

MDM2 gene amplification and protein expressions in colon carcinoma: is targeting MDM2 a new therapeutic option?

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Abstract The aim of this study was to investigate murine double minute-2 (MDM2) gene copy number changes in colon carcinoma and to correlate these findings with an immunohistochemical analysis of MDM2 protein expression and histopathologic prognostic indicators of the tumors. The study included 80 cases of sporadic colon carcinomas. MDM2 protein expression was assessed by immunohistochemistry, and MDM2 gene status by fluorescence in situ hybridization. MDM2 gene amplification was detected in 18% of the 80 cases examined. A strong correlation was found between MDM2 gene amplification and the presence, intensity, and staining proportion of cytoplasmic MDM2 protein expression ($p=0.01$). No correlation was found between MDM2 gene amplification and the well-established histopathologic prognostic factors. Given the correlation with gene amplification, we clearly demonstrated that cytoplasmic expression of MDM2

protein is true and relevant and that this finding has to be taken into account when immunohistochemistry would be used as a screening for MDM2 gene amplification in the near future. Targeting MDM2 could be a new approach in colon cancer therapy. The amplification status could be a predictive factor of the response to MDM2-targeted therapy.

Keywords MDM2 · MDM2 protein expression · MDM2 gene amplification · MDM2 in colon carcinoma · Colon carcinoma · Targeted treatment in colon carcinoma

Introduction

The p53 tumor suppressor protein is a transcription factor that is activated in response to cellular stress. Depending on the severity of the threat to genome integrity, p53 then imposes cell-cycle arrest or apoptosis [1–4]. Because p53 has strong growth-suppressive activity, it must be tightly regulated to allow normal cells to function. This is achieved to a large extent by a protein known as murine double minute-2 (MDM2), it is so called because its gene function was first discovered in DNA associated with paired acentric chromatin bodies, termed double minutes, in spontaneously transformed mouse 3T3 fibroblasts. The functions of p53 are ablated in cancer to avoid apoptosis, which can be achieved through mutation or deletion of the TP53 gene, but also by alteration of the MDM2 function. One example of MDM2 alteration is overexpression, which is found in 7% of cancers. MDM2 overexpression due to gene amplification is especially frequent in sarcomas [5]. Reports in the literature on MDM2 gene status in colon cancer are controversial. Apart from one recent study, which was

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mainly based on PCR/ligase detection of MDM2 gene status, most studies could not demonstrate amplification in colon carcinoma [5, 6]. In our fluorescence in situ hybridization (FISH) study, however, we found a significant proportion of cases with MDM2 amplification. Another interesting finding is the correlation of MDM2 amplification with cytoplasmic overexpression of MDM2 protein. These findings could change the therapeutic strategy in a certain subset of colon cancer patients. To our knowledge, this is the first study to examine MDM2 status by FISH in a large series of formalin-fixed, paraffin-embedded colon carcinomas.

Materials and methods

Tissue

Our study was conducted on 80 sporadic colon carcinomas which included 69 cases retrieved from the archives and 11 cases that were sent for K-ras mutation analyses, at Pathology department of Ghent University Hospital between 2006 and 2009. These latter 11 cases did not include information on the pathological stage and histopathologic features. The other 69 tumors were classified according to TNM sixth edition [7]. The patients' data and histopathologic characteristics of the tumors are summarized in Table 1.

Immunohistochemistry

Immunohistochemical staining for MDM2 was performed on formalin-fixed, paraffin-embedded material using a standardized and automated immunostainer (Nexes; Ventana Medical Systems, Tucson, AZ). Four-micrometer-thick sections were deparaffinized in a series of graded alcohol and microwaved in EDTA buffer for 2×10 min at 450 W. After cooling to room temperature and rinsing with Tris buffer, endogenous peroxidase was blocked by preincubation of the slides with 0.3% H_2O_2 , followed by incubation with anti-MDM2 antibody (Dilution, SMP-14; DAKO, Hamburg, Germany) in an immunostainer. IgG2b-stained sections were used as negative controls, and sections from dedifferentiated liposarcoma were used as positive controls. MDM2 protein expression was assessed by a single pathologist (MH). Nuclear staining was recorded separately from cytoplasmic staining. For each staining pattern, MDM2 expression was evaluated based on staining intensity and proportion of positive tumor cells, the latter by calculating the percentage of stained cells in 30 high-power fields. We classified MDM2 expression into three different staining intensities, namely weak, moderate, and strong. In cases where variable staining intensities were noticed, possibly due to tumor heterogeneity, the predomi-

Table 1 Patient data and tumor characteristics

	No. (%)
Sex	80
Male	45 (56%)
Female	35 (44%)
Median age (years) (range)	72 (29–90)
TNM stage	
T stage	69
T1	1 (1%)
T2	7 (10%)
T3	41 (59%)
T4	20 (30%)
N stage	69
N0	36 (52%)
N1	23 (33%)
N2	10 (15%)
Differentiation	69
Good	7 (10%)
Moderate	43 (62%)
Poor	19 (28%)
Lympho-vascular invasion	69
Present	29 (42%)
Absent	40 (58%)
Perineural invasion	69
Present	12 (17%)
Absent	57 (83%)

nant staining intensity determined the overall intensity of MDM2 expression in the tumors.

MDM2 amplification

MDM2 gene amplification status was detected by dual-color FISH. The FISH assay was performed on all the 80 colon tumors according to established laboratory protocol by using Poseidon repeat-free fluorescent-labeled DNA probes specific for MDM2 (12q15) and probes specific for the centromeric region of chromosome 12 (KREATECH Diagnostics, Amsterdam, The Netherlands). The MDM2 FISH assays were scored blindly by counting a minimum of 20 nuclei per case at $\times 100$ magnification with a DAPI/Green/Red triple band pass filter. Only nuclei with at least two CEP12 signals were evaluated to minimize nuclear truncation artifact, and overlapping tumor nuclei were excluded from evaluation to decrease false-positive scoring. The average number of MDM2 and CEP12 signals was then determined and a MDM2/CEP12 ratio was calculated for each case. A ratio of more than 2.0 was considered amplified for the MDM2 gene, whereas a ratio of less than 2.0 was considered nonamplified. A ratio of less than 2.0 with ≥ 2 signals of both probes was considered polysomic for chromosome 12.

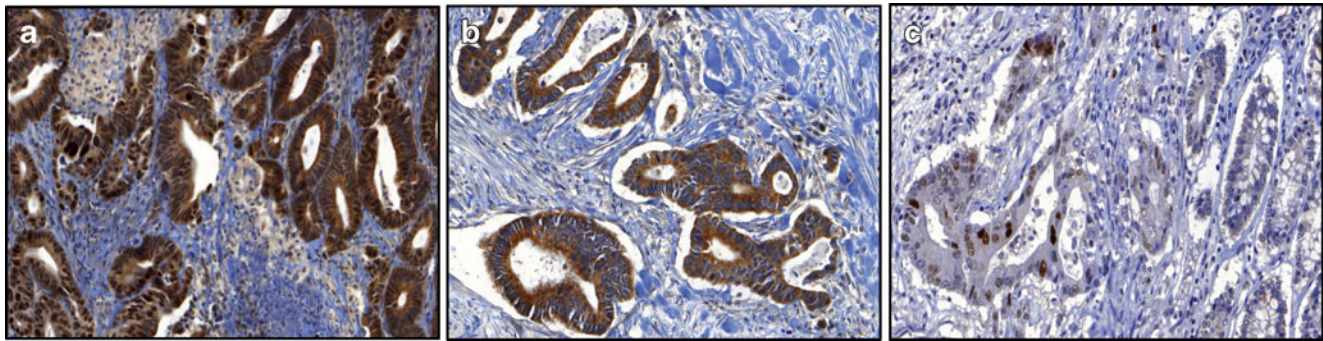


Fig. 1 **a** Case with both nuclear and cytoplasmic MDM2 expression ($\times 10$ magnification); **b** case with only cytoplasmic MDM2 expression ($\times 20$ magnification); **c** case with only nuclear MDM2 expression ($\times 20$ magnification)

Statistical evaluation

The significance of associations between MDM2 gene amplification status, protein expression, and histopathologic features was measured statistically with Chi-squared and Mann–Whitney test, when appropriate. Statview 5.0.1 statistical software system (SAS Institute, Inc., Cary, NC) was used. A probability value of less than 0.05 was considered significant.

Results

MDM2 protein expression

Of the 80 tumors, nuclear MDM2 expression was seen in 18 cases (23%), whereas cytoplasmic expression was present in 29 cases (36%). Only seven cases (11%) showed both nuclear and cytoplasmic MDM2 expression. The different patterns of MDM2 protein expression are illustrated in Fig. 1. We observed an inverse correlation between MDM2 nuclear expression and differentiation grade of the tumors ($p=0.03$). Tables 2 and 3 summarize the different expression patterns of MDM2 protein and their correlation with histopathologic prognostic factors.

Table 2 MDM2 protein expressions in the 80 colon carcinoma

	MDM2 expression patterns			
	Number of cases	Nuclear (%)	Number of cases	Cytoplasmic (%)
MDM2 expression	80		80	
Positive		18 (23%)		29 (36%)
Negative		62 (77%)		51 (64%)
Mean proportion of tumor cells stained	(%)	4.87%		18.62%
	(range)	(0–70%)		(0–100%)
Staining intensity	18		30	
Weak		2 (11%)		17 (57%)
Moderate to strong		16 (89%)		13 (43%)

MDM2 gene amplification

FISH analyses revealed that 14 (18%) out of 80 colon carcinomas showed MDM2 gene amplification (Fig. 2). This amplification significantly correlated with the presence, intensity and staining proportion of cytoplasmic expression of MDM2 protein (Table 4). No association was found between MDM2 gene amplification and any of the major histopathologic prognostic parameters (Table 5).

Discussion

Proper p53 control is critical for its tumor suppressive function. Under homeostatic conditions, p53 is maintained at low levels by MDM2, which catalyzes p53 ubiquitination, marking it for degradation by the proteasome [8, 9]. MDM2 also binds and inhibits the p53 transcriptional activation domain [10]. The importance of MDM2 in p53 regulation was shown in vivo by the lethality of MDM2 $^{-/-}$ embryos at 3.5 days post coitum whereas p53 $^{-/-}$ MDM2 $^{-/-}$ mice develop without abnormalities [11, 12]. Deletion of MDM2 in vivo results in apoptosis. In cancer, inactivation of the tumor suppression function of p53 can be achieved by different mechanisms.

Table 3 Correlation between MDM2 protein expressions and histopathologic parameters

	Number of cases	Nuclear expression			Cytoplasmic expression		
		Positive (%)	Negative (%)	<i>p</i> value	Positive (%)	Negative (%)	<i>p</i> value
TNM stage				0.55			0.27
I	7	3 (43%)	4 (57%)		1 (14%)	6 (86%)	
II	27	5 (19%)	22 (81%)		6 (22%)	21 (78%)	
III	25	5 (20%)	20 (80%)		9 (36%)	16 (64%)	
IV	10	2 (20%)	8 (80%)		5 (50%)	5 (50%)	
Differentiation				0.03 ^a			0.23
Good	7	4 (57%)	3 (43%)		2 (29%)	5 (71%)	
Moderate	43	6 (14%)	37 (86%)		16 (37%)	27 (63%)	
Poor	19	5 (26%)	14 (74%)		3 (16%)	16 (84%)	
LV invasion				0.68			0.24
Present	29	7 (24%)	22 (76%)		11 (38%)	18 (62%)	
Absent	40	8 (20%)	32 (80%)		10 (25%)	30 (75%)	
PN invasion				0.21			0.10
Present	12	1 (8%)	11 (92%)		6 (50%)	6 (50%)	
Absent	57	14 (25%)	43 (75%)		15 (26%)	42 (74%)	

LV lympho-vascular; PN perineural

^a Significance value

Mutational inactivation of p53 occurs frequently. Loss of p53 function also occurs by overexpression of MDM2. This can result from small nucleotide polymorphisms (SNPs) in the MDM2 gene. Bond et al. showed that, in cell culture, SNP309 in the promoter of the MDM2 gene increased the binding affinity of the transcriptional activator Sp1, resulting in high levels of MDM2 RNA and protein [13]. A novel role of the MDM2 SNP309 locus in regulating pancreatic ductal adenocarcinoma formation has recently been suggested and described by Grochola et al. [14]. MDM2 protein overexpression can also be due to gene amplification and the concomitant

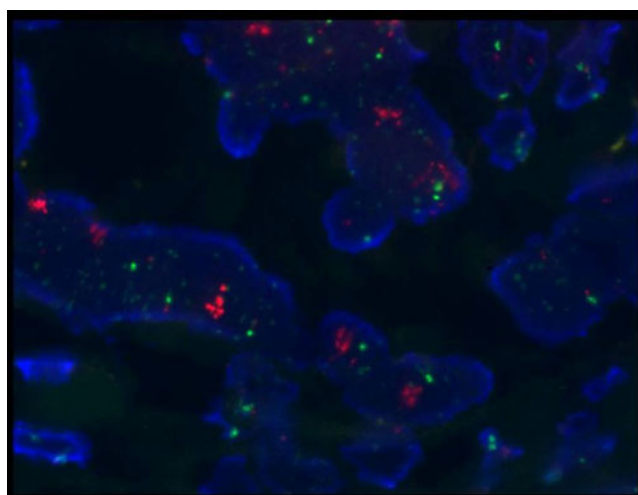


Fig. 2 Representative photomicrograph of the dual-color FISH analysis of MDM2 gene (red) and centromere 12 (green) in a paraffine section of one of our colon carcinoma cases, showing MDM2 amplification with clusters of MDM2 gene copies

appearance of double minutes, hence the name murine double minute [15]. MDM2 gene amplification has been described in different tumors, such as well and dedifferentiated liposarcoma. In fact, more than 95% of these tumors show MDM2 amplification [16]. MDM2 gene amplification has also been reported in glioma, osteosarcoma, hematological malignancies, and carcinomas such as non-small cell lung cancer [5, 17].

In our study of colon carcinoma, we found MDM2 amplification in 18% of our cases, which is 9% higher than previously reported [6]. This might be explained by the use of different methodologies (FISH vs qPCR). This has also been demonstrated in other studies. Vanden Bempt and colleagues conducted a study on polysomy 17 and HER-2 expression in breast cancer and also found discrepancies between HER-2 status shown by FISH and qPCR [18]. Our finding can be of therapeutic interest for several reasons. In most studies, MDM2 amplification occurred only in p53 wild-type tumors [17]. Indeed, a study of MDM2 gene amplification in tumors of 28 different types comprising more than 3,000 tumors supported this notion [5]. A recent study by Forslund et al., however, showed MDM2 amplification in 8% of p53-mutated cases, almost all of which were low-level amplified cases [6]. It seems that concurrent p53 mutation and MDM2 gene amplification is a rare event. A series of genetic studies in mouse models have shown that loss of wild-type p53 induces tumor formation, whereas restoration of p53 leads to a rapid and impressive tumor regression. This provides strong evidence for the benefits of designing anticancer drugs that restore p53 functions [2]. Inhibiting MDM2 activity in tumors with wild-type p53 and amplified MDM2 genes has been

Table 4 Correlation between MDM2 gene amplification and protein expression

		MDM2 gene amplification status			<i>p</i> value
		Number of cases	Amplified (%)	Non-amplified (%)	
All cases	80		14 (18%)	66 (82%)	–
Protein expression patterns					
Nuclear					0.91
Negative	62		11 (18%)	51 (82%)	
Positive	18		3 (17%)	15 (83%)	
Staining intensity					
Weak	2		1 (33%)	1 (67%)	0.17
Moderate to strong	16		2 (8%)	14 (92%)	
Staining proportion					
Mean (range)			10% (0–70%)	3.78% (0–60%)	0.93
Cytoplasmic					
Negative	51		5 (10%)	46 (90%)	0.01 ^a
Positive	29		9 (31%)	20 (69%)	
Staining intensity					
Weak	17		2 (12%)	15 (88%)	0.01 ^a
Moderate to strong	13		7 (54%)	6 (46%)	
Staining proportion					
Mean (range)			43.21% (0–100%)	13.40% (0–100%)	0.01 ^a

^a Significance value

considered an attractive anticancer strategy for many years [19]. The aim is to reactivate wild-type p53 by regressing the inhibitory function of MDM2. One of the approaches is to find small molecules that target MDM2 in such a way that it can no longer bind to p53. In a screening to identify small-molecule inhibitors of MDM2-p53 interaction, Nutlin 3a, MI-219 and MI-319 were identified [20–24]. MI-219 and MI-319 have been shown to disrupt the MDM2-p53 interaction and activate the p53 pathway in cells with wild-

type p53, thereby leading to cell-cycle arrest in both normal and tumor cells and selective apoptosis in the latter. In a recent study in pancreatic cancer, these two MDM2 small-molecule inhibitors synergistically augmented anti-tumor effects of therapeutic drug gemcitabine both in terms of cell-growth inhibition as well as apoptosis [25]. Similarly, mice bearing human tumor transplants that were orally administered with Nutlin 3a showed a decrease of 90% in tumor growth, without evident side effects. Treatment of

Table 5 Correlation between MDM2 gene amplification and histopathologic parameters

	Mdm2 gene amplification status			<i>P</i> value
	Number of cases	Amplified (%)	Non-amplified (%)	
TNM stage				0.22
I	7	1 (14%)	6 (86%)	
II	27	3 (11%)	24 (89%)	
III	25	4 (16%)	21 (84%)	
IV	10	4 (40%)	6 (60%)	
Differentiation				0.93
Good	7	1 (14%)	6 (86%)	
Moderate	43	8 (19%)	35 (81%)	
Poor	19	3 (16%)	16 (84%)	
Lymphovascular invasion				0.53
Present	29	6 (21%)	23 (79%)	
Absent	40	6 (15%)	34 (85%)	
Perineural invasion				0.10
Present	12	4 (30%)	8 (70%)	
Absent	57	8 (14%)	49 (86%)	

wild-type p53 containing HCT116 cells, a colon carcinoma cell line, with Nutlin 3a resulted in increased level of p53, MDM2 and p21, which is consistent with activation of the p53 pathway. Results confirmed that p53 accumulation is caused by a decrease in degradation rather than an increase in expression. How does MDM2 amplification influence the response to MDM2 antagonists such as Nutlin-3a? Tovar et al. tested the effects on cell-cycle arrest and apoptosis in several cancer cell lines and interestingly found that the degree of induction of apoptosis by Nutlin-3a varied among cancer cell lines. Osteosarcoma cell lines (SJSA-1 and MHM) with MDM2 gene amplification were the most sensitive, whereas HCT116 (colon cancer) and A549 (lung cancer cell lines) harboring no MDM2 gene amplification were the least sensitive to MDM2 antagonists [26]. Therefore, it appears that MDM2 amplification is a predictive marker for response to anti-MDM2 therapy [27, 28].

In contrast to Forslund et al. [6], we found no correlation between MDM2 amplification and the well-recognized histopathologic prognostic predictors including tumor stage. However, we observed a significant correlation between the presence, intensity, and staining proportion of cytoplasmic MDM2 protein expression and MDM2 gene amplification, implying that MDM2 gene amplification could have prognostic value in this specific subgroup of patients since cytoplasmic MDM2 proteins have been shown to play an important role in p53 inactivation [29]. There are conflicting data concerning whether cytoplasmic expression of MDM2 protein is true or false positive [30–33]. Given the correlation with gene amplification, we clearly demonstrated that cytoplasmic expression of MDM2 protein is true and relevant. Therefore, this finding has to be taken into account when immunohistochemistry would be used as a screening for MDM2 gene amplification in the near future, assuming that clinical studies validate the in vitro and animal findings regarding small-molecule inhibitors of MDM2.

Conclusion

Our study is the first MDM2 FISH study in colon carcinoma, correlating the amplification status with protein expression detected by immunohistochemistry. MDM2 amplification was demonstrated in 18% of cases and significantly correlated with presence, intensity, and staining proportion of cytoplasmic MDM2 protein expression. No correlation was found between MDM2 gene amplification and histopathologic prognostic factors. These findings suggest that targeting MDM2 could be a new approach in colon cancer therapy, and amplification status could be a predictive factor of response to MDM2-targeted therapy.

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Disclosure/conflict of interest None of the authors has conflict of interests to declare.

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