FULL PAPER

BJC

British Journal of Cancer (2015) 112, 883–890 | doi: 10.1038/bjc.2015.27

Keywords: pleural mesothelioma; MDM2; P14/ARF; Nutlin-3; targeted therapy; qPCR

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

R F H Walter*[,1,2,7], F D Mairinger[2,7], S Ting[2], C Vollbrecht[3], T Mairinger[4], D Theegarten[3], D C Christoph[5,6], K W Schmid[2] and J Wohlschlaeger[2]

[1]*Ruhrlandklinik, West German Lung Center, University Hospital Essen, University of Duisburg-Essen, Tüschener Weg 40, Essen D-45239, Germany;* [2]*Institute of Pathology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany;* [3]*Institute of Pathology, University Hospital Cologne, Cologne, Germany;* [4]*Department of Pathology, Helios Klinikum Emil von Behring, Berlin, Germany;* [5]*Department of Medical Oncology, West German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Essen, Germany and* [6]*Department of Medicine, Division of Medical Oncology, University of Colorado Denver, Aurora, CO, USA*

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: $P \leqslant 0.002$, 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.

*Correspondence: RFH Walter; E-mail: robert.walter@ruhrlandklinik.uk-essen.de
[7]These authors contributed equally to this work.

Received 1 August 2014; revised 1 January 2015; accepted 12 January 2015; published online 10 February 2015

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 (Weill *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 (Tomek and Manegold, 2004; Kindler, 2008). In that therapeutical 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*, 2010; Christoph *et al*, 2012; Lustgarten *et al*, 2013; Mairinger *et al*, 2013a,b).

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; Ringshausen *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 epitheloid MPM or the epitheloid 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 miRNeasy 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 $-80\,^{\circ}$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 $-20\,^{\circ}$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

Table 1A. Summary of the applied chemicals and volumes for qPCR experiments

Mastermix for hydrolysis probes (TaqMan probes)	
Reagent	**Volume (μl)**
Mastermix 2 ×	5
Assay on Demand (AoD) 20 ×	0.5
Aqua test	3.5
Sample cDNA	1
Total reaction volume	10

Abbreviation: qPCR = quantitative PCR.

Table 1B. Summary of the cycler program for qPCR analysis using a Roche Light Cycler 480II

Programme	Temperature	Duration (s)	Heating rate	PCR cycles
Activation	50 °C	00:02:00	4.8	1
Initial Incubation	95 °C	00:10:00	4.8	1
Amplification	95 °C 60 °C	00:00:15 00:01:00	4.8 2.5	50
Cooling	40 °C	00:00:10	2.5	1

Abbreviation: qPCR = quantitative PCR.

Table 2. Clinical characterisation of the investigated sub-collectives

	Essen, overall collective	Essen, IHC results	Essen, qPCR results
Number of patients	72	65	48
Male patients	61	57	41
Female patients	11	8	7
Unknown gender	0	0	0
Mean age at diagnosis (months)	63.8	63.9	64.6
Median age at diagnosis (months)	62.6	63.2	64.5
MPM subtype			
Epithelioid	59	54	38
Biphasic	6	5	5
Sarcomatoid	4	3	2
Overall survival			
Deceased	59	51	43
Alive	13	13	5
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.

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 normalisation 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, a proportional hazards model was calculated.

OS and PFS were visualised by creating single-factorial and combined Kaplan–Meier curves (also called product limit estimator). Kaplan–Meier curves with a confidence interval of 95% (CI: 95%) were calculated based on existing survival data.

The Spearman's rank correlation coefficient (also called the Spearman's rho) was used to calculate correlations between the expression levels of the tested genes. Additionally, 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 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 $P \leqslant 0.05$.

RESULTS

A collective of 72 patients was investigated. Sixty-five patients (90%) gave evaluable IHC data with respect to MDM2 immunoexpression. 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 four sarcomatoid (6%) MPM subtype. For three patients, the histological subtype was not available (4%). 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).

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.

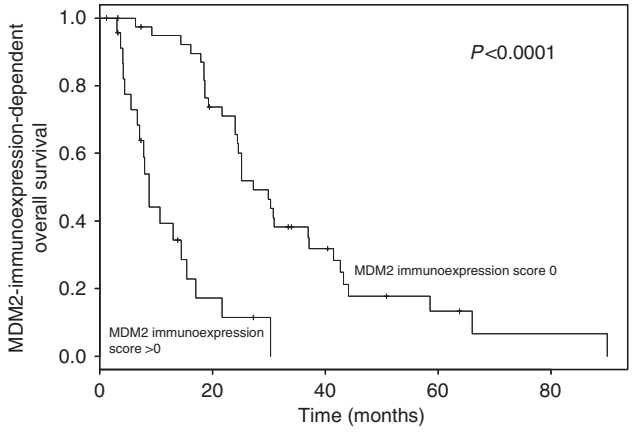

Figure 1. MDM2-immunoexpression-dependent overall survival of the patients from Essen.

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: 0.2–4.2), with faster progression in biphasic and sarcomatoid MPM (data not shown).

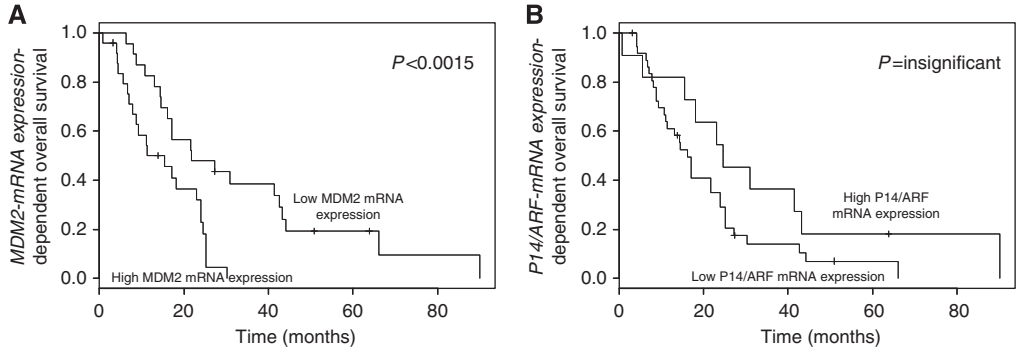

Figure 2. (**A**) *MDM2*-dependent overall survival and (**B**) *P14/ARF*-dependent overall survival of the patients from Essen.

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; $P=0.0004$, CI: 95%, range: 1.4–3.7, HR: 2.3 for patients with MDM2 expression and logarithmic scale Score (logrank) test; $P=0.0002$, 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 ($P<0.0005$).

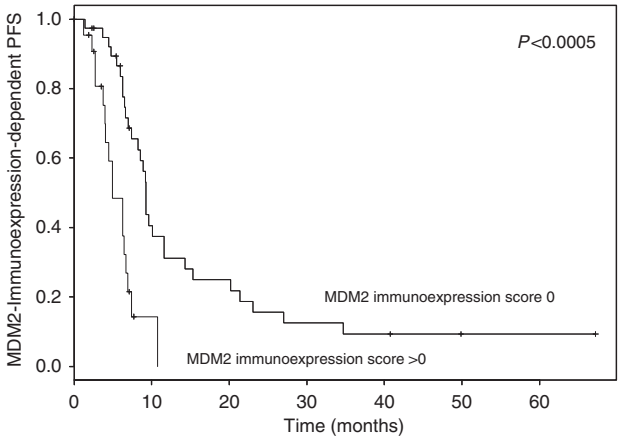

Figure 3. MDM2-immunoexpression-dependent progression-free survival of the patients from Essen.

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; $P=0.0072$, 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 ($P=0.057$).

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)

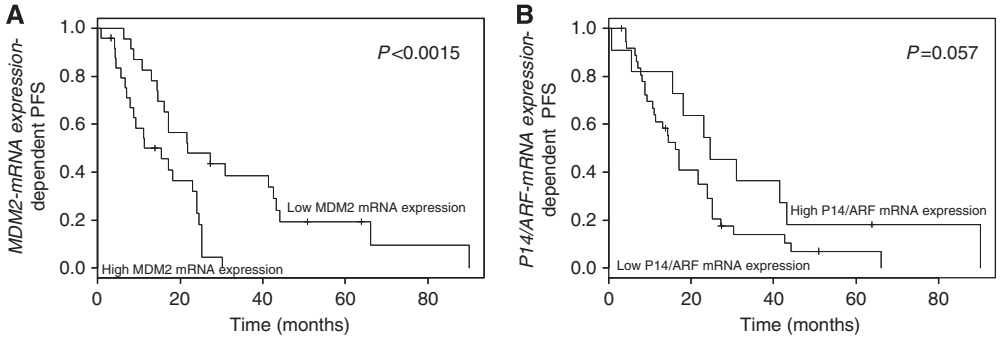

Figure 4. (**A**) *MDM2*-dependent and (**B**) *P14/ARF*-dependent progression-free survival of the patients from Essen.

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*, 2014; Sun *et al*, 2014), and P53's activity and stability is negatively regulated by overexpression of MDM2 in approximately 20% of epithelioid mesotheliomas and the epithelioid component of biphasic MPM (Mairinger *et al*, 2014). 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 immunoexpression. 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 that 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 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* (Kanellou *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 (Kanellou *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; Krasinskas *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 (Kanellou *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 RO5045337) (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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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)