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Analysis of the p53-hMDM2-p21 (WAF1/CIP1) Cell Cycle Regulation Pathway in Malignant Fibrous Histiocytomas

ULRICH BRINCK[1], THILO SCHLOTT[2], CARLOS CORDON-CARDO[3], JERZY STACHURA[4], MARTIN WALZ[5], GÖSTA FISCHER[1] and MONIKA KORABIOWSKA[1]

[1]Department of Pathology, Reinhard Nieter Hospital, Friedrich Paffrath Str. 100, 26389 Wilhelmshaven, Academic Hospital of the University Göttingen, Göttingen;
[2]Department of Pathology, University Göttingen, Robert Koch Str. 40, 37075 Göttingen, Germany;
[3]Division of Molecular Pathology, Memorial Sloan-Kettering Cancer Center, 1075 York Avenue, 10021 New York, U.S.A.;
[4]Department of Pathology, Jagiellonian University, Grzegorzecka 16, 30531 Krakow, Poland;
[5]Department of Traumatic and Reconstructive Surgery, Kliniken Uelzen & Bad Bevensen, Hagenskamp 34, 29525 Uelzen, Germany

Abstract. *This study was undertaken to analyze patterns of expression of critical cell cycle regulators (CCR) involved in the p53 pathway in malignant fibrous histiocytomas (MFH). Protein expression was assessed using immunohistochemistry analyzing p53, hMDM2 and p21 (WAF1/CIP1) phenotypes. p53- and hMDM2-positive phenotypes were found to be associated with low p21 levels ($p<0.01$). Positive hMDM2 phenotype did not correlate with any hMDM2 mutations, which in our tumor collective were not found. High-grade MFH differed from MFH grade I and II concerning higher p53 and lower p21 levels, while hMDM2 expression was independent of grade. Inclusion of categorized values into a Cox regression study proved the independent prognostic relevance of p53, hMDM2 and p21 phenotypes.*

Malignant fibrous histiocytoma (MFH) occurs with a high frequency among malignant soft tissue tumors in late adulthood (1,2). Approximately 43% of primary MFH tumors are localized in the lower extremities, 19% in the upper extremities, 14% in the trunk, 13% in the retroperitoneum and mesentery and 10% in the head and neck region (1). The prognosis of MFH has been mainly based on the assessment of the histopathological grade of the lesion and the size of the tumor (3). However, it is well known that tumors presenting different stages and grades might behave with different clinical outcomes, and that the use of molecular markers could assist in the stratification of these neoplasms (4,5).

Correspondence to: PD Dr. Monika Korabiowska, Department of Pathology, Reinhard Nieter Hospital, Friedrich Paffrath Str. 100, 26389 Wilhelmshaven, Germany. Tel 00494421892782, Fax: 00494421892771, e-mail: Ubrinck@aol.com

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p53 mediates G1 arrest of tumor cells in certain circumstances by transactivating p21, a cyclin-dependent kinase inhibitor (6-8). Analysis of the partial crystal structure of p53 has demonstrated that the fragment between amino acid residues 94 through 312 contains the DNA-binding domain crucial for the biological activity of p53 (9). Mutations of p53 are among the most common genetic abnormalities identified in human cancer, including soft tissue sarcomas, and more specifically MFH (4, 10-12). Moreover, detection of p53 point mutation by polymerase chain reaction followed by single strand conformation polymorphism (PCR-SSCP) and sequencing have been found to be associated with nuclear accumulation of altered p53 products, as determined by immunohistochemistry (12-13).

As part of its own autoregulatory feedback loop, p53 transactivates hMDM2 (14). It has been shown that hMDM2 binds to p53, inhibiting its transcription regulatory activity and presenting p53 for ubiquitin-mediated degradation (15,16). hMDM2 maps to an amplicon on 12q13-14, and has been shown to be amplified and overexpressed in soft tissue sarcoma (10).

The main objective of this study was to determine the potential prognostic significance of critical genes involved in the G1-induced p53 arrest, including p53, hMDM2 and p21, in malignant fibrous histiocytoma, as well as to correlate their patterns of expression with hMDM2-mutations and clinical outcome.

Materials and Methods

Patient characteristics and tissues. A well-characterized cohort of 161 primary malignant fibrous histiocytomas, corresponding to patients seen at the teaching hospitals of the University of Göttingen, Germany and the University of Krakow, Poland between 1992 and

2001, was the focus of this study. There were no differences between patients treated in Poland (n=116) and Germany (n=45) with respect to disease-related survival (log-rank test). The age of the patients ranged between 4 and 90 years (mean 61 years). Forty-five % of MFH patients were females and 55% males.

The anatomic localization of these tumors was distributed as follows: head and neck (n=27), upper extremities (n=19), lower extremities (n=86) and trunk (n=29). Thirty-six tumors (22%) showed superficial localization, while 117 (73%) had deep localization. Tumor depth could not be determined in 8 cases. The size of the tumors ranged between 0.6 and 36 cm (median=8cm). The histopathological grade was determined as previously (17). Thirty-nine (24%) of the neoplasms were assigned to pathological stage pT1, and 122 (76%) tumors to pT2. In 43 patients (27%), distant metastases were clinically present at the time of the primary diagnosis. In 2 patients, locoregional lymph node metastases were assessed by histopathology. All patients were observed from the time of diagnosis up to the end of the study period (October 2001) and disease-related survival within this period was documented. Non-MFH-related fatalities were excluded from the investigation.

Antibodies and immunohistochemistry. The following primary antibodies were utilized for immunohistochemical analyses: mAb D07 to wild-type and p53 mutant proteins (Dako, Carpinteria, USA); mAb 1B10 to hMDM2 (murine double minute or hMDM2 gene product, Novocastra Laboratories, Newcastle Upon Tyne, UK); mAb 2G12 to human p21 (Pharmingen, San Diego, USA).

Four micron-thick tissue sections were pretreated with microwave heating in citrate buffer (720 W) for antigen retrieval (three times for 5 min for immunohistochemical detection of p53 and hMDM2). The tissue was incubated with the primary antibodies at dilutions of 1:50 (hMDM2), 1:10 (p53), and at a concentration of 20 mg/ml (p21) for 2 h at 20˚C (p53) and for 24 h at 4˚C (hMDM2 and p21). The immunohistochemical reactions were carried out using the alkaline phosphatase-antialkaline phosphatase (APAAP) method for p53. The Biotin Streptavidin Amplification (B-SA) Detection System (Biogenex, Hamburg, Germany) was used to detect p21 and biotinylated anti-mouse IgM (Dianova, Hamburg, Germany) and the avidin-biotin- peroxidase complex (ABC) technique for hMDM2. Hereby, 3-amino-9-ethylcarbazole was used as the final substrate. The sections were counterstained with hematoxylin. Squamous cell carcinomas of the oral cavity known to overexpress p53, sarcoma specimens with overexpression of hMDM2, and intestinal mucosa with known immunoreactivity for p21 and proliferation markers were used as positive controls. As a negative control, sections from all cases were incubated in the absence of primary antibodies.

Immunohistochemical evaluation was done by at least two independent investigators. Each observer determined the percentage of tumor cells displaying nuclear immunolabelling reactivities in 10 high-power fields (areas measured at x 400 magnification) per histological specimen. Minor quantitative differences of results between observers (mostly below 5%) allowed us to use mean values of results for statistical analysis

Analysis of hMDM2 mutations

DNA preparation. DNA was isolated for hMDM2 PCR from sections of routinely-fixed, paraffin-embedded archival tissues using the QIAamp Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Before DNA isolation, paraffin-embedded material was processed by standard methods: deparaffination of tissue sections by washing twice in xylene at 60˚C for 20 min, rehydration in absolute ethanol, and desiccation.

Amplification of β-globin gene. β-globin gene was amplified using a 3 μl aliquot of DNA in a final volume of 50 μl. After an initial 95˚C denaturation step for 7 min, 40 cycles were performed at 95˚C (1 min), 60˚C (1 min), and 72˚C (1 min), followed by a final extension step at 72˚C for 7 min. PCR products were separated on a 3% (w/v) agarose gel and stained with ethidium-bromide. The reaction mixture contained 40 pmol of primer 5'-ACTCTCTCTGCCTATTGGTC-3' (Sense) and 5'-ACTCACCCTGAAGTTCTCAG-3' (Anti-Sense), 1 μl dNTP (10mM each), 5 μl PCR buffer (10x), and 1 U Taq polymerase (Pharmacia, Germany). The primers yield a 270 bp product. Only samples were used for hMDM2 exon 12 analysis that had given positive results in β-globin PCR.

Amplification of hMDM2 exon12. hMDM2 exon 12 was amplified using a 3 μl aliquot of DNA in a final volume of 50 μl. After an initial 95˚C denaturation step for 7 min, 40 cycles were performed at 95˚C (45 sec), 50˚C (50 sec), and 72˚C (50 sec), followed by a final extension step at 72˚C for 7 min. PCR products were separated on a 3% (w/v) agarose gel and stained with ethidium-bromide. The reaction mixture contained 20 pmol of primer 5'-GACTATTGG AAATGCACTTC-3' (Sense) and 5'-ATTGGTTGTCTACATAC TGG-3' (Anti-Sense), 1 ml dNTP (10mM each), 5 ml PCR buffer (10x), and 1 U Taq polymerase (Pharmacia). The primers yield a 545 bp product (Figure 1).

Sequencing of PCR fragments. hMDM2 PCR fragments were purified using QIA Quick PCR Purification Kit (QIAGEN). The fragments were labelled with the PRISM-Ready Reaction Dye Deoxy-TM Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) and analysed in an ABI 310 analyzer. Sense or anti-sense oligonucleotides served as sequencing primers.

Statistical analyses. Results were expressed as the percentages of tumor cells presenting nuclear immunostaining (expression index = index). In general, all index-related data were rank-scaled (*i.e.*, no means or standard deviations were calculated since indices are not metrical data *per se*). The Mann-Whitney U-test was used for the paired group comparisons (between malignancy grades).

Cox regression was the multivariate method used for predicting the survival rate based on several parameters (18). The Kaplan-Meier method was employed to calculate survival rates (19). The significance of the differences between the survival curves was calculated using the log-rank test.

Results

Patterns of p53 expression. All p53 immunoreactive MFH cases showed a characteristic intense and selective nuclear localization of the reaction product (Figure 2A-B). p53 immunoreactivities were detected in 32% of the cases analyzed, ranging from 1% to 70% labelling tumor cells (Table I). Staining was predominantly observed in grade III tumors. Grade I lesions showed p53 immunostaining in only

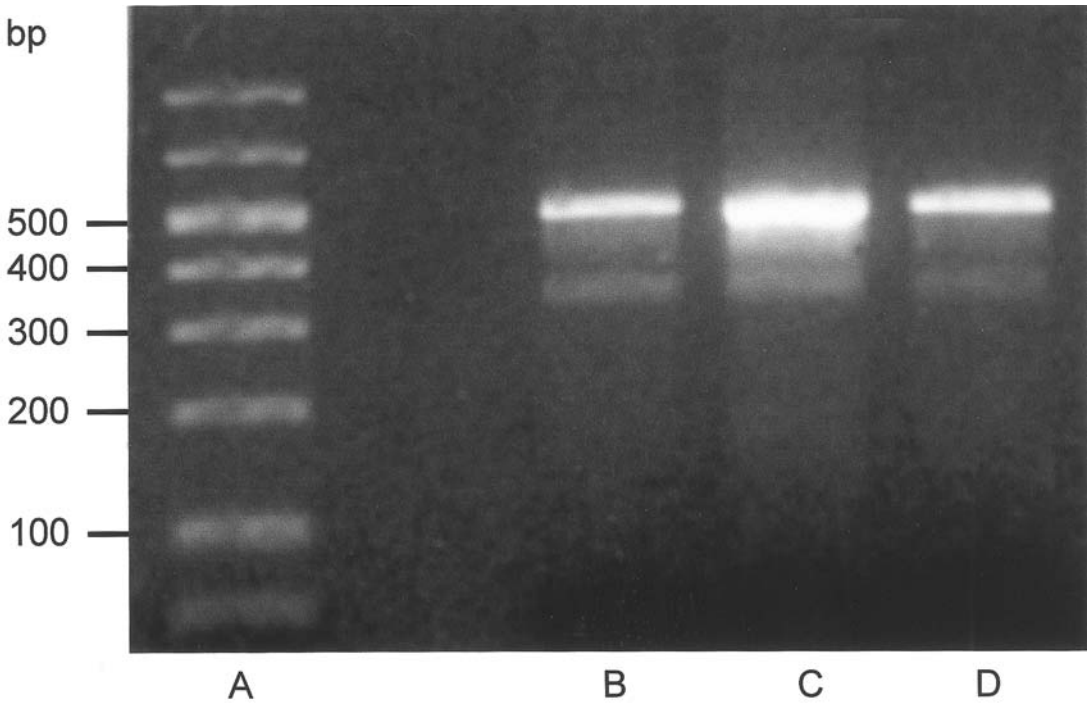

Figure 1. *Detection of hMDM2 exon 12 on 3% agarose gel. Lane A shows molecular weight standard. Lanes B-D contain PCR results of four MFH revealing hMDM2 overexpression. The bands with a length of 545 base pairs were identified by automatic DNA sequencing.*

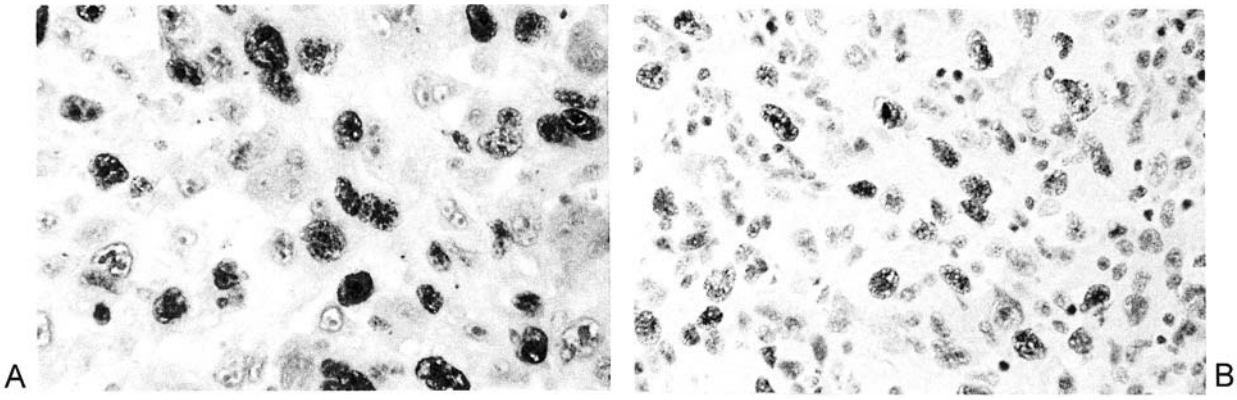

Figure 2. *Photomicrographs of 2 MFH primary tumors, storiform-pleomorphic subtype, (A-B) with unfavorable (A-high >20% p53 expression) and favorable (B- low p53 expression <20%) prognosis.*

5 of the 31 investigated cases. The p53 index was significantly lower in grade I and grade II tumors than in grade III lesions (Table I).

We considered high p53 expression, or detection of a p53-positive phenotype, when >20% tumor cells displayed nuclear immunoreactivities. Cases presenting with this p53 expression pattern had a lower probability of survival than cases showing undetectable or low p53 staining (index 0-20), both considering the whole cohort (including UICC stages I-IV; Figure 3A, C) as well as in the group of localized primary MFH lesions (UICC stages I-III) ($p<0.01$). The applied cut-off point, *i.e.* >20% positive tumor cells, had the highest discriminatory value with respect to survival as judged on the basis of obtained *p*-values in log-rank tests.

Table I. *Expression of cell cycle regulators in malignant fibrous histiocytomas investigated.*

Parameter	Grade	Minimum	25th Percentile	Median	75th Percentile	Maximum
p53	All cases	0	0	0	1	70
index	I	0	0	0	0	8
	II	0	0	0	0	11
	III	0	0	0	23.5	70
hMDM2	All cases	0	0	10	40	99
index	I	0	3	13	26	85
	II	0	0	5	30	70
	III	0	0	14	74.75	99
p21	All cases	0	0	6	23	90
index	I	0	9	22	29	85
	II	0	1	9.5	23.75	90
	III	0	0	1	15	62

Patterns of hMDM2 expression. All hMDM2 immunoreactive cases showed intranuclear labelling. hMDM2 nuclear immunostaining was detected in 73% of the MFH lesions, ranging from 1% to 99% (Table I). The median hMDM2 index was 14% for grade III tumors, 5% for grade II, and 13% for grade I lesions. These differences were not statistically significant. We determined the cut-off point by defining the value that had the highest discriminatory significance with respect to prognosis, being in this study >33% positive tumor nuclei. Cases displaying this phenotype had a lower probability of survival than cases with undetectable or low hMDM2 expression levels, both considering the whole cohort (UICC stage I-IV; Figure 3A, B) as well as in the group of localized primary MFH lesions (UICC stage I-III) ($p<0.01$).

Analysis of hMDM2 mutations. The sequence located near the stop codon of the hMDM2 gene was screened for mutations. This region representing exon 12 codes for a zinc

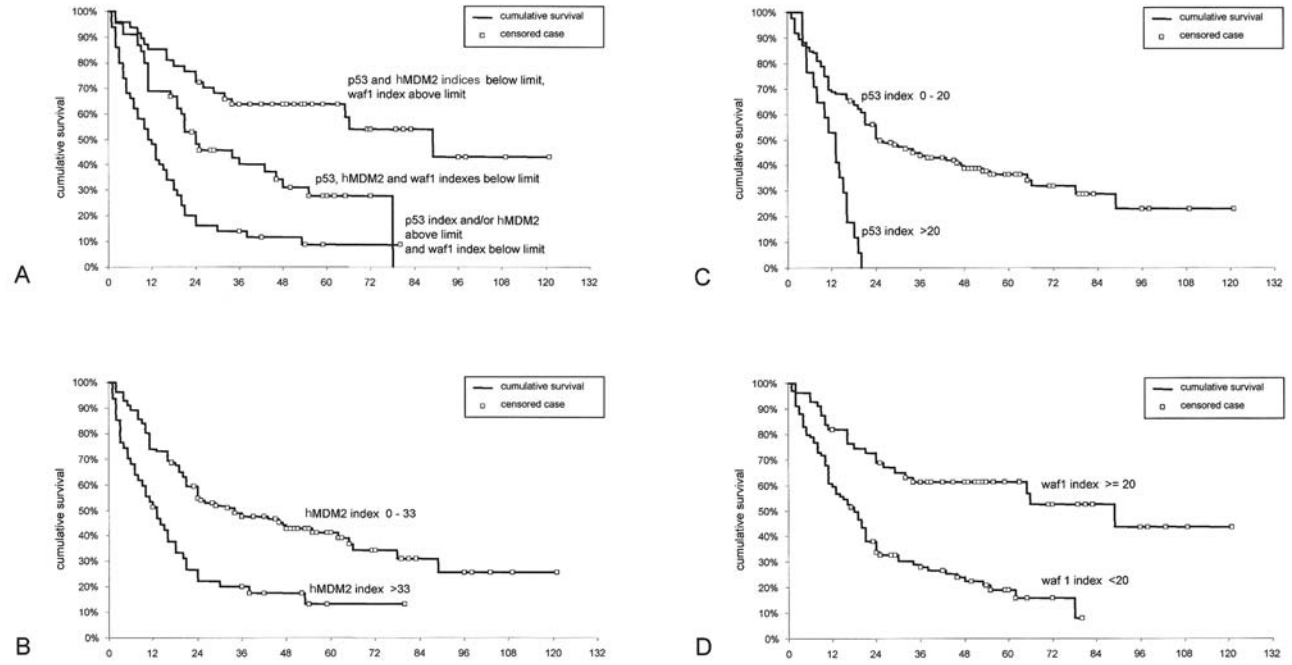

Figure 3. *A: Kaplan Meier survival curves for MFH cases without deregulated expression of CCR (p53- and hMDM2- negative phenotypes, p21 (waf1/cip1)- positive phenotype), deregulated expression of p21 (p53-, hMDM2- and p21- negative phenotypes) and deregulated expression of p53 or/and hMDM2 coincident with a p21- negative phenotype. Survival is given in months.*
B: Kaplan Meier survival curves for patients with hMDM2- negative phenotype (index 0-33) as well as with hMDM2- positive phenotype (index>33) of the MFH primary tumors. Survival is given in months.
C: Kaplan Meier survival curves for patients with p53- negative phenotype (index 0-20) as well as with p53- positive phenotype (index >20) of the MFH primary tumors. Survival is given in months.
D: Kaplan Meier survival curves for patients with p21 (waf1/cip1)- negative phenotype (index 0-19) as well as with p21- positive phenotype (index ≥20) of the MFH primary tumors. Survival is given in months.

Table II. *Results of the Cox regression for predicting survival based on categorized CCR indices (p53, hMDM2, p21).*

Remaining independent prognostic parameters	*P*-value	Change of hazard rate per unit of parameter	Lower bound of 95% confidence	Upper bound of 95% confidence
p53 index	$p=0.003$	+54%	+16%	+104%
hMDM2 index	$p=0.031$	+27%	+2%	+58%
p21 index	$p<0.001$	-27%	-50%	-16%

finger/ RING finger domain involved in RNA binding and was selected as a target because previous studies had indicated mutational hot spots in various tumor entities The present study shows that malignant fibrous histiocytomas are not affected by gene mutations in hMDM2 exon 12.

Patterns of p21 expression. p21 was selectively detected in the nuclei of tumor cells in certain cases. The percentage of p21- immunoreactive cases was 68%, ranging from 1% to 70% (median=6) (Table I). The median of p21 expression was higher in grade I and II tumors than in grade III MFH lesions (Table I). As for the p53 index, the applied cut-off point for the p21 phenotype that had the highest discriminatory value with respect to prognosis was that of >20% tumor cells displaying nuclear immunoreactivities. Cases of MFH with this positive phenotype showed a higher probability of survival than cases with undetectable or low p21 expression, both considering the whole cohort (UICC stage I-IV; Figure 3A, D) as well as in the group of localized primary MFH tumors (UICC stage I-III) ($p<0.01$).

Predictability of survival based on the expression of p53, hMDM2 and p21 (Cox regression and multivariate analysis). Inclusion of categorized cell cycle regulator indices, as defined above, into a Cox regression resulted in the finding that each of the markers conferred independent prognostic relevance, both considering the whole cohort (UICC stage I-IV; Table II) as well as in the group of localized primary MFH lesions (UICC stage I-III) ($p<0.05$).

Discussion

Data from the present study reveals that altered patterns of expression of critical genes involved in the p53 pathway are associated with increased tumor proliferative activity and adverse prognosis for patients affected by MFH. These observations are in support of other previously published studies (10-11,17).

The impact of deregulation of the p53-hMDM2-p21 cell cycle regulation pathway has been analyzed in the context of clinical studies (20). It appears that the accumulation of alterations affecting several of the genes in the p53 pathway confers cooperative effects (10). This could be explained by the multiple effects that the expression of these genes target. For example, it has been reported that hMDM2 acts as an ubiquitin ligase and that it can recognize a variety of substrates, including other critical cell cycle regulators such as retinoblastoma gene protein (21).

Results from this study demonstrate the dependency of p21 expression patterns on both p53 and hMDM2 status. These relationships could be explained by the fact that p21 is a transactivation target of p53, and that hMDM2 inactivates p53 function (14,15). However, the finding that low levels of p21 were found in the absence of p53 or hMDM2 alterations suggests that alternative mechanisms participate in the regulation of p21. This observation is consistent with previous findings in transitional cell carcinoma of the urinary bladder (22). With respect to p53, our data are in accordance with previous reports on sarcoma series, composed of different histological types, which suggest that p53 overexpression is related to functional inactivation of this protein (23,24).

The genetic basis and functional relation to growth control for hMDM2 overexpression in sarcomas is only partly known. In about 20% of malignant soft tissue tumors, hMDM2 overexpression is related to amplification of the gene (25). Protein overexpression does not necessarily correlate with increased hMDM2 mRNA expression in soft tissue sarcomas (26). Additionally, to enhanced translation of hMDM2 mRNA, reduced hMDM2 protein degradation is considered a possible cause for immunohistochemically detectable increased hMDM2 expression (27-29). Recent studies suggest that the hMDM2 protein consists of growth stimulatory and inhibitory domains (30). In the present study, an antibody to the C-terminal end of hMDM2 (clone 1B10) was used, which recognizes a sequence of amino acids distant to the p53-binding domain of the protein. A recent study, on a mixed collection of sarcoma types reported that immunoreactivity to this part of the hMDM2 protein has prognostic impact, but not immunoreactivity with antibodies to the p53- binding domain of hMDM2, thus suggesting that the C-terminal domain may have a prognostically unfavorable function (31,32). The data presented here show that the vast majority (89%) of MFH cases with hMDM2 overexpression had a loss of p21 immunoreactivity and that hMDM2 overexpression exerts an independent unfavorable prognostic influence in addition to loss of p21 immunoreactivity. Hence, it is suggested that hMDM2 overexpression may act both in a p53-p21- related and independent manner. The present study demonstrated that high grade MFH (grade III) differed from MFH of grade I and II concerning higher p53 and lower p21

indices, whereas hMDM2 immunoreactivity was not related to grade. These results suggest that deregulation of hMDM2 is an early molecular alteration and may also be relevant in low grade sarcomas.

As its ultimate objective, this study dealt with the question of the prognostic relevance of cell cycle regulators of the p53 pathway. In univariate analyses (log-rank test), we were able to show that patients with MFH primary tumors that were positive for p53 (index>20), hMDM2 (index>33) or were immunonegative for p21 (index<20) had significantly reduced median survival rates compared to patients with tumors that did not meet the respective criterion. These data confirm the prognostic significance of p53 and hMDM2 overexpression previously found in investigated series, composed of heterogeneous histological types of soft tissue sarcomas (10, 31-33). The presented results also confirm the prognostic significance of immunodetection of these CCR in paraffin-embedded tissue.

The unfavorable prognostic impact of the p53- positive phenotype has also been shown for MFH (11). Additionally, the independent prognostic impact of all 3 investigated cell cycle regulators (p53, hMDM2, p21) was demonstrated in multivariate analyses. With respect to p53 and hMDM2 overexpression, these data are in accordance with previous studies on sarcoma series, composed of different histological types (31-32).

The unfavorable prognostic impact of the p21- negative phenotype in MFH is a new finding, which, to our knowledge, has not been examined in any other type of soft tissue sarcomas. Previous studies on various tumor types suggest that loss of p21 protein expression is not due to mutations of the p21 gene (34-35). p21 expression is also known to be regulated independently of p53 and hMDM2 by other factors, *e.g.* growth factors (36-40). Consequently, this influence may account for its prognostic impact, unrelated to p53 and hMDM2 expression.

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