cell cultures *in vitro*. Information in the literature concerning the coexpression of p53 and mdm2 mRNA in tumour tissue is scarce. Additionally, there is only one published study on p53 and mdm2 coexpression and its prognostic significance in colorectal carcinomas [14]. There is a lack of information about the coexpression of p53 and mdm2 at the mRNA and the protein level.

In order to investigate the regulatory mechanism mentioned above, the coexpression of p53 and mdm2 at the mRNA and protein level was examined in tumour specimens as well as in adjacent normal colorectal tissue of 81 patients suffering from colorectal carcinoma. Furthermore, correlation between p53 and mdm2 expression and histopathological parameters (TNM classification and tumour grade) was also investigated.

PATIENTS AND METHODS

Tissue samples

81 patients were included in this study (44 male, 37 female), who underwent surgery for colorectal adenocarcinoma between June 1994 and February 1996 in our clinic. The mean age of patients was 69.0 years (range: 43–90 years). Tumour staging and histological grading were performed using the UICC criteria [15]. Sixteen per cent (13/81) of the patients suffered from a stage I tumour, 31% (25/81) from a stage II, 22% (18/81) from a stage III and 31% (25/81) from a stage IV tumour. Most of the patients (79%, 64/81) had a moderately differentiated carcinoma (G2), only 21% (17/81) had a poorly differentiated (G3) tumour.

For RNA isolation, 60 tumour samples and 12 tissue samples of corresponding normal mucosa (control group) were immediately collected after resection and frozen in liquid nitrogen at − 180°C. For immunohistochemical staining, formalin-fixed and paraffin-embedded tissue blocks from all 81 colorectal carcinomas as well as 15 samples of corresponding normal mucosa were used. Slides from all specimens were stained with haematoxylin and eosin in order to include only samples showing predominantly tumour areas or normal mucosa.

RNA-extraction and reverse transcription (RT)

Total RNA was extracted from 100 µg of homogenised tumour or normal tissue samples by treatment with Trizol® reagent (Gibco BRL, Berlin, Germany) according to the manufacturer's recommendations. RNA concentrations were quantified by spectrophotometry (E = 260 nm). Three micrograms of the prepared RNA were reverse transcribed with 1 µl (dT) primer using the Superscript II kit (Gibco BRL) for cDNA synthesis.

Polymerase chain reaction (PCR) amplification

β2 microglobulin PCR was performed for quantitation of the cDNA templates to the internal standard (HeLa cells = 1.0). The primer sequences for β2 microglobulin were: sense: 5′-ATC CAG CGT ACT CCA AAG AAT-3′; antisense: 5′-CAT GTC TCG ATC CCA CTT AAC TAT-3′ (Eurogenetic, Brussels, Belgium) resulting in a cDNA fragment of 254 bp. The results of the p53 and mdm2 mRNA expression in tumour tissues and adjacent normal mucosa were equalised with the β2 microglobulin mRNA content observed in the same samples. Only samples with a positive β2 microglobulin control PCR were evaluated.

Five microlitres of cDNA were used for the PCR with specific primers for *TP53* and *mdm2*. We used the following *TP53* primer sequences: sense: 5′-GGG ACA GCC AAG TCT GTG-3′ (position 596–618); antisense: 5′-GGA GTC TTC CAG TGT GAT-3′ (position 1007–1026) [16] (Stratagene, Heidelberg, Germany) resulting in a 432 bp cDNA fragment. The primers bind to stable exons of the *TP53* gene, which are part of the wild-type as well as the mutated gene. The primer sequences for *mdm2* were as follows: sense: 5′-AAT CAT CGG ACT CAG GTA CA-3′; antisense: 5′-GTC CAG CTA AGG AAA TTT CAG G-3′ [17] (Eurogenetic). The product was a 585 bp cDNA fragment (nucleotides 650–1214 of the *mdm2* sequence). The PCR reaction was performed in 100 µl of reaction medium containing 72.5 µl aqua bidest, 10 µl tricine buffer, 2 µl dNTP (10 mM), 5 µl of each primer (20 µM), 5 µl cDNA (template) and 0.5 µl *Taq* DNA polymerase (5 U/µl) (Gibco BRL). H_2O served as the negative control, 5 µl of cDNA of HeLa cells served as the positive control.

For *TP53* PCR, 35 rounds of amplification were performed in a Perkin–Elmer thermal cycler TC1 (Applied Biosystems, Weiterstadt, Germany), each cycle for a period of 2 min denaturing at 96°C, 2 min annealing at 58°C and 3 min extension at 72°C. For *mdm2* PCR, 32 rounds were performed in the same manner, except using an annealing temperature of 56°C.

The amplified DNA fragments were first analysed by standard agarose gel electrophoresis, stained with ethidium bromide and then quantified by the computer program MultiD (MWG Biotech, Ebersberg, Germany). The computer measured the digitally stored intensity of the bands in positive tumours in relation to the internal standard (HeLa cells = 1.0). For statistical evaluation we classified the results as follows: 0 = no mRNA expression; 1 = very low expression (0.01–0.2); 2 = low expression (0.21–0.4); 3 = moderate expression (0.41–0.6); 4 = medium expression (0.61–0.8); 5 = high expression (0.81–1.0); 6 = very high expression (1.01–1.2); 7 = excessive expression (> 1.2).

Immunohistochemistry

Prepared 4 µm sections of the specimens were dewaxed in xylene, rehydrated in graded acetone (100%, 70%, 40%) and then pretreated for antigen retrieval in citrate buffer using a microwave. After quenching the endogenous peroxidase activity with hydrogen peroxide (10 min), non-specific binding of the antibodies was blocked by 20% normal goat serum for 20 min. The specimens were incubated with the p53 mouse monoclonal antibody (MAb) DO-1 (Oncogene Science, Uniondale, New York, U.S.A.) at a dilution of 1:100 overnight at 4°C, or with the mdm2 mouse MAb IF-2 (Oncogene Science) at a dilution of 1:5 for 30 min at room

temperature, followed by 90 min at 37°C. The MAb DO-1 recognises an epitope of both wild-type as well as mutant p53. The MAb IF-2 is able to recognise an epitope at the amino acid end of the 90 kDa human mdm2 protein [17]. For visualisation of p53 and mdm2 protein, we used the Strept-ABC kit (Dako, Hamburg, Germany) and 3-amino-9-ethylcarbazol (AEC) as the chromogene. The nuclei were counterstained with haematoxylin. p53- and mdm2-expressing colorectal carcinomas were used as positive controls, whereas the primary antibody was omitted for negative controls. Any section with at least one stained cell was determined to be positive. The percentage of positive tumour cells per slide was estimated in areas of maximal concentration of stained cells. Therefore, stained cells were counted in 3×100 square grids, placed in the microscope's eyepiece (magnification 400×). For statistical analysis the results were classified as follows: 0 = no expression; 1 = low expression (1–20% of cells stained); 2 = moderate expression (21–40% of cells stained); 3 = medium expression (41–60% of cells stained); 4 = high expression (61–80% of cells stained); 5 = very high expression (81–100% of cells stained).

Statistical analysis

Statistical evaluation of the different variables (p53 and/or mdm2 mRNA and protein expression, tumour stage and grade) was performed with the SAS statistical program (SAS Institute, Heidelberg, Germany). For univariate analysis the modified chi-square test with Yates correction was used. P values ≤ 0.05 were taken to be significant.

RESULTS

p53 and mdm2 mRNA expression

p53 mRNA was detected in 80% (48/60) of the tumour samples and in 67% (8/12) of the adjacent normal tissue ($P > 0.05$). Compared with standard HeLa cells (arbitrarily given the value of 1) the mean intensity of the bands was 0.76 in tumour tissue (range: 0.7–1.67), whereas the mean in normal tissue was 0.57 (range: 0.38–0.87) (Figure 1).

Eighty-seven per cent 87% (52/60) of tumour samples had detectable mdm2 mRNA in contrast to only 17% (2/12) in corresponding normal tissue ($P < 0.00001$). The intensity of the mdm2 bands was little different between tumour and normal tissue: the mean value was 0.52 in tumour samples (range: 0.05–1.35) and 0.56 in normal tissue (range: 0.48–0.64) (Figure 1).

Eight per cent (5/60) of the tumour samples expressed p53 only, 15% (9/60) mdm2 only. Seventy-two per cent (43/60) had both, and 5% (3/60) showed no p53 or mdm2 mRNA expression.

p53 and mdm2 protein expression

Only nuclear and no cytoplasmic p53 staining was observed in these specimens (Figure 2). Fifty-two per cent (42/81) of the tumour samples were positive for p53, whereas none of the 15 normal tissue specimens were positive. In tumour tissue, the mean proportion of stained nuclei was 68%, ranging from 4 to 98%. In contrast, mdm2 protein expression was both nuclear and cytoplasmic (Figure 2), but in a lower number of cells. Thirty-one per cent (25/81) of the tumour samples had nuclear mdm2 expression, whereas all 15 normal tissue specimens were negative. The difference between cytoplasmic mdm2 staining of tumours and normal tissue was highly significant ($P < 0.00001$): 86% (70/81) in tumours versus 13% (2/15) in normal mucosa.

In contrast to p53 only a few mdm2-positive cells were counted per section. Some sections contained only five to six stained cells, with a mean value of stained nuclei below 1%. As the difference between samples appeared unremarkable, we did not determine the percentage of mdm2 stained cells per section.

Thirty-five per cent (28/81) of the tumour samples were only p53 positive, 14% (11/81) were only mdm2 positive, 35% (28/81) were p53 and mdm2 negative and 17% (14/81) were both p53 and mdm2 positive.

Correlation between p53 and mdm2 expression and clinicopathological parameters

There was a significant correlation ($P = 0.03$) between the overexpression of both proteins and the occurrence of lymph node metastases. Only 8% (3/38) of tumour specimens from patients without lymph node metastases (N0) were positive for both p53 and mdm2, whereas 26% (11/43) of the samples from patients with lymph node metastases (N1–3) expressed both proteins. A significant association was also found between p53 expression and the occurrence of distant metastases ($P = 0.007$): 16/25 (64%) patients with distant metastases showed > 20% p53 expression in contrast to 18/56 (32%) M0 patients (Table 1). There was not significant association between p53 mRNA expression and the occurrence of distant metastases (Table 1), but the number of cases in the groups with an excessive mRNA expression (> 120%) was quite small.

No further significant association has been observed to other clinicopathological parameters such as tumour staging and grading, localisation (colon versus rectum) and tumour size (< 5 cm versus ≥ 5 cm) (data not shown).

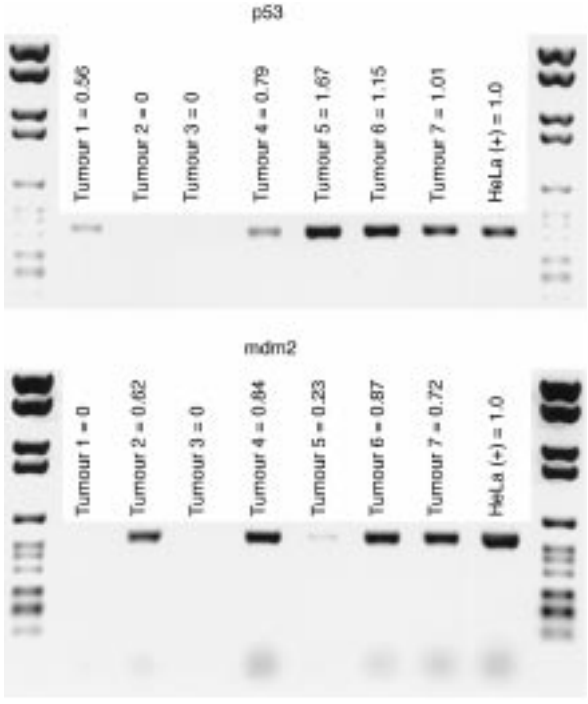

Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) products of p53 and mdm2 mRNA of seven selected tumour specimens. HeLa cells served as a standard (1.0).

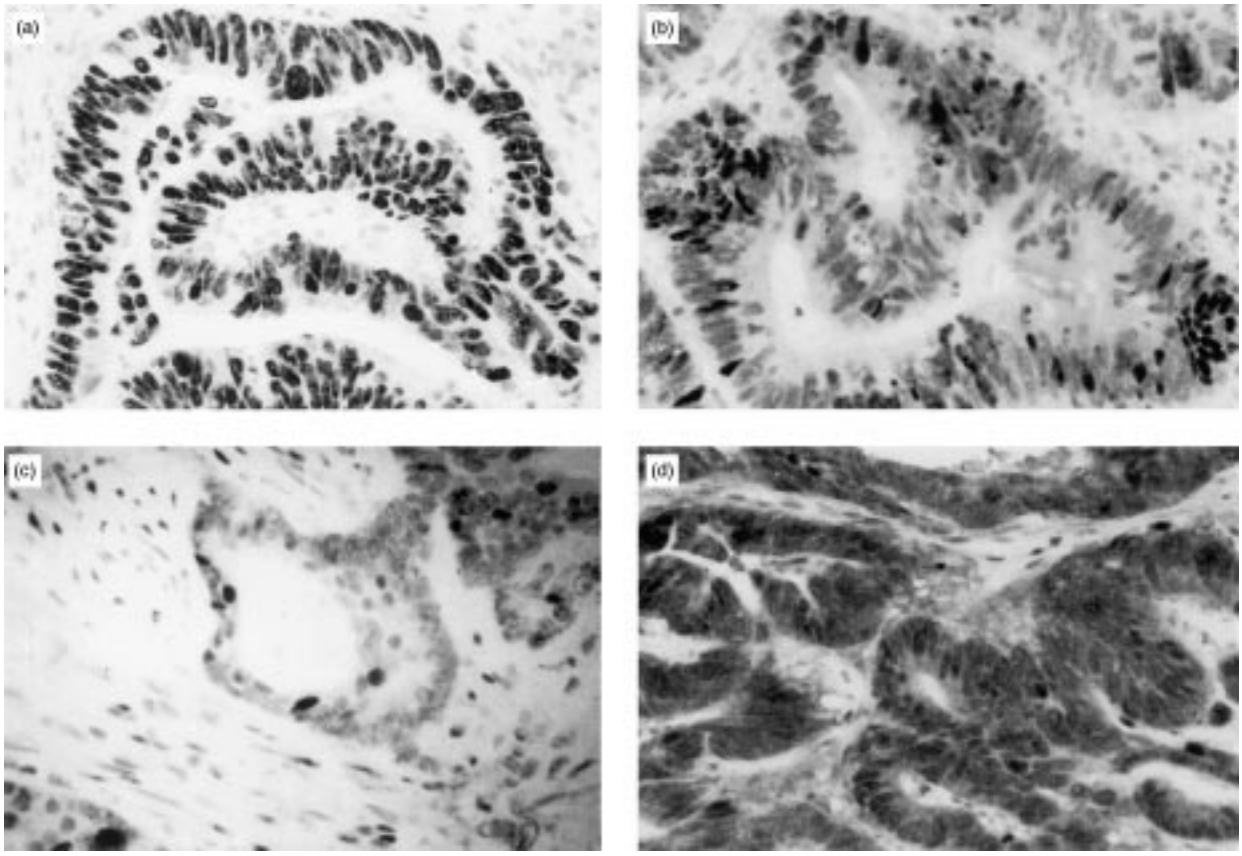

Figure 2. Immunohistochemically stained tumour specimens. Nuclear staining with anti-p53 antibody DO-1 with (a) high expression and (b) low expression. Nuclear staining (c) and cytoplasmic and nuclear staining (d) with anti-mdm2 antibody IF-2. All original magnifications 400×.

Correlation between p53 and mdm2 mRNA or protein expression

p53 mRNA expression showed a significant correlation to p53 protein expression, mdm2 mRNA and mdm2 protein expression, supporting the regulatory mechanism of p53 and mdm2 (Table 2). mdm2 mRNA was significantly associated with nuclear and cytoplasmic mdm2 protein expression. We did not find any association between p53 and mdm2 at the protein level and between p53 protein and mdm2 mRNA.

DISCUSSION

In contrast to the many studies concerning p53 in colorectal carcinomas, only one study has been published on p53 mRNA expression [18], in which 63% of the tumour specimens overexpressed p53 mRNA. In comparison, we found a higher number of tumour specimens (80%) as well as corresponding normal tissue (67%) expressing p53 mRNA. Interestingly, only 52% of the tumour specimens and none of the normal mucosa demonstrated p53 nuclear protein. This difference between mRNA and protein expression has been observed previously in leukaemia cells by Fu and colleagues [19]. They identified a region in human p53 mRNA which is able to inhibit further translation of the protein. This indicates post-transcriptional regulation of p53 protein expression. We found a highly significant ($P = 0.002$) association between p53 mRNA (> 80%) and p53 protein (> 40%) expression in our study.

Reviewing the literature, there are contradictory results on p53 protein expression in colorectal cancer, with results ranging from 25 to 80% [20, 21]. This might be due to the different antibodies used in these studies. We used the MAb DO-1, which recognises wild-type p53 as well as mutant p53 protein and which is especially suitable for paraffin-embedded tissue [22]. In accordance with Greenblatt and associates [23] all normal adjacent colon tissue specimens were completely negative for p53 protein due to the very small amount of wild-type p53 protein they contain.

Data on the occurrence of distant metastases and p53 overexpression in colorectal carcinomas vary in the literature. Whereas some authors have found no significant correlation [21, 24, 25], we observed a highly significant association ($P = 0.007$) between p53 protein expression (> 20%) and the occurrence of distant metastases. This is in accordance with Bertorelle and colleagues [26] and Yamaguchi and associates [27]. In contrast to protein expression, there was no significant correlation between p53 mRNA expression and distant metastases.

Table 1. *Correlation between p53 protein and mRNA expression and distant metastasis*

	p53 protein expression		p53 mRNA expression	
	≤20%	> 20%	≤1.2	> 1.2
M0	68% (38/56)	32% (18/56)	93% (38/41)	7% (3/41)
	$P = 0.007$		n.s.	
M1	36% (9/25)	64% (16/25)	79% (15/19)	21% (4/19)

n.s., not significant.

Table 2. Correlation between p53 and mdm2 mRNA or protein expression in colorectal tumours

	p53 protein expression		mdm2 mRNA expression		mdm2 protein expression			
	>40%	≤40%	>20%	≤20%	Nuclear		Cytoplasmic	
p53 mRNA protein expression								
>80%	13	8	20	1	7	14	13	8
≤80%	10	29	28	11	13	26	22	17
	$P=0.005$		$P=0.02$		$P=1$*		$P=0.68$*	
>60%	15	13	27	1	12	16	19	9
≤60%	8	24	21	11	8	24	16	16
	$P=0.02$		$P=0.03$		$P=0.14$*		$P=0.15$*	
>20%	19	24	37	6	18	25	27	16
≤20%	4	13	11	6	2	15	8	9
	$P=0.13$		$P=0.059$*		$P=0.02$		$P=0.26$*	
mdm2 mRNA expression								
>60%					12	6	14	4
≤60%					8	34	21	21
					$P=0.00045$		$P=0.043$	
>20%					19	29	30	18
≤20%					1	11	5	7
					$P=0.038$		$P=0.18$*	
mdm2 protein nuclear expression								
Yes							25	0
No							$P=0.01$	

*Not significant.

Eighty-seven per cent of our tumour specimens expressed mdm2 compared with only 17% of adjacent normal specimens ($P<0.00001$). Although comparable studies do not exist for colorectal cancer, this is remarkable, as mdm2 mRNA expression has so far only been detected in 7–10% of breast tumours [28] and in glioblastomas and anaplastic astrocytomas [29].

In contrast to this, mdm2 protein expression was much lower, with only 31% of tumour specimens showing nuclear staining, which is nearly the same rate revealed by the study of Öfner and colleagues [14]. No mdm2 protein was detected in normal tissue samples. However, we noticed a significantly higher rate of cytoplasmic mdm2 protein staining in tumour tissue (86%) than in normal mucosa (13%) ($P<0.00001$). Cytoplasmic mdm2 staining has also been described by Wiethege and associates [30] in normal human bronchial epithelium and corresponding tumour epithelium and additionally by Maxwell [31]. The latter author found a selective compartmentalisation of different mdm2 proteins in various non-small cell lung carcinoma cell types, a 76K species was observed exclusively in the cytoplasmic fraction.

As shown in Table 2, a significant association was observed between mdm2 mRNA expression (>60%) and nuclear *or* cytoplasmic mdm2 protein expression as well as between nuclear and cytoplasmic protein expression. Considering that in normal tissue nuclear mdm2 protein is not detectable, cytoplasmic staining is only demonstrable in a small number of cases and mdm2 mRNA expression is also very low, it must be concluded that only a high level (>60%) of mdm2 mRNA leads to a immunohistochemically detectable protein expression.

Regarding the autoregulatory feedback loop, we found a significant association between p53 mRNA and p53 protein expression and also between p53 mRNA and mdm2 mRNA expression, supporting the hypothesis of the loop. Interestingly our results showed no correlation between p53 protein and mdm2 mRNA or protein expression. This could be cautiously interpreted as an existing translational p53 regulation leading to different protein variants, which partly lose the ability to bind specifically to DNA of the mdm2 promoter. It is currently unknown whether the DO-1 p53 antibody is able to detect different p53 protein variants.

In contrast to p53 expression, we did not find any correlation of mdm2 mRNA or protein expression with histopathological parameters. This is in accordance with examinations of Öfner and colleagues [14] at the protein level in colorectal carcinomas and Marchetti and associates [17] at the DNA and protein level in breast carcinomas, but in contrast to studies at the DNA level of Courjal and coworkers [28] in breast tumours, Ladanyi and colleagues [32] in osteosarcomas and Patterson and associates [33] in leiomyosarcomas, in which appropriate associations were found.

Our observation that mdm2 expression does not correlate with common tumour parameters suggests that mdm2 might play an important role in a very early stage of colorectal tumour genesis, mainly in the form of p53 inactivation.

Considering the coexpression of mdm2 and p53, our study showed a significant ($P=0.03$) association between the coexpression of *both* proteins and the occurrence of lymph node metastases. This observation has not been described in the literature before for colorectal carcinomas.

In conclusion, our data support the possibility of a regulatory mechanism between p53 and mdm2 in colorectal cancer, with significant associations between p53 mRNA and mdm2 mRNA expression, between p53 mRNA and mdm2 protein expression and between mdm2 mRNA and mdm2 protein expression. However, these associations could only be demonstrated for p53 mRNA but not for the p53 protein, and there was no significant association between p53 and mdm2 protein expression nor between p53 protein and mdm2 mRNA expression.

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