MDM2 is an important prognostic and predictive factor for platin–pemetrexed therapy in malignant pleural mesotheliomas and deregulation of P14/ARF (encoded by CDKN2A) seems to contribute to an MDM2-driven inactivation of P53

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Background: Malignant pleural mesothelioma (MPM) is a highly aggressive tumour that is first-line treated with a combination of cisplatin and pemetrexed. Until now, predictive and prognostic biomarkers are lacking, making it a non-tailored therapy regimen with unknown outcome. P53 is frequently inactivated in MPM, but mutations are extremely rare. MDM2 and P14/ARF are upstream regulators of P53 that may contribute to P53 inactivation.

Methods: A total of 72 MPM patients were investigated. MDM2 immunoexpression was assessed in 65 patients. MDM2 and P14/ARF mRNA expression was analysed in 48 patients of the overall collective. The expression results were correlated to overall survival (OS) and progression-free survival (PFS).

Results: OS and PFS correlated highly significantly with MDM2 mRNA and protein expression, showing a dismal prognosis for patients with elevated MDM2 expression (for OS: Score (logrank) test: \( P \leq 0.002 \), and for PFS: Score (logrank) test: \( P < 0.007 \)). MDM2 was identified as robust prognostic and predictive biomarker for MPM on the mRNA and protein level. P14/ARF mRNA expression reached no statistical significance, but Kaplan–Meier curves distinguished patients with low P14/ARF expression and hence shorter survival from patients with higher expression and prolonged survival.

Conclusions: MDM2 is a prognostic and predictive marker for a platin–pemetrexed therapy of patients with MPMs. Downregulation of P14/ARF expression seems to contribute to MDM2-overexpression-mediated P53 inactivation in MPM patients.

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Malignant pleural mesothelioma (MPM) is a highly aggressive tumour, which is linked to prior exposure to asbestos with a latency period of 20–30 years in approximately 80% of all cases (Weil et al, 2004; Hazarika et al, 2005; Goudar, 2008). Systemic therapy represents the primary treatment option for most patients (Treasure and Sedrakyan, 2004; Tsao et al, 2009), but standard MPM therapy is still deficient and decisions for radiotherapy, surgery or combined approaches are based on a case-by-case decision leading to a palliative treatment approach for most patients (Guyatt et al, 2006; Muers et al, 2008; Stahel et al, 2009; Astoul et al, 2012). Gender, histological subtype and haematological parameters have been identified as important prognostic parameters (Flores et al, 2007; Rusch et al, 2012).

Combination of antifolates (e.g. pemetrexed) and platin derivatives is considered as the most effective regimen for MPM (Tomék and Manegold, 2004; Kindler, 2008). In that therapeutic setting, patients show response rates of approximately 40% (stable disease and partial response) with a progression-free survival (PFS) of 5.7 months (Vogelzang et al, 2003). As reason for these low response rates and short PFS, expression differences of members of the folic acid pathway, folic acid transport and activation, which are important for the uptake and metabolism of pemetrexed, were discussed controversially in the past (Righi et al, 2008). Gender, histological subtype and haematological parameters have been identified as important prognostic parameters (Flores et al, 2007; Rusch et al, 2012).

Additional investigations on the gene level showed that inactivation of tumour-suppressor genes are frequent in MPM (Frew et al, 2009). In contrast to other solid tumours, mutations of the TP53 gene are extremely rare in MPM, so other mechanisms such as deletion of the locus or methylation contribute to inactivation of P53 (Papp et al, 2001a,b; Toyooka et al, 2008). Numerous noxious stimuli activate the P53 protein by posttranslational modifications resulting in cell cycle arrest, cellular senescence or apoptosis (Harris and Levine, 2005).

Among other factors, P53 activity and stability is tightly controlled by the E3 ubiquitin ligase (MDM2; also HDM2). Overexpression of MDM2 in some tumour types can lead to a loss of P53 regulatory function by increased proteasomal degradation of P53 (Jones et al, 1995; Montes de Oca Luna et al, 1995; Parant et al, 2001; Marine et al, 2006; Ringhausen et al, 2006). This pathomechanism is considered to be of importance in a variety of malignant tumours, including lung, breast, colon, stomach and hepatocellular carcinomas (Toledo and Wahl, 2006). Approximately 20% of all MPM show strong nuclear MDM2 expression, restricted to epithelial MPM or the epithelial components of biphasic MPM, and these MDM2-positive MPM show significantly decreased overall survival (OS) (Mairinger et al, 2014).

The physiological inhibitor of MDM2 is P14/ARF, and loss of P14/ARF activity may have a similar effect as loss of P53 (Kanellou et al, 2009). P14/ARF is recognised as a tumour suppressor inducing cell cycle arrest in a P53-dependent and P53-independent manner (Huang et al, 2003; Chen et al, 2005; Miao et al, 2010). Thereby, P14/ARF may control TP53 transcription, repress P53 degradation that is not MDM2-mediated and stimulate P53 activity (Van Maerken et al, 2011). Additionally, loss of P14/ARF activity seems to occur in a reciprocal manner to P53 loss and seems to be typical for tumours that are TP53 wild type (Huang et al, 2003).

In sum, reliable predictive biomarkers in MPM are lacking. Additionally, a personalised therapeutic concept is eagerly needed.

In the present study, we sought to determine whether decreased activity of the physiological MDM2 inhibitor P14/ARF contributes to MDM2-mediated inactivation of P53 in MPM.

**MATERIALS AND METHODS**

**Patient collective.** From the MPM database of the Institute of Pathology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, 72 formalin-fixed, paraffin-embedded (FFPE) specimens from patients harbouring a MPM were selected for quantitative real-time PCR (qPCR) and IHC analysis. Samples from 2004 to 2010 were investigated. Inclusion criteria for this study were complete data with respect to follow-up, treatment and sufficient FFPE tissue. The study design was approved by the ethical committee of the University Hospital Essen (ID: 14-5775-BO). The investigations conform to the principles of the Declaration of Helsinki.

FFPE tissue preparation was performed according to the institutional standards. The fresh tissue was fixed in 4% buffered formalin for 24 h and subsequently embedded in paraffin. For diagnostic classification, multiple 1–4-μm thick sections were used for IHC and stained with haematoxylin and eosin. The most representative part of the tumour was used for subsequent analysis.

**Immunohistochemistry.** Tissue microarrays were constructed from FFPE blocks. Three cores with a diameter of 0.6 mm were taken from different areas of each tumour to take possible tumour heterogeneity into account. When feasible, a core containing only normal, non-malignant pleura was taken from every specimen, which served as a negative control.

IHC for MDM2 was newly established. After validation on reference tissues (liposarcoma as a malignant mesenchymal tumour with a consistent strong expression of MDM2), the immunohistochemical investigations were performed with an antibody directed against MDM2 (clone IF2, Calbiochem, Darmstadt, Germany; dilution: 1:80). Protein expression was assessed using a four-stage semiquantitative IHC scoring system based on the percentage of tumour cell nuclei with a positive immunoreaction (Score 0: no immunohistochemical signal; Score 1 (weak expression): 1–25%; Score 2 (moderate expression): 26–50%; Score 3 (strong expression): >50%). MPM were only considered positive for MDM2 when a strong nuclear staining could be observed comparable to the positive controls (liposarcoma). Tumour cells with a weak immunohistochemical nuclear or only cytoplasmic signal for MDM2 were not counted.

**RNA isolation and qPCR.** Expression levels of ACTB (actin, beta; reference gene), MDM2 and P14/ARF were investigated by using hydrolysis probes (also known as TaqMan probes) for qPCR. Therefore, RNA was isolated from FFPE tissue. Only tumour tissue was used for mRNA extraction. This was carried out by macrodissection (cutting only the tumour region). Three-to-five sections of 4 μm were cut from one FFPE block by using a microtome (Leica, SM 2000 R, Wetzlar, Germany). Total RNA was isolated by using the miRNAeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, except for two modifications (proteinase K digestion overnight; elution in a final volume of 25 μl in RNase-free water). RNA concentrations were measured using the Nanodrop UV/VIS spectrometry (NanoDrop ND-1000, PEQLAB Biotechnologie GmbH, Erlangen, Germany). RNA concentrations ranged from 73 ng μl−1 to 3789 ng μl−1. Until further use, RNA was stored at −80 °C. For cDNA synthesis, the iScript Select cDNA Synthesis Kit and protocol from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used. Approximately 1 μg of the total RNA was used with a final concentration of 100 ng μl−1 per reaction. cDNA was stored for short term (<1 week) at 4 °C and otherwise at −20 °C.

For qPCR, the TaqMan Gene Expression Assays on Demand (Aod) for ACTB (HS03023943_g1), MDM2 (HS01066942_m1) and P14/ARF encoded by CDKN2A (HS99999189_m1) were used (Thermo Fisher Scientific, Waltham, MA, USA). The primer–probe volumes were modified by using 50% of the total reaction volumes that were recommended by the manufacturer. For each reaction, 50 ng cDNA were applied. The Aod were chosen because of their short amplicon size (ACTB: 93 nt, MDM2: 89 nt and P14/ARF/CDKN2A: 72 nt) and, additionally, because they spanned
exon–exon boundaries to circumvent simultaneous detection of genomic DNA. Each target was measured in triplicates for each patient. Non-template controls were processed for each AoD on each reaction plate as negative control. Table 1A summarises the applied chemicals and volumes for the qPCR and Table 1B shows the qPCR conditions. ACTB is the standard reference gene for investigation of MPM at our institution. That is based on previous experiments testing several potential reference genes using the geNorm and NormFinder algorithms. ACTB showed robust and stable expression in MPM and hence was processed for normalis-ation purposes and as reference gene. Ct-values of MDM2 and P14/ARF were normalised to the mean values of ACTB. Data analysis and qPCR were performed on a Roche LightCycler 480 II (Roche, Basel, Switzerland) and the corresponding software. All qPCR experiments were performed in concordance with the MIQE-guidelines (Bustin et al, 2009).

**Statistical analysis.** Statistical analysis was performed using the statistical computer language R (r-project.org; version R i386 2.15.1).

**Gene expression analysis.** Analysis of OS and PFS were done by using the proportional hazards model (also called Cox-regression (COXPH-model)), and statistical significance was determined using the likelihood ratio test, Wald test and Score (logrank) test. With respect to functional scale-differences in biological systems, proportional hazards model analysis was done in a linear and logarithmic scale for MDM2 mRNA and protein expression and for P14/ARF mRNA expression. For association between either protein expression or mRNA expression with respect to gender, patients’ age at time of diagnosis, age of the paraffin blocks and histological subtype of MPM to OS and PFS was analysed. The investigated sub-collectives are proportional hazards model was calculated.

OS and PFS were visualised by creating single-factorial and dichotomous variables (e.g., gender). This test was also used to rule out a possible association between expression and age of the patients, age of the FFPE tissue, gender and clinical data.

The relationship of the expression of the tested markers with OS and PFS was analysed via qPCR for the mRNA expression of MDM2 and P14/ARF. The Mann–Whitney U (also called the Wilcoxon rank-sum test) was used, for example, to test associations between the mean protein expression obtained from three cores after IHC or mRNA expression and dichotomous variables (e.g., gender).

The level of statistical significance was defined as $P \leq 0.05$.

**RESULTS**

A collective of 72 patients was investigated. Sixty-five patients (90%) gave evaluable IHC data with respect to MDM2 immuno-expression. Out of the overall collective, 48 specimens were analysed via qPCR for the mRNA expression of MDM2 and P14/ARF. The relationship of the expression of the tested markers with OS and PFS was analysed. The investigated sub-collectives are summarised in Table 2.

**Clinicopathological and survival data of patients from Essen Clinicopathological analysis.** Seventy-two patients harbouring a MPM were selected. Eleven female (15%) patients and 61 male (85%) patients were investigated. The mean age of the patients was 63.8 years (median age 62.6 years, range 48–80 years). For 69 out of the 72 patients, the histological MPM subtype was available. Fifty-nine patients showed an epithelioid (82%), six biphasic (8%) and nine patients showed an epithelioid (82%), six biphasic (8%) and nine patients showed an epithelioid (82%), six biphasic (8%) and Sarcomatoid (4%) histological subtype. Table 2 summarises the characteristics of the investigated patient collective.

**Analysis of OS.** Survival data of 72 patients were available, and 59 (81.9%) were reported dead and 13 (18.1%) were still alive when survival data were assessed. Median survival was 18.5 months (mean without censored patients: 21.9 months; range: 8.8–29.9 months).

| Table 1A. Summary of the applied chemicals and volumes for qPCR experiments | Table 1B. Summary of the cycler program for qPCR analysis using a Roche Light Cycler 480II |
|---|---|
| **Mastermix for hydrolysis probes (TaqMan probes)** | **Programme** |
| Reagent | Temperature | Duration (s) | Heating rate | PCR cycles |
| Mastermix 2 × | 50°C | 00:02:00 | 4.8 | 1 |
| Aquastat | 95°C | 00:10:00 | 4.8 | 1 |
| Assay on Demand (AoD) 20 × | 95°C | 00:00:15 | 4.8 | 50 |
| Sample cDNA | 60°C | 00:01:00 | 2.5 | 1 |
| Total reaction volume | 40°C | 00:00:10 | 2.5 | 1 |
| Abbreviation: qPCR = quantitative PCR |

| Table 2. Clinical characterisation of the investigated sub-collectives |
|---|
| **MPM subtype** |
| Epithelioid | 59 | 54 | 38 |
| Biphasic | 6 | 5 | 5 |
| Sarcomatoid | 4 | 3 | 2 |
| **Overall survival** |
| Deceased | 59 | 51 | 43 |
| Alive | 13 | 13 | 11 |
| Median OS (months) | 18.5 | 19.3 | 17.1 |
| **Progression-free survival** |
| Progression-free survival | 53 | 47 | 35 |
| Progression | 16 | 14 | 11 |
| Unknown PFS | 3 | 3 | 2 |
| Median PFS (months) | 6.3 | 6.6 | 6.1 |

| Abbreviations: IHC = immunohistochemistry, MPM = malignant pleural mesothelioma; OS = overall survival; PFS = progression-free survival; qPCR = quantitative PCR. |
The histological subtype correlated significantly with OS (Score (logrank) test; \( P = 0.021 \)), with a better prognosis for patients with an epithelioid subtype (Supplementary Figure S1).

**Analysis of OS with respect to MDM2 immunoexpression.** IHC gave evaluable results for 65 out of the 72 investigated patients. Fifty-four patients showed an epithelioid subtype (83.1%), five biphasic (7.7%), three sarcomatoid (4.6%) and three remained inconclusive (4.6%). Fifty-one (80%) patients were reported dead, and 13 were still alive (20%). The median OS was 19.3 months (Table 2).

OS analysis showed that gender was statistically significant (Score (logrank) test; \( P = 0.016 \)), with shorter survival for male patients (HR: 3.35; 95% CI, range: 1.2–9.5; data not shown). Age of the patients (Score (logrank) test; \( P = 0.15 \)) and age of the FFPE specimens (Score (logrank) test; \( P = 0.48 \)) showed no statistical correlation to OS.

According to the described scoring system, 48 patients showed Score 0 (65%), 19 Score 1 (29%), 4 Score 2 (6%) and none of the patients’ specimens had a Score 3. Of note, MDM2 expression was present in epithelioid and the epithelioid component of biphasic MPM only. All MDM2-positive MPM specimens, regardless of the score, were calculated against all MDM2-negative MPM and correlated with OS. Lacking MDM2 expression significantly associated with longer OS with respect to the linear (Score (logrank) test; \( P < 0.0001 \), CI: 95%, range: 2.5–7.2, HR: 4.2 for patients with MDM2 protein expression) and the logarithmic scale (Score (logrank) test; \( P < 0.0001 \), CI: 95%, range: 4.5–25.6, HR: 10.7 for patients with MDM2 protein expression) (Figure 1). MDM2-negative MPM showed a 3-year survival of 29%; no patients with MDM2-positive MPM were alive after 3 years.

Figure 1 shows a Kaplan–Meier plot for OS in correlation to the MDM2 immunoexpression in the patient collective from Essen. The x axis shows the survival time in months. On the y axis, the survival rate in percentage is shown. MDM2-positive MPM (regardless of the score) showed a significantly decreased survival time compared with MDM2-negative MPM (\( P < 0.0001 \)).

Analysis of OS with respect to mRNA expression. Out of these 72 samples, 48 specimens were subjected to qPCR analysis. Thirty-eight patients (79%) showed an epithelioid, five (10%) a biphasic and two (4%) a sarcomatoid MPM subtype. For three (6%), the histological subtype remained inconclusive. Five patients (10%) were reported alive, and 43 (90%) had succumbed to the disease. The median OS time was 17.1 months (Table 2).

Sufficient amounts of mRNA for subsequent cDNA synthesis and qPCR analysis could be extracted from all samples. All tested samples returned evaluable qPCR data and were subjected to normalization, and all negative controls (non-template controls) showed no detectable signal.

Neither MDM2 (\( P = 0.54 \)) nor P14/ARF (\( P = 0.27 \)) mRNA expression showed a significant correlation with respect to the patients’ age. P14/ARF mRNA expression showed no significant correlation with respect to sample age (\( P = 0.089 \)). MDM2 mRNA expression showed a statistical trend with respect to sample age (\( P = 0.0505 \)), but the positive rho-value (rho = 0.28) indicated a direct correlation between sample age and mRNA leading to the assumption that with increasing age of the sample the mRNA amount would increase. This result was considered as an irrelevant contingency.

OS correlated significantly with MDM2 mRNA expression with respect to the logarithmic scale (Score (logrank) test; \( P = 0.0014 \)) with a hazard ratio of 3.2 for elevated expression (CI: 95%, range: 1.5–6.9). With respect to the linear scale, a significant correlation was found (Score (logrank) test; \( P = 0.0001 \)) confirming the hazard ratio for elevated expression. The results are summarised in Figure 2A.

Statistical analysis of P14/ARF-mRNA expression showed no significant correlation with respect to OS (linear scale; Score (logrank) test; \( P = 0.38 \), logarithmic scale: Score (logrank) test; \( P = 0.13 \)), but Kaplan–Meier curves separated patients having a low expression from patients with high expression as shown in Figure 2B. Elevated P14/ARF expression correlated with prolonged survival.

The age of the patients (Score (logrank) test; \( P = 0.048 \)) correlated significantly with OS with a hazard ratio of 1.04 (CI: 95%, range: 0.99–1.1) for older patients (data not shown). Gender (Score (logrank) test; \( P = 0.066 \)) showed no significant correlation for OS (data not shown).

Figure 2 shows a Kaplan–Meier plot for OS in correlation to the mRNA expression of (A) MDM2 and (B) P14/ARF. The x axis shows the survival time in months. On the y axis, the survival rate in percentage is shown. Elevated MDM2 expression was associated with significantly decreased survival rates (\( P < 0.0015 \)). P14/ARF expression showed no significant relationship to OS, but Kaplan–Meier curves separated patients with elevated expression from patients with low expression and higher expression correlated with prolonged survival.

**Analysis of PFS.** All of the investigated patients received cisplatin in combination with pemetrexed. During therapy, 53 (74%) patients showed progression of the disease and 16 (22%) were free of progression. For three patients (4%), no PFS data were available. Median PFS was 6.4 months (mean without censored patients: 9.4 months, range: 4.0–9.3 months) (Table 2). Histological subtype showed a significant correlation with respect to PFS (\( P < 0.0001 \)) with shorter PFS for patients with biphasic and sarcomatoid subtypes (CI: 95%, range: 0.005–0.2, HR: > 3.3 for biphasic and sarcomatoid MPM; data not shown). Male patients showed shorter PFS than female patients (\( P = 0.037 \), CI: 95%, range: 1.0–5.8, HR: 2.4 for male patients; data not shown).

Analysis of PFS with respect to MDM2 immunoexpression. Out of the 65 patients subjected to IHC analysis, 47 (72%) showed progression of the disease, 14 (22%) showed no progression and for 4 (6%) patients no PFS data were available. Median PFS was 6.6 months (Table 2).

Male patients showed a statistically significant shorter PFS than female patients (Score (logrank) test; \( P = 0.037 \), CI: 95%, range: 1.0–5.8) with a hazard ratio of 2.4 (data not shown). The histological subtype showed a statistically significant correlation to PFS (Score (logrank) test; \( P < 0.0001 \), CI: 95%, range: 2.0–4.2), with faster progression in biphasic and sarcomatoid MPM (data not shown).
Any MDM2 protein expression (Scores 1–3) correlated with shorter PFS, whereas a lack of MDM2 expression (Score 0) associated with prolonged PFS (linear scale Score (logrank) test; \( P = 0.0004, \text{ CI: } 95\% \), range: 1.4–3.7, HR: 2.3 for patients with MDM2 expression and logarithmic scale Score (logrank) test; \( P = 0.0002, \text{ CI: } 95\% \), range: 1.99–10.4, HR: 4.5 for patients with MDM2 expression). The results are summarised in Figure 3.

Age of the patients (Score (logrank) test; \( P = 0.75 \)) and age of the specimens (Score (logrank) test; \( P = 0.51 \)) showed no statistical correlation to PFS.

Figure 3 shows a Kaplan–Meier plot for PFS in correlation to the protein expression of MDM2. On the x axis, the survival time in months is shown. The y axis shows the survival rate in percentage. Higher MDM2 expression (regardless of the score) was significantly associated with shorter PFS (\( P < 0.0005 \)).

**Analysis of PFS with respect to mRNA expression.** Out of the 48 patients who were investigated by qPCR, 38 (73%) showed progression of the disease, whereas 11 (23%) were free of progression and for 3 (6%) no PFS data were available. Median PFS was 6.1 months (Table 2). For PFS, a significant correlation for MDM2-mRNA expression was found with respect to the logarithmic scale (Score (logrank) test; \( P = 0.0072, \text{ CI: } 95\% \), range 1.3–5.4) and also with respect to the linear scale (Score (logrank) test; \( P = 0.0049 \)) as shown in Figure 4A. Elevated expression showed a hazard ratio of 2.6 with shorter PFS in patients with elevated MDM2 expression.

Statistical analysis of P14/ARF mRNA expression showed a statistical trend with elevated expression in patients with prolonged PFS (logarithmic scale: Score (logrank) test; \( P = 0.057 \) and linear scale: Score (logrank) test; \( P = 0.62 \)). Figure 4B shows the Kaplan–Meier curves for P14/ARF expression with respect to PFS.

The patients’ age (Score (logrank) test; \( P = 0.63 \)) and age of the specimens (Score (logrank) test; \( P = 0.15 \)) did not show any impact on PFS (data not shown).

Figure 4 shows a Kaplan–Meier plot for PFS in correlation to the mRNA expression of (A) MDM2 and (B) P14/ARF. On the x axis, the survival time in months is shown. The y axis shows the survival rate in percentage. Higher MDM2 expression was significantly associated with shorter PFS (\( P < 0.0015 \)). P14/ARF expression showed a statistical trend correlating prolonged PFS with elevated expression (\( P = 0.085 \)).

**DISCUSSION**

The standard first-line therapy for MPM is a combination of cisplatin or carboplatin with pemetrexed (Ramalingam and Belani, 2008; Ray and Kindler, 2009), and this treatment results in a median OS of 11–12 months (Papa et al, 2013). Our investigated patients showed a comparable median OS of 18.5 months. The investigated patients showed a median PFS of 6.4 months that is comparable to previously reported PFS of 5.7 months (Ramalingam and Belani, 2008; Ray and Kindler, 2009). The efficacy of platin-based therapies depends on several DNA-repair enzymes, which determine the potential of a neoplasia to respond to platin-induced damage (Ting et al, 2013). These rather short OS and PFS rates imply that the standard MPM therapy can be considered deficient (Tomek and Manegold, 2004; Guyatt et al, 2006; Muers et al, 2008; Stahel et al, 2009; Astoul et al, 2012), and predictive biomarkers for cisplatin–pemetrexed-based therapy concepts are lacking or discussed controversially (Tomek and Manegold, 2004; Stahel et al, 2009; Astoul et al, 2012; Mairinger et al, 2013a). Additionally, no approved second-line therapy exists (Papa et al, 2013). Therefore, a recent guideline emphasises the need of an innovative and novel therapy strategy (Astoul et al, 2012) that should be based on reliable, predictive and prognostic biomarkers.

The tumour-suppressor TP53 gene locus is mutated in approximately 50% of all human cancers. In the remaining 50% of all malignant tumours, TP53 is wild type but inactivated. TP53 mutations are extremely rare events in MPM, but the P53 protein can be inactivated by several other molecular mechanisms (Papp et al, 2001a,b; Toyooka et al, 2008). One of these putative mechanisms is an amplification and/or overexpression of MDM2, which exerts an E3 ubiquitin protein ligase function and thus is a physiological repressor of the P53 functional protein (Jones et al, 1995; Montes de Oca Luna et al, 1995; Parant et al, 2001; Marine et al, 2006; Ringshausen et al, 2006). Furthermore, MDM2 expression is regulated by transcriptionally active P53 showing that both MDM2 and P53 control each other in a (negative)
feedback loop manner (Wang et al., 2011; Rao et al., 2013). This is an important physiological interplay, keeping P53 levels low under non-cancerous, physiological cell conditions and inducing P53 activity in the presence of cellular stress (Hopkins-Donaldson et al., 2006; Herce et al., 2013). Overexpression of MDM2 can lead to a loss of P53 regulatory function in cancer cells (Jones et al., 1995; Montes de Oca Luna et al., 1995; Parant et al., 2001; Marine et al., 2006; Ringshausen et al., 2006). Of note, enzymes involved in DNA repair, such as P53, are associated with patients’ response and outcome to platinum-based therapy regimens (Barckhausen et al., 2006; Ringshausen et al., 2006). In the recent manuscript, we confirmed this previous finding and additionally found that MDM2 has prognostic value on the mRNA and protein level. Prolonged survival and PFS in MPM patients was associated with lower expression of MDM2 on the mRNA level and absent MDM2 immunoeexpression. Furthermore, MDM2-negative MPM showed a 3-year survival of 29%; no patients with MDM2-positive MPM were alive after 3 years. This is in line with previous findings (Mairinger et al., 2014). Expression analysis on the protein and mRNA level showed that the statistical results were highly consistent between both methods indicating that IHC and qPCR could be used interchangeably to test MPM patients for the expression of MDM2. FFPE tissue is a challenging source for molecular biological analysis, but if the assay design takes potential FFPE-specific pitfalls into account, reliable and reproducible results can be achieved (Walter et al., 2013).

Another important tumour suppressor in this setting is P14/ARF, which stabilises P53 (Rao et al., 2013). P14/ARF is the physiological inhibitor of MDM2 and, when bound to MDM2, prevents P53 degradation (Rao et al., 2013). Additionally, P14/ARF can increase P53 synthesis, inhibit other negative regulators of P53 than MDM2, regulate P53 influencing pathways, increase the transcriptional activity of P53 and has a critical role as a tumour suppressor (Huang et al., 2003; Chen et al., 2005; Rocha et al., 2005; Miao et al., 2010). Loss of P14/ARF is expected to have a similar impact on cancer development and maintenance as inactivation of TP53 (Kanelloiu et al., 2009). P14/ARF is encoded from the CDKN2A gene locus that also encodes P16/INK2A (Chen et al., 2005). P16/INK2A and P14/ARF are controlled by distinct promoters and differ in one exon leading to two unique proteins that are structurally different (Kanelloiu et al., 2009). Deletion of the whole CDKN2A locus, on chromosome 9p21, is present in 79–90% of all malignant mesotheliomas; in the sarcomatoid subtype, the prevalence is up to 100% (Frew et al., 2009; Kräsiniskas et al., 2010; Altomare et al., 2011; Monaco et al., 2011; Bahnassy et al., 2012; Matsumoto et al., 2013; Tochigi et al., 2013). The inactivation of the CDKN2A locus occurs also without deletion. Epigenetic inactivation including DNA methylation (Kanelloiu et al., 2009; Fujii et al., 2012) and miRNA regulation were reported (Guled et al., 2009; Ivanov et al., 2010). In this study, P14/ARF expression reached no statistical significance, but Kaplan–Meier curves separated patients with low expression and poor prognosis from patients with high expression and favourable prognosis. Furthermore, P14/ARF showed a statistical trend with respect to PFS, and low expression was associated with faster progression of the disease. In an in vitro experiment, P14/ARF was identified as a marker for response to Nutlin-3A treatment, which is a potent and selective MDM2 inhibitor (Van Maerken et al., 2011). This substantiates the notion that MDM2-mediated P53 inactivation may benefit from inactivation of P14/ARF.

Nutlin-3A (a cis-imidazoline analogue) is a newly developed, potent and selective MDM2 inhibitor with an IC_{50} value in the 90–300 nM range (Vassilev et al., 2004; Shangary and Wang, 2009) that prevents MDM2–P53 interaction by binding to the hydrophobic P53-binding pocket of MDM2 (Vassilev et al., 2004; Gamble et al., 2012). It is a non-genotoxic drug that can restore P53 activity leading to subsequent senescence or apoptosis in a cell-type-dependent manner (Vassilev et al., 2004; Gamble et al., 2012; Voltan et al., 2013). Additionally, Nutlin-3A shows a low risk of inducing therapy resistance and is currently being tested in a phase I clinical trial (protocol ID: NCT00623870 as substance ROS045337) (Voltan et al., 2013). In summary, MDM2 overexpression seems to mediate an inactivation of P53 leading to more aggressive MPM that are less sensitive to current therapies resulting in poor outcome, and additional downregulation of its physiological inhibitor P14/ARF may intensify this effect. In a previous study, P53 failed as prognostic factor in MPM patients (Mairinger et al., 2014), but the assessment of MDM2 may be a helpful tool in a subset of MPM patients to identify patients who would have the largest benefit from a platin–pemetrexed therapy and to predict the outcome.

**CONCLUSION**

Despite being wild type with respect to TP53, P53-mediated cell cycle control or apoptosis are lacking in MPM, which might be explained by inactivation of functional P53 protein by different mechanisms, such as proteasomal degradation via overexpression of MDM2. Overall survival and PFS showed a significant correlation between increased MDM2 expression and decreased survival, making MDM2 a reliable and robust prognostic and predictive biomarker in MPM.

P14/ARF mRNA expression is significantly decreased in many MPM, which might further contribute to deregulation and hyperfunction of MDM2. Kaplan–Meier curves were able to separate patients with low P14/ARF expression with poor outcome from patients with higher expression and favourable outcome.

In summary, a substantial proportion of MPM show a MDM2-mediated inactivation of P53 and concomitant downregulation of P14/ARF that can predict the response to pemetrexed and platinum-based chemotherapy regimens.
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