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mdm2 mRNA Level is a Prognostic Factor in Soft Tissue Sarcoma

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Abstract

Background: The oncogenic properties of murine double minute-2 (*mdm2*) protein over-expression, which mostly results from the interaction with the tumor suppressor p53, are well described and their negative impacts on the prognosis of affected patients is well characterized. However, clinical relevance of *mdm2* mRNA expression is poorly investigated.

Materials and Methods: In this study, 65 soft tissue sarcoma (STS) samples were analyzed for *mdm2* mRNA expression by a quantitative reverse transcription polymerase chain reaction (RT-PCR) approach using available validated ready-to-use assays based on the TaqMan® technology (PE Applied Biosystems, Weiterstadt, Germany). *Mdm2* data were correlated to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression calculated from the same sample.

Results: For patients with a *mdm2*/*GAPDH* mRNA ratio below 50 zmol/amol the survival was strikingly reduced in comparison to patients with a ratio of ⩾50 ($p = 0.0241$). Multivariate Cox analysis showed that the difference in prognosis for patients with tumor stage 2 and 3 became even more pronounced between patients with a ratio of <50 zmol/amol and patients with a ratio of ⩾50 ($p = 0.0041$; $RR = 5.6$). To test if the group with an *mdm2* mRNA expression ⩾50 is homogenous concerning the prognosis, the group was divided into three subgroups with values of 50 to <100, 100 to <500 and ⩾500. The subgroup with values of 100 to <500 showed the best prognosis ($p = 0.0164$); whereas, the one with values of 50 to <100 showed the worst prognosis in this group and, in between, was the one with values of ⩾500. After omitting patients of stage 1 and 4, the subgroup with values of 100 to <500 showed an even more striking best prognosis ($p = 0.0015$); the other subgroups remained in the same sequence. The risk of tumor-related death over 5 years was most conspicuous in patients with *mdm2* mRNA expression <50 than in those with ratios of 100 to <500 displaying a 13.3-fold higher risk. In a comparison between *mdm2* mRNA levels and P53 protein expression or *p53* mutational status, no relationship was found.

Conclusions: In our study, the *mdm2* mRNA level appears to be an independent prognostic factor for STS patients, marking its role in STS genesis and as a potential factor for gene therapeutical approaches.

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Introduction

The *mdm2* gene was originally isolated by virtue of its amplification in a tumorigenic derivative of NIH-3T3 cells (1,2). Beside localiza-

tion of the human *mdm2* gene to chromosome 12q13–14, *mdm2* gene amplifications were detected in a variety of human malignancies and particularly in sarcomas (3–8). The oncogenic properties of the human *mdm2* gene product have been attributed mostly to its interaction with the tumor suppressor gene *p53* (9). *Mdm2* promotes inactivation of *p53* by its rapid degradation, inhibition of *p53*-mediated apoptosis/growth arrest and by masking the transactivation domain of *p53,* thus, impairing the interaction with the transcriptional machinery (3,10–19). The effect of *mdm2* can be modulated by other proteins as RB-1 or $p19^{ARF}$ (20,21). On the other hand, mdm2 can act independently from p53, for example, it interacts with transcription factors of the *E2F*-family and the human TATA-binding protein (22,23), inhibits Rb growth regulatory function (24), contributes to tumorigenesis in $p53^{-/-}$ mammary epithelial cells (25), mediates TGF-β1 resistance (26), and inhibits the G_0/G_1-S-phase transition in normal human diploid cells (27). In numerous tumor cell lines and malignant tumors, particularly sarcomas, the human MDM2 protein overexpression is a characteristic feature (28) which can be correlated to poor prognosis (7,29). However, overexpression may occur independently of gene amplification and might correlate with an increased transcription and/or a different translation efficiency of human *mdm2* transcripts (30–35). Occurrence of different mRNA transcript levels and splice products in malignant tumors is well described (31,36,37), but only recently was an impact on tumor behavior and prognosis uncovered (38,39). Comparably less is known about *mdm2*-mRNA expression in sarcomas, beside correlating it to *mdm2* gene amplification (7,35,40). Therefore, the aim of this study was to investigate the level of human *mdm2* transcription, if there is a relationship to *p53* mutational state or P53 protein expression, and if the *mdm2* mRNA level has a prognostic impact for soft tissue sarcoma (STS) patients.

Materials and Methods

Tissue Specimens and Histopathological Data

We examined 65 frozen tumor samples from 65 adult, non-selected soft tissue sarcoma STS patients (Institute of Pathology, University of Halle, Germany and Surgical Clinic 1, University of Leipzig, Germany); consisting of 18 malignant fibrous histiocytoma, 13 liposarcomas, 11 malignant neural tumors, 7 fibrosarcomas, 5 leiomyosarcomas, 4 rhabdomyosarcomas, 4 synovial sarcomas and 3 other STS. They comprised 44 primary tumors and 21 relapses. Tumors originated from different locations: at the extremities, 63%; intraabdominal/retroperitioneal, 23%; trunk wall, 9%; head/neck, 5%. Insofar as possible, surgical therapy for all patients was localization dependent: compartment resection, wide excision or multivisceral resection with tumor free resection margins for all samples confirmed in histological examination. Histoprognostic staging of the tumors showed: 5 (7.7%) stage 1; 33 (50.8%) stage 2; 19 (29.2%) stage 3; and 8 (12.3%) stage 4. Out of 65 STS patients, 25 (38%) died of the tumor after an average of 27 months (range 2 to 201); whereas, 40 (62%) of the patients are alive after an average observation period of 38 months (range 4 to 104).

RNA Preparation and cDNA Synthesis from Clinical Samples and Cell Lines

Ten to 20 cryosections (40 μm in thickness) of each STS sample were transferred to RNase-free 1.5 ml Eppendorf tubes and homogenized in 1 ml of "Reagent 14" (Integrated Separation Systems, Natick, MA). Whole RNA was isolated by running cycle program 805 using an Autogen 540 nucleic acid extraction robot (Integrated Separation Systems). cDNA was synthesized from 1 µg aliquots of purified, resuspended and ultraviolet (UV) absorption–measured RNA samples in a 20 μl standard reaction mixture containing AMV reverse transcriptase buffer (250 mM Tris/HCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM spermidine), 5 U AMV reverse transcriptase, 0.5 mM of each dNTP (Promega, Madison, WI, U.S.A.), 10 U recombinant RNase inhibitor (AGS, Heidelberg, Germany), and 200 ng oligo(dT) (Amersham Pharmacia Biotech, Uppsala, Sweden) at 42°C for 1 hr. For this, a GeneAmp®9600 thermal cycler and 0.2 ml-MicroAmp® reaction tubes (PE Applied Biosystems, Weiterstadt, Germany) were used. RNA and cDNA samples were stored at −80°C until use.

Automated mdm2 *and* GAPDH *Transcript Analysis by Quantitative Fluorescence PCR*

Two commercially available, validated polymerase chain reaction (PCR) assays for quanti-

tation of murine double minute-2 (*mdm2*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene transcripts (ZeptoQuant Nukleinsäure-Diagnostika, Leipzig, Germany) were used in our laboratory (41). The *mdm2* assay detects all but the shortest splice variant of *mdm2* mRNA, previously described by Sigalas et al. (38). Briefly, conventional 96-well bases were loaded with an 8-well ready-to-use standard DNA strip either coated with eight different amounts of reference-DNA for quantitation *mdm2* or *GAPDH* transcripts, respectively, foreward and reverse primer, and the TaqMan® probe. The double labeled probes were either 5'-labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) for detection of *mdm2* or 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) for *GAPDH,* and the common 3'-fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) in order to generate the respective *mdm2* or *GAPDH* standard reference curves for each run. The remaining free base positions were loaded with the required number of sample tubes containing just the respective TaqMan® oligonucleotide sets. Reaction premixes containing PCR buffer, which was supplemented with the passive fluorescence dye 6-carboxy-tetramethyl-rhodamin (ROX), dNTPs, and 1.25 U of AmpliTaq® "GOLD" (PE Applied Biosystems) were assembled according to the manufacturers instructions. Aliquots of the mixes were added to each reaction tube by using a BIOMEK® 2000 laboratory automation workstation (Beckman Instruments Inc., Fullerton, CA, U.S.A.). Sample and standard reactions (final volume of 50 μl) differed only by the addition of 2 μl aliquots of the analyzed cDNA sample. PCR amplification and detection was performed with an ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems). Sample cDNA amounts were calculated from data obtained with the simultaneously amplified reference DNA strips. Mdm-2 data were correlated to *GAPDH* cDNA (zeptomoles [zmol, 10^{-21}] *mdm2* mRNA per attomole [amol, 10^{-18}] GAPDH mRNA) calculated from the same cDNA sample.

p53 *Mutational Analysis*

The tumor samples were examined for mutations in the *p53* gene by nonradioactive PCR-SSCP-sequencing. DNA was isolated from frozen tumor samples and the p53 gene (exons 4 to 9) was amplified in PCR reactions as described previously (42). In an SSCP-pre-screen for mutations, PCR products were investigated in 6 or 10% (PAA)-ready-made gels (Novex, Heidelberg, Germany) for abnormal single strand DNA shifts and striking cases were cycle-sequenced on an ABI 373 using the Dye Terminator Kit (PE Applied Biosystems).

Western Blot Analysis

Thirty μg of total protein were separated on 10% polyacrylamide/SDS gel (Minigel system; Biometra, Göttingen, Germany). Afterwards, proteins were transferred to a PVDF Immobilon membrane (Millipore; Eschborn, Germany) at 200 mA for 90 min (Miniblotter; Biometra). Afterwards, the membrane was blocked with 0.1% Tween 20 containing 3% bovine serum albumin (BSA) and incubated for 1 hr with anti-P53 antibody (DO-7; 1:500; Dianova, Hamburg, Germany) or with anti-mdm2 antibody (1B10; 1:500; Loxo, Dossenheim, Germany) and 1 hr with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:1000; Dako, Denmark) at room temperature. For protein detection, the membrane was placed for 1 min in ECL-substrate (Amersham, Braunschweig, Germany) and exposed to Biomax film (Kodak, Germany). The amount of P53 protein was compared with an internal positive control (p53 mutated RD cells/ATCC CCL 136 with a P53 over-expression) and standardized to the β-actin band determined in the same sample by densitometry (Imagemaster VDS 3.0; Pharmacia, Braunschweig, Germany). P53 and MDM2 expression were characterized according to a semiquantitative scale as zero, modest, marked, or strong expression. For the evaluation of the MDM2 protein expression, the 90kD, 85kD, 76kD, 74kD and 58kD bands were considered as relevant.

Statistical Analysis

Prognostic analysis began with a descriptive presentation of the cumulative survival functions according to the Kaplan-Meier method and a univariate evaluation of prognostic differences with a log-rank test. The Cox regression model, which was used to estimate the effect of *mdm2* mRNA expression on prognosis, was adjusted to stage, localization and type of surgical resection. A probability (p) of < 0.05 was defined as significant and a relative risk (*RR*) was calculated. The statistic analyses

were carried out using software from SPSS Inc. (SPSS 8.0). The cut points for *mdm2* mRNA expression levels represent prognostic relevant thresholds, which were set after continously sliding cut points in increment values of 10.

Results

mdm2 mRNA Expression

We investigated 65 STS samples for human mdm2 mRNA expression using the ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems) and quantitative ready-to-use fluorescence PCR assays. The assays, which were by automation using mostly robotic workstations supporting the 96-well format, allowed precise and reproducible data recovery combined with excellent sensitivity at low zeptomole detection levels. Dynamic ranges of usually 4–6 logs without prior sample dilution (up to $>10^6$ copies per tube) were easily achieved. The slopes of the individual reference curves were very close to the mean slopes (SD ± 2–5%), at simultaneous correlation coefficients of the calculated linear fits usually >0.99 (Fig. 1).

The correspondence of data with an in-house competitive GAPDH PCR protocol was found to be 99% (41). Human *mdm2* cDNA amounts were calculated from an external reference curve obtained with eight known amounts of *mdm-2* standard reference DNA, calibrated as described earlier (43). Data were normalized to the number of GAPDH transcripts measured in the same cDNA sample, which were reported to be constant in several heterogenous tumors, tissues and cell lines (44–47). Thus, the calculated ratios of both cDNAs reflected the initial ratios of the mRNAs in the sample.

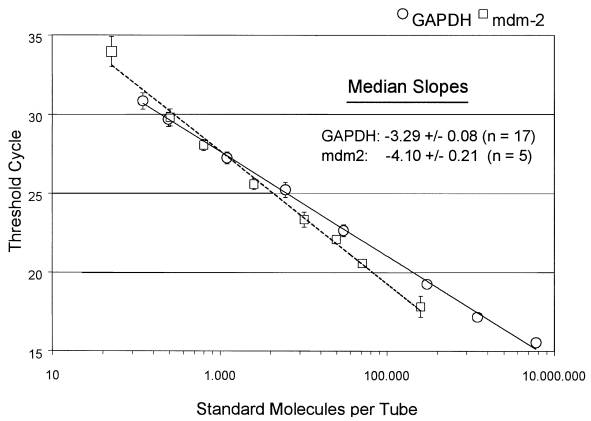

Fig. 1. Dynamic ranges of the applied ready-to-use quantitative RT-PCR assays. The calculated linear fits of the reference curves are usually >0.99, the standard deviations (SD) from the mean of individual slopes are usually 2–5%. *n* = number of independent experiments. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mdm-2, murine double-minute-2.

At first, a threshold for the ratio of *mdm2*/*GAPDH*-mRNA was determined after cut point sliding. A value of 50 (zmol mdm2/amol GAPDH mRNA) as the most relevant cut point. In 15 STS samples, the ratio *mdm2*/*GAPDH*-mRNA was below 50; whereas, the other 50 samples showed values ⩾50, with a maximum of 7571. In a Kaplan-Meier curve, patients with *mdm2* mRNA values below 50 showed a highly decreased average survival time (18 months), compared with the patients with *mdm2* mRNA values ⩾50 (>60 months). In an univariate log rank test, a significantly better survival for patients exhibiting *mdm2* expression values ⩾50 (p = 0 .0241) was found.

For a multivariate analysis, a Cox regression model was applied to examine whether *mdm2* mRNA expression was a prognostic factor independent of other known risk factors, such as tumor stage, kind of tumor resection and localization. As shown in Table 1, *mdm2* mRNA expression (<50 versus ⩾50) did not seem to be an independent prognostic factor (p = 0.1355). But after excluding patients with tumors of stage 1 and 4, the prognostic relevance became clearly visible (p = 0.0041, RR=5.6) (Table 1, Fig. 2). This can be reasoned by the fact that survival of both patient groups might be rather independent of *mdm2* mRNA levels (i.e. patients with stage 1 tumors had mostly a good survival; 4 out of 5 patients with a ratio ⩾50 survived); whereas, for patients with stage 4 tumors, poor prognosis is determined by the occurring manifested metastases. Patients with stage 2 and 3 tumors are of especially high interest in clinical practice, because their tumors may have a similar histo-morphological appearance; whereas, tumor behavior and prognosis can differ dramatically. When primary tumors and relapses were investigated, no difference in the *mdm2* mRNA content was found (data not shown).

Next, we investigated if elevated human *mdm2* transcript levels may be generally correlated with better survival or if this patient group might consist of several subpopulations with a diverging prognosis. When the group of patients with a *mdm2*/*GAPDH*-mRNA ratio ⩾50 was subdivided into three groups (50 to

Table 1. Cox-Regression model (adjusted to tumor stage, kind of tumor resection and localization) for mdm2 mRNA expression and survival, after setting a threshold for mdm2 mRNA expression at a ratio of 50

mdm2 mRNA Expression	Number of Samples per Tumor Stage							Number of Samples per Tumor Stage		
	1	2	3	4	total	*p*	*RR*	2 and 3	*p*	*RR*
<50	0	6	6	3	15	*	2.2	12	*	5.6
⩾50	5	27	13	5	50	0.1355	*	40	0.0041	*
total	6	33	19	9	65			52		

p = probability
RR = Relative risk
*reference group

<100; 100 to <500; and ⩾500; Table 2), the group with a ratio of 100 to <500 was distinguished by significantly better survival, compared with the reference group showing a ratio of <50 ($p = 0.0164$). The remaining two groups still showed a better survival than the reference group, but no statistical significance ($p = 0.1981$ and $p = 0.2003$) was found. Again, excluding patients with stage 1 and 4 tumors, the group with a ratio *mdm2/GAPDH*-mRNA of 100 to <500 showed the best survival (76% of patients alive) at an increased significance level ($p = 0.0015$; Fig. 3), compared with the reference group (42% of patients alive). Most striking was that the risk of tumor-related death was 13.3-fold increased in the patients with a *mdm2* mRNA expression <50, comparison with those with a ratio of 100 to <500 (Table 2).

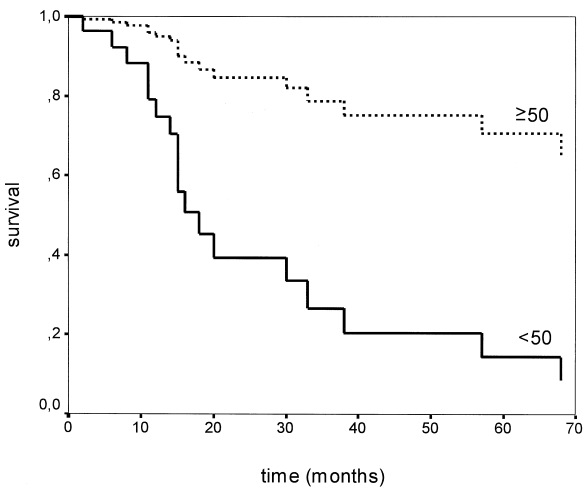

Fig. 2 Multivariate Cox model for *mdm2* mRNA expression and survival of STS patients (stage 2 and 3; *n* = 52). The threshold for the ratio of *mdm2/GAPDH* mRNA was set at a value of 50, after threshold sliding in Cox regression analyses. Curves for patients with an *mdm2* mRNA level below 50 (−) and ⩾50 (...) are significantly different ($p = 0.0041$). A relative risk of 5.6 of tumor-related death is associated with a *mdm2* mRNA level below 50, compared with a higher *mdm2* mRNA expression level.

P53 and MDM2 Protein Expression

P53 protein expression was detected by Western blots analysis and normalized to actin expression. A gross differentiation between four P53 expression groups divided into zero, modest, marked or strong expression was made. The group with zero and with strong expression showed the highest number of cases, which was not surprising since tumors may have lost *p53* or may show abnormally high P53 protein expression levels. However, no relationship between the *mdm2* RNA level and the detectable P53 protein expression, independent of considering tumors stage was observed (Table 3). In previous studies, a significant relationship between MDM2 protein expression, detectable immunohistochemistry, and a poor prognosis was found (29). However, the MDM2 expression, detected immunohistochemically or in Western blots, did not correlate with either the *mdm2* mRNA level or survival (data not shown).

p53 Mutational Analysis

Fifty-five out of 65 STS samples were investigated for *p53* mutations (exons 4-9). In eight

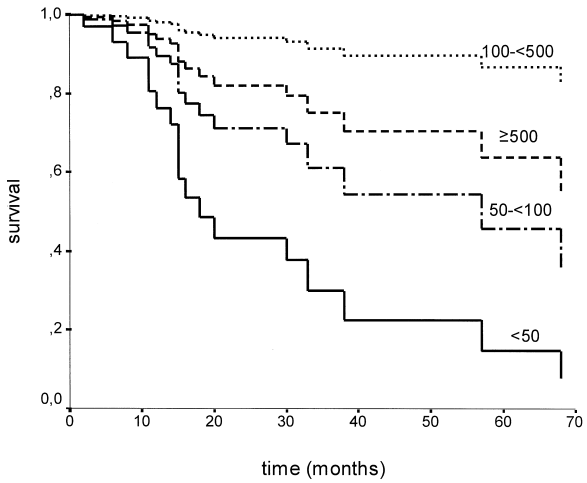

Fig. 3 Multivariate Cox model for *mdm2* mRNA expression and survival in STS patients (stage 2 and 3; $n = 52$). The group of patients with a *mdm2/GAPDH*-mRNA ratio ⩾50 was further subdivided into three groups, 50 to <100; 100 to <500 and ⩾500. The group with a ratio of 100 to <500 showed the best prognosis, compared with the reference group characterized by a ratio of <50 ($p = 0.0015$). The other two groups still showed a better survival than the reference group ($p = 0.1981$ and $p = 0.2003$, respectively). The relative risk of tumor-related death was 13.3 times higher in the patients with a *mdm2* mRNA expression <50, compared with those with a ratio of 100 to <500.

cases, a *p53* mutation was detected as previously described (48). Independent of the type of mutation, all patients but one showed a higher *mdm2* mRNA expression (>50), four cases were in the range of 50 to <100, and three in the range of 100 to <500. Concerning survival in the group with a *mdm2* mRNA expression of 50 to <100, two out of four patients died and, in the group with 100 to <500, one out of three patients died (Table 4). This followed the observed trend that patients with *mdm2* mRNA expression 100 to <500 had an increased overall survival. But we suggest, that rather the type of *p53* mutation, i.e., patients with non-frameshift mutations had a poorer survival than those with frame-shift mutations, seems to correlate with prognosis (48).

Discussion

Our results show that human *mdm2* mRNA expression, measured with an automated quantitative RT-PCR protocol, is an independent molecular prognostic factor for STS patients. In a Kaplan Meier test, the survival rate of patients with a *mdm2/GAPDH*-mRNA ratio below 50 was strikingly reduced (average, 18 months survival time), compared with patients with a ratio ⩾50 (>60 months average survival time) ($p = 0.0241$). In a multivariate Cox model confined to patients with tumor stage 2 and 3, the difference in prognosis became even more pronounced between patients with a ratio of <50 and patients with a ratio of ⩾50 ($p = 0.0041$; $RR = 5.6$).

The result that diminished, rather than increased, human *mdm2* mRNA expression correlates with a poor survival is somewhat surpris-

Table 2. Cox-Regression model (adjusted to tumor stage, kind of tumor resection and localization) for mRNA expression and survival after subdividing patients in four groups according to *mdm2* mRNA expression levels

mdm2 mRNA expression	Number of Samples per Tumor Stage 1 to 4	*p*	*RR*	Number of Samples per Tumor Stage 2 and 3[1]	*p*	*RR*
<50	15	*	3.7	12	*	13.3
50 to <100	14	0.1981	1.8	11	0.2035	5.4
100 to <500	26	0.0164	*	21	0.0015	*
⩾500	10	0.2003	1.5	8	0.0892	3.2
total	65			52		

p = probability
RR = Relative risk
[1]stage 2: 33 patients, stage 3: 19 patients
*reference group

Table 3. Comparison of *mdm2* RNA expression and P53 protein expression

P53 protein expression	*mdm2* mRNA expression tumor samples stages 1 to 4				*mdm2* mRNA expression tumor samples stages 2 and 3			
	<50	50 to <100	100 to <500	≥500	<50	50 to <100	100 to <500	≥500
none	7	7	9	1	5	7	8	1
moderate	1	1	2	1	1	1	2	1
marked	3	3	5	4	3	2	3	3
strong	4	3	10	4	3	1	8	3
total	15	14	26	10	12	11	21	8

ing. To our knowledge, only one group described a very similar observation made in ovarial carcinomas (49). Comparable with our data, a higher *mdm2* mRNA expression was associated with a better prognosis in patients with ovarial carcinomas. On the other hand, there are several reports that describe significantly higher levels of *mdm2* mRNA expression that are associated with an unfavourable prognosis in (AML) patients (30), with a subset of aggressive breast tumors (50) and with high invasiveness of hepatocellular carcinomas (51). Furthermore, it is well known that many malignant tumors, and particularly sarcomas, are characterized by elevated levels of *mdm2* protein (28), which can be associated with a poor survival for sarcoma patients (7,29,52). However, our data suggest that a protein overexpression does not neccessarily correlate with increased *mdm2* mRNA expression.

Therefore, it would be of high interest to find out if increased MDM2 protein expression is based on increased mRNA stabilization rather than on upregulation of transcription. There are several reports showing RNA stabilization as a major reason for increased mRNA levels, for example the heat shock protein hsp 70, insulin–like growth factor binding protein IGFB-BP3 and *waf*-1 (53–55). Noticeably, the latter two genes are like *mdm2* target genes of *p53* and, for *waf*-1, mRNA stabilization, can be *p53*-dependent and *p53*-independent (53). We did not find any relationship between *mdm2* mRNA level and P53 protein expression. However, the relationship between P53 protein level and *mdm2* mRNA expression is controversial. On one hand, there are several reports showing no relationship (6,37,56,57). On the other hand, some studies concluded that tumors with *p53* mutations may have an decreased *mdm2* mRNA expression or that tumors without *p53* mutations were characterized by higher *mdm2* mRNA expression (40,51). In our study, seven out of eight tumor samples with

Table 4. Comparison of *mdm2* mRNA expression and *p53*-mutational status in relation to survival

Case	*p53*-mutational status	*mdm2* mRNA expression	Survival/ observ. time (mo.)	Survival
51/92	non-fs-dp	57.2	40	a
M44	ts	78.5	6	d
G25/92	non-fs-del	94.2	20	d
M42	non-fs-del	230.9	24	d
US8-93	nonsense	45.8	15	d
G14-93	fs-del	70.3	31	a
G54-92	fs-del	138.5	39	a
L56	fs-del	193.3	46	a

Abbreviations: a, alive; d, dead; del, deletion; dp, duplication; fs, frameshift; non-fs, non-frameshift; observ., observational time; ts, transition.

p53 mutations did not show a striking decrease of *mdm2* mRNA levels (50 to <100 and 100 to <500; Table 4). One possible explanation could be that one remaining wild type *p53* allele is capable of maintaining *mdm2* gene activity (58). Alternatively, *mdm2* mRNA expression can occur independently from *p53* (26,37,59). Furthermore, tumor cell lines with high levels of transcriptionally inactive *p53* only might be unable to induce the expression of the MDM2 protein (60). Recently, a correlation between alternatively spliced *mdm2* transcripts (missing the *p53*-binding site) and stabilized wild type *p53* protein could be shown in glioblastoma cells, but its significance still remains unclear (61). Although we could detect short alternatively spliced forms of the *mdm2* mRNA in STS, the correlation between the occurrence of spliced transcripts and the *p53* gene status (wild-type/mutant) and the P53 protein expression level remains unclear (Bartel et al., submitted). However, the relationship between *mdm2* mRNA level and P53 protein expression is not yet clear and needs further investigation (28). A comparable negative result was obtained by the attempt to correlate MDM2 protein expression previously evaluated by immunohistochemistry (52) or by Western blot analysis (data not shown) to *mdm2* mRNA expression level. Furthermore, neither P53 nor MDM2 expression could be correlated to prognosis of the investigated 65 STS patients in a multivariate Cox regression analysis, which might be due to the relatively small number of patients.

Nevertheless, it seems neccessary to stress here that only alterations in the gene, transcript or/and translational level may result in an oncogenic potential of *mdm2. mdm2* normally acts as a cell cycle regulator (27), co-transcriptional factor (22,23) and a cellular regulator of several P53 protein functions (16,62,63). We suggest that moderately increased *mdm2* transcript levels (100 to <500) might be related to a normal *mdm2* function rather than a reduced (<50) or an extreme high level (>500). In summary, the human *mdm2* RNA level appears as an independent prognostic factor for STS patients, especially the potential role of *mdm2* alterations in STS tumorigenesis, in STS diagnosis and as an target for gene therapeutical approaches.

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