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Research Paper

Screening of Pleural Mesotheliomas for DNA-damage Repair Players by Digital Gene Expression Analysis Can Enhance Clinical Management of Patients Receiving Platin-Based Chemotherapy

Robert Fred Henry Walter[1, 2]; Claudia Vollbrecht[3]; Robert Werner[4]; Thomas Mairinger[4]; Jan Schmeller[2]; Elena Flom[1]; Jeremias Wohlschlaeger[2,5]; Nikolaos Barbetakis[6]; Dimitrios Paliouras[6]; Fotios Chatzinikolaou[7]; Vasilis Adamidis[8], Kosmas Tsakiridis[8]; Paul Zarogoulidis[8]✉; Georgia Trakada[9]; Daniel Christian Christoph[2]; Kurt Werner Schmid[2]; Fabian Dominik Mairinger[2]

1. Ruhrlandklinik, West German Lung Centre, University Hospital Essen, University of Duisburg-Essen, Essen, Germany;
2. Institute of Pathology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany;
3. Institute of Pathology, Division of Molecular Pathology, Charité, Berlin, Germany;
4. Department of Pathology, Helios Klinikum Emil von Behring, Berlin Germany;
5. Institute of Pathology, Ev.-Luth. Diakonissenkrankenhaus Flensburg, Flensburg, Germany;
6. Thoracic Surgery Department, Theagenio Cancer Hospital, Thessaloniki, Greece;
7. Pathology Department, "Theagenio" Cancer Hospital, Thessaloniki, Greece;
8. Pulmonary Department-Oncology Unit, "G. Papanikolaou" General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece;
9. Division of Pulmonology, Department of Clinical Therapeutics, National and Kapodistrian University of Athens School of Medicine, Alexandra Hospital, Athens, Greece;
10. Department of Medical Oncology, West German Cancer Centre, University Hospital Essen, University of Duisburg-Essen, Essen, Germany.

✉ Corresponding author: Paul Zarogoulidis, M.D, Ph. D. Pulmonary Department-Oncology Unit, "G. Papanikolaou" General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece. Fax: 00302310992424 Mobile: 00306977271974 E-mail: pzarog@hotmail.com.

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Abstract

Background: Malignant pleural mesothelioma (MPM) is a rare, predominantly asbestos-related and biologically highly aggressive tumour leading to a dismal prognosis. Multimodality therapy consisting of platinum-based chemotherapy is the treatment of choice. The reasons for the rather poor efficacy of platinum compounds remain largely unknown.

Material and Methods: For this exploratory mRNA study, 24 FFPE tumour specimens were screened by digital gene expression analysis. Based on data from preliminary experiments and recent literature, a total of 366 mRNAs were investigated using a Custom CodeSet from NanoString. All statistical analyses were calculated with the R i386 statistical programming environment.

Results: *CDC25A* and *PARP1* gene expression were correlated with lymph node spread, *BRCA1* and *TP73* expression levels with higher IMIG stage. *NTHL1* and *XRCC3* expression was associated with TNM stage. *CHECK1* as well as *XRCC2* expression levels were correlated with tumour progression in the overall cohort of patients. *CDKN2A* and *MLH1* gene expression influenced overall survival in this collective. In the adjuvant treated cohort only, *CDKN2A*, *CHEK1* as well as *ERCC1* were significantly associated with overall survival. Furthermore, *TP73* expression was associated with progression in this subgroup.

Conclusion: DNA-damage response plays a crucial role in response to platin-based chemotherapeutic regimes. In particular, *CHEK1*, *XRCC2* and *TP73* are strongly associated with tumour progression. *ERCC1*, *MLH1*, *CDKN2A* and most promising *CHEK1* are prognostic markers for OS in MPM. *TP73*, *CDKN2A*, *CHEK1* and *ERCC1* seem to be also predictive markers in adjuvant treated MPMs. After a prospective validation, these markers may improve clinical and pathological practice, finally leading to a patients' benefit by an enhanced clinical management.

Key words: pleural mesothelioma, NanoString nCounter, digital gene expression analysis, DNA-damage repair, platin-based chemotherapy.

Introduction

Malignant pleural mesothelioma (MPM) is a rare, predominantly asbestos-related and biologically highly aggressive tumour leading to a dismal prognosis [1, 2]. In the US, approximately 2500 new cases of mesothelioma are diagnosed each year and the incidence of mesothelioma is expected to decline steadily [1, 3, 4]. In contrast, the incidence of mesothelioma in Europe continues to rise. Its peak is expected in the next two decades and may account for as many as 250,000 European deaths in the next 35 years [1, 2, 5-7].

Multimodality therapy consisting of chemotherapy, surgery and/or radiotherapy is centred on surgical resection in early stages. In clinical practice, the antifolate pemetrexed is used in combination with cisplatin [8] or carboplatin [9-12]. Cisplatin resulted in a response rate of merely 14% and a median survival of below 7 months [13]. Carboplatin resulted in similar response rates ranging from 6 to 16% [13, 14]. The reasons for the rather poor efficacy of platinum-compounds remain largely unknown.

Platinum cytotoxicity is based on forming bulky DNA adducts by chemically altering DNA bases by covalent binding of platinum [12], leading to both DNA inter- and (1,2 or 1,3)-intra-strand cross-linking [15-22]. Platinum-compounds prevent normal cell replication and trigger apoptosis [17, 21, 23], unless adducts from genomic DNA are repaired [20]. Even a single DNA cross-link, if not repairable, can be lethal [24]. There are also several local treatment modalities that have been used such as gene therapy, zoledronic acid and photodynamic therapy [25-30].

Nucleotide excision repair (NER) is capable of removing numerous types of DNA helix-distorting lesions, which are induced by platinum [15, 16, 31]. The endonuclease excision repair cross-complementing 1 (*ERCC1*) performs an essential late step in the NER process, where it nicks the damaged DNA strand at the 5′ site of the helix-distorting lesion [31], and is the rate-limiting member of the NER pathway [15, 16, 20].

In contrast, mismatch repair (MMR) is one of the major DNA repair pathways, which is responsible for the repair of single-base or nucleotide mismatches and insertion-deletion loops that results from slippage during replication of repetitive sequences or during recombination [32-34]. The MutS homologue 2 (*MSH2*) protein belongs to the MMR pathway and is crucially involved in the repair of DNA cross-links. MSH2 also recognizes and binds to platinum-induced DNA interstrand cross-links, thereby initiating their excision and repair [24] whilst building a MSH2/MSH6 heterodimer [35, 36]. Defects in DNA MMR have been shown to be a mechanism of resistance to cisplatin both in vivo and in vitro [15, 37].

Base excision repair (BER) corrects small base alterations without distorting the DNA helix structure [38-40] by using two distinct ways. In the short patch pathway a single base replacement is performed by DNA polymerase b (*POLB*), DNA ligase3 and the X-ray cross complementing group 1 (*XRCC1*) protein, the latter as a scaffold protein to form the complex [41]. In the long patch pathway a DNA synthesis of multiple nucleotides (2–10 bases) occurs by the DNA pold/e, proliferating cell nuclear antigen (*PCNA*), the flap endonuclease 1 (*FEN1*) and DNA ligase1 [41]. Interestingly, BER components are also used to repair single-stranded DNA breaks (SSB), whereat *XRCC1* and poly (ADP-ribose) polymerase (*PARP1*) serve as SSB sensors [38, 42]. As DNA lesions occurring in mesothelioma are in majority single strand breaks, the BER pathway plays a crucial role in survival of these cells [43, 44]. Further important pathways are the recombination repair system (Homologous recombination repair (HR), Non-homologous end-joining (NHEJ) and Fanconi anemia (FA) pathway) and the downstream target cascade that contributes to HR, including *RAD52*, *RAD54*, *RPA* and Breast Cancer 1 and 2 (*BRCA1/2*) [38, 45, 46]. These are of high interest for the understanding of the resistance mechanism to platin-compounds. Although impaired DNA repair could promote mutagenesis, an intact HR repair system may increase resistance to radiotherapy and chemotherapy [47].

In consideration of these aspects we aimed to investigate the impact of DNA-damage response and repair key players on platin-compound based therapy. Therefore, we analysed twenty-four MPM patients, whereof twelve underwent adjuvant and twelve neoadjuvant treatments. The selection of targets was based on recent literature and preliminary results of our group.

Material and Methods

Study Cohort and Experimental Design

For this exploratory mRNA study, twenty-four formalin-fixed paraffin-embedded (FFPE) tumour specimens were screened. Tumour classification was based on the *WHO Classification Of Tumours* guidelines (2004) [48] and TNM-staging was based on the *UICC Classification of Malignant Tumours* [49]. Those were confirmed by two experienced pathologists (JWO, KWS). The study included only MPM patients treated at the West German Cancer Centre or the West German Lung Centre between

2005 and 2009. All patients received platinum-based chemotherapy. Half of the collected samples were taken in an adjuvant and the other half in a neoadjuvant situation. The clinical stage was determined according to the criteria of the *International Mesothelioma Interest Group* (IMIG) tumour-node-metastasis staging system for MPM [50]. Radiologic response rate was assessed by modified Response Evaluation Criteria in Solid Tumors (modRECIST), which have been validated in MPM [51, 52]. Clinicopathological data including age, gender, histology and TNM are summarized in table 1. Surveillance for this study was stopped on August 31, 2014. Complete follow up was available for all patients with reported deaths in more than 96% (23/24). Progression under therapy was observed in approximately 80% (19/24) of patients. The retrospective study was approved by the Ethics Committee of the Medical Faculty of the University Duisburg-Essen (identifier: 14-5775-BO). The investigation conforms to the principles outlined in the declaration of Helsinki.

RNA Isolation and RNA Quantity Assessment

Three to five 10µm thick paraffin sections per sample were de-paraffinised using xylene prior to total RNA isolation including miRNAs. RNA was isolated by using the miRNeasy FFPE kit (Qiagen, Venlo, Netherlands) as recommended by the supplier except for proteinase K digestion performed overnight. Total RNA quantity was assessed using a Qubit flouormetric quantification system and Broad Range RNA Assay Kit (Thermo Fisher Scientific, MA, USA).

CodeSet Design

Based on preliminary experiments, recent literature and *in silico* predicted miRNA-targets, a total of 366 mRNAs (including reference genes) were investigated using a Custom CodeSet from NanoString. The nCounter standard chemistry was used and the preparation was carried out as recommended by the manufacturer. For each sample, 200 ng total RNA were processed. The high-sensitivity protocol was chosen; the cartridge was measurement at 555 fields of view.

NanoString Data Processing and Statistical Analysis

All statistical analyses were calculated using the R i386 statistical programming environment (v3.2.3). A technical normalization of the counts was done by subtracting the mean counts plus two-times standard deviation from the CodeSet inherent negative controls. Afterwards, a biological normalization using the included reference genes was performed [53-55]. In brief, the gene expression stability measures (M) were calculated by using the NormFinder algorithm and all sample counts were normalized against the geometric mean of the normalization gene counts. *ACTB*, *MAPK14* and *TCEB1* were identified as most stable reference genes.

Table 1. Overview of therapeutic scheme, clinicopathological parameters as well as clinical outcome for each patient

Therapy-Concept	Therapy-Regimen	Age (Years)	Gender	T-Stage	N-Status	M-Status	IMIG-Stage	Time to Death (Months)	Time to Progress (Months)	Progression	Outcome
adjuvant	CARBO+PEM	76	m	2	0	0	2	44,2	14,3	Yes	Death
adjuvant	CARBO+PEM	80	m	3	2	0	3	13,0	6,9	Yes	Death
adjuvant	CIS+PEM	51	m	x	x	x	x	8,0	6,2	Yes	Death
adjuvant	CIS+PEM	52	m	2	2	0	3	9,3	5,5	Yes	Death
adjuvant	CIS+PEM	56	m	3	0	1	4	43,2	5,5	No	Death
adjuvant	CIS+PEM	61	m	2	2	1	4	3,1	1,2	Yes	Death
adjuvant	CIS+PEM	65	m	2	2	0	3	8,8	4,9	Yes	Death
adjuvant	CIS+PEM	68	f	2	0	1	4	3,7	3,5	No	Death
adjuvant	CIS+PEM	70	m	1	2	0	3	14,5	6,7	Yes	Death
adjuvant	CIS+PEM	73	m	2	0	0	2	18,0	4,8	Yes	Death
adjuvant	CIS+PEM	75	m	3	0	0	3	21,7	6,4	Yes	Death
adjuvant	CIS+PEM	77	m	2	0	0	2	5,6	3,8	Yes	Death
neoadjuvant	CIS+CARBO+PEM	69	m	1	0	0	1	7,1	7,7	No	Death
neoadjuvant	CIS+PEM	54	m	3	2	1	4	13,8	4,1	Yes	Alive
neoadjuvant	CIS+PEM	56	m	2	0	0	2	37,0	23,1	Yes	Death
neoadjuvant	CIS+PEM	58	m	3	1	0	3	18,5	9,3	Yes	Death
neoadjuvant	CIS+PEM	58	m	2	0	0	2	6,7	2,8	Yes	Death
neoadjuvant	CIS+PEM	61	m	3	0	0	3	25,2	11,6	Yes	Death
neoadjuvant	CIS+PEM	62	m	2	2	0	3	41,5	34,7	Yes	Death
neoadjuvant	CIS+PEM	62	m	2	0	0	2	29,9	10,1	Yes	Death
neoadjuvant	CIS+PEM	62	m	2	2	0	3	4,4	4,0	Yes	Death
neoadjuvant	CIS+PEM	69	m	3	0	0	3	42,6	40,8	No	Death
neoadjuvant	CIS+PEM	69	f	2	0	0	2	27,3	9,7	Yes	Death
neoadjuvant	CIS+PEM	73	m	4	2	0	4	24,0	5,9	No	Death

CARBO … Carboplatin; CIS … Cisplatin; PEM…Pemetrexed.

For dichotomous variables (e.g. gender) the Wilcoxon Mann-Whitney rank sum test was used. Correlations between mRNA-expression and TNM-criteria were tested by using the Spearman's rank correlation test. Kaplan-Meier analysis was done for the assessment of associations between gene expression and progression-free survival (PFS) or overall survival (OS). Significant differences in PFS or OS between groups were verified by COXPH-model using Wald-test, likelihood-ratio test and Score (logrank) test.

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

Results

Overall Expression of Targets

The 366 analysed targets were narrowed down by their profile resulting in 30 members important for DNA-damage recognition and repair. In their overall expression, two markers stick out as they show a high expression of more than 2,500 median detected counts (*AKT1*: median counts 3513, range 1,804-7,493; *PRDX5*: median counts 2,873, range 417-10,850). Seven markers showed low expression less than 500 counts in median (*BRCA1*, *CDC25A*, *CDKN2A*, *MNAT1*, *NTHL1*, *TP73*, *XRCC2*), in particular *NTHL1* (median: 37 counts; range 0-113) and *XRCC2* (median: 68 counts; range 0-194) show a basal to absent gene expression but still differences between the tumours were detected. The remaining 21 targets revealed medial count numbers, ranging from 500 to 2,500 median counts. Median, mean and range of all 30 markers are summarized in table 2 and results are graphically illustrated in figure 1.

Correlation to Clinicopathological Data

High mRNA levels of *BRCA1* and *TP73* were significantly correlated to higher IMIG-Stage (p=0.0067; p= 0.0284). Furthermore, a low *CDC25A* gene expression was correlated to lymph node invasion (p=0.0358). In contrast, high *PARP1* expression levels were significantly associated with lymph node spread (p=0.0078).

Similarly, lower *NTHL1* gene expression (p=0.0478) and higher *XRCC3* gene expression levels were significantly associated with higher T-stage (p=0.0026). An increase of gene expression during platin-based chemotherapy was determined for *H2AFX* (p=0.0100), *PCNA* (p=0.0242) and *XRCC1* (p=0.0387).

With increasing patients age, *MSH2* (p=0.0267), *RBX1* (p=0.0390) as well as *XRCC1* (p=0.0030) expression decreased. No correlation between one of the investigated markers and the appearance of distant metastasis was found. No significant association to patient's gender or storage duration of the analysed paraffin tissues was detected.

Results are summarized and illustrated in figure 2.

Table 2. Summary of median count number, mean count number and count range of each measured target. Of note, *AKT1*, *PRDX5* and *TP53* show outstanding high, and *BRCA1*, *CDC25A*, *CDKN2A*, *MNAT1*, *NTHL1*, *TP73* and *XRCC2* a low to basal gene expression.

	Median	Mean	Minimum	Maximum
AKT1	3,513	3,969	1,804	7,493
ATM	1,920	1,921	758	3,128
ATR	1,006	960	456	1,291
BRCA1	480	479	0	900
CCNH	739	925	206	2,066
CDC25A	278	275	31	633
CDK7	607	580	213	939
CDKN2A	108	153	0	890
CHEK1	830	887	332	1,654
CHEK2	446	501	195	1,120
ERCC1	1,191	1,164	639	1,965
H2AFX	1,284	1,389	762	2,497
MGMT	934	1,026	223	2,012
MLH1	924	911	213	1,541
MNAT1	127	155	0	388
MSH2	834	871	427	2,021
MYC	1,724	1,952	1,001	4,821
NBN	1,091	1,110	470	1,696
NTHL1	37	46	0	113
OGG1	767	730	313	1,234
PARP1	1,769	1,740	912	2,899
PCNA	809	948	377	1,540
PRDX5	2,694	2,996	1,043	5,678
RAD52	1,013	1,045	320	1,915
RBX1	1,817	1,985	938	3,551
TP53	2,446	2,435	897	4,128
TP73	327	347	0	943
XRCC1	589	599	230	969
XRCC2	68	84	0	194
XRCC3	503	536	0	900

Associations to Clinical Outcome

In the overall cohort, associations between gene expression level and objective tumour progression were found for *CHEK1* (p=0.0362) and *XRCC2* (p=0.0053). No associations with PFS were observed; nevertheless, *CDKN2A* (p=0.0387) as well as *MLH1* (p=0.0344) gene expression levels influenced OS independently.

In the cohort of adjuvant treated patients, some predictive and prognostic markers were identified. Increased *TP73* gene expression was significantly associated with progression of the tumour during therapy (p=0.0303). *CDKN2A* (p=0.0297) as well as *CHEK1* (p=0.0162) and *ERCC1* (p=0.0455) gene expression levels were significantly correlated to OS.

Results are graphically illustrated in figure 3 and a list of all significant p-values is given in table 3.

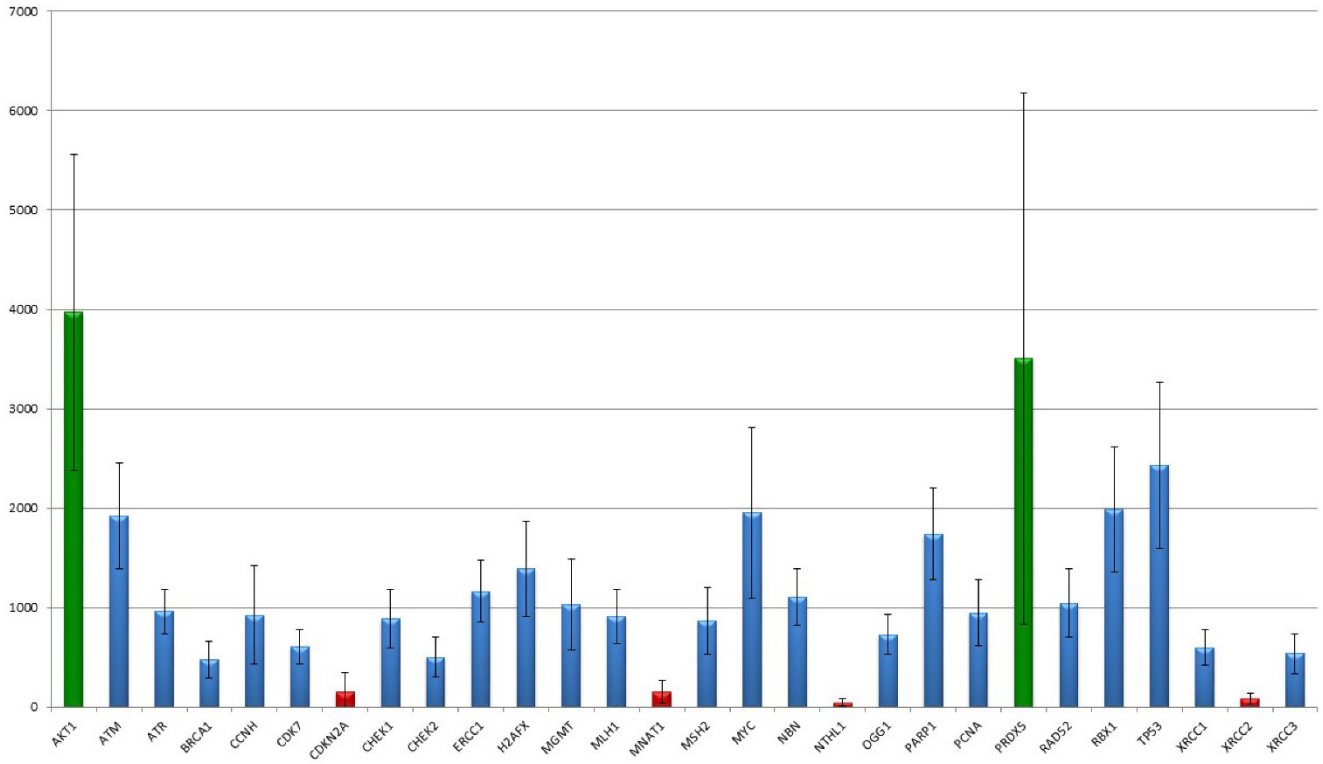

Figure 1. The figure shows the distribution of NanoString mRNA counts over for each target. AKT1 as well as PRDX5 showed the strongest gene expression level. By the way, the disparities of PRDX5 are notable ranging between 1,000 and 5,800 counts. CDKN2A, MNAT1, NTHL1 and XRCC2 show the lowest mean expression levels. Interestingly, for both CDKN2A and XRCC2 the standard deviation clearly exceeds the median count number, indicating differences between the samples.

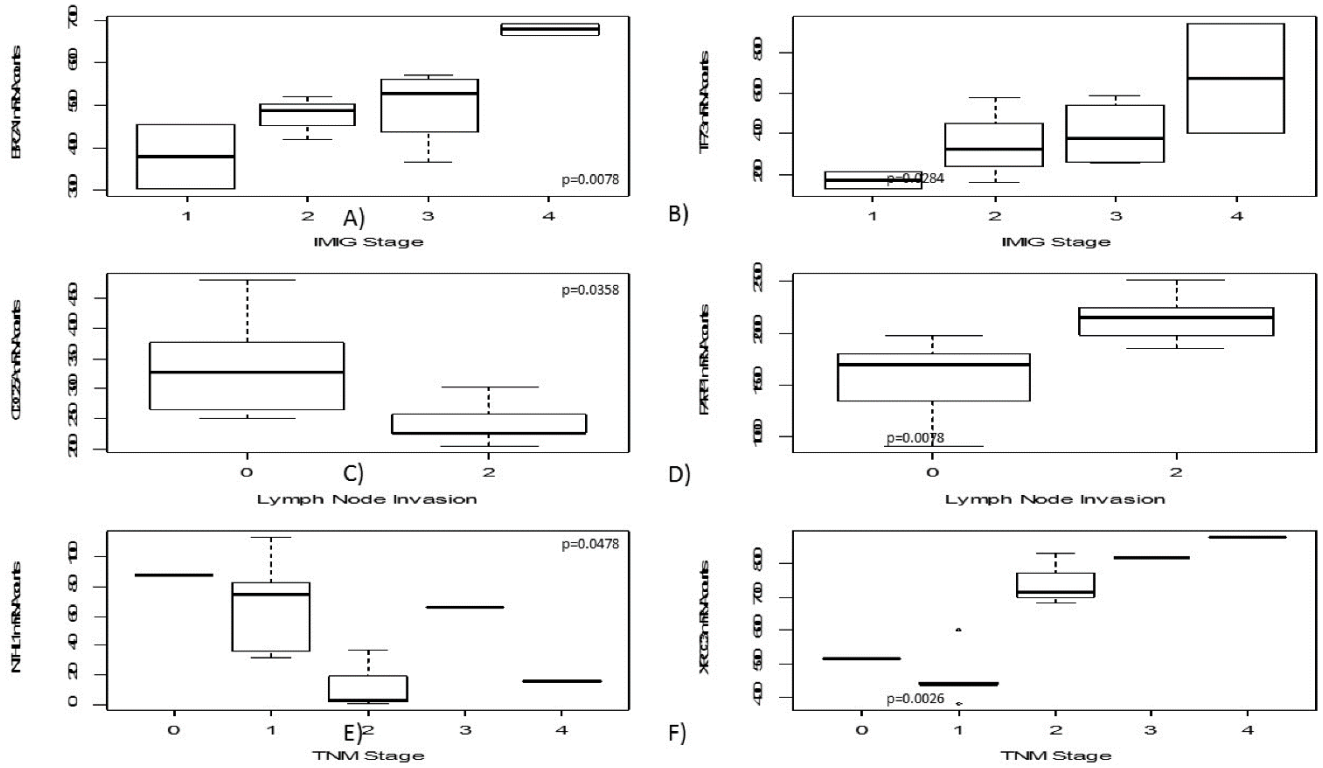

Figure 2. In this figure boxplots regarding clinicopathological parameters are shown, including IMIG-stage (A+B), N-status (C+D) as well as TNM-stage (E+F). Both BRCA1 and TP73 show increasing gene expression with higher IMIG-stage. CDC25A gene expression decreases in lymph-metastatic tumours whereas PARP1 gene expression increases in those. Interestingly, strong XRCC3 gene expression was just found in pT2 or higher tumours.

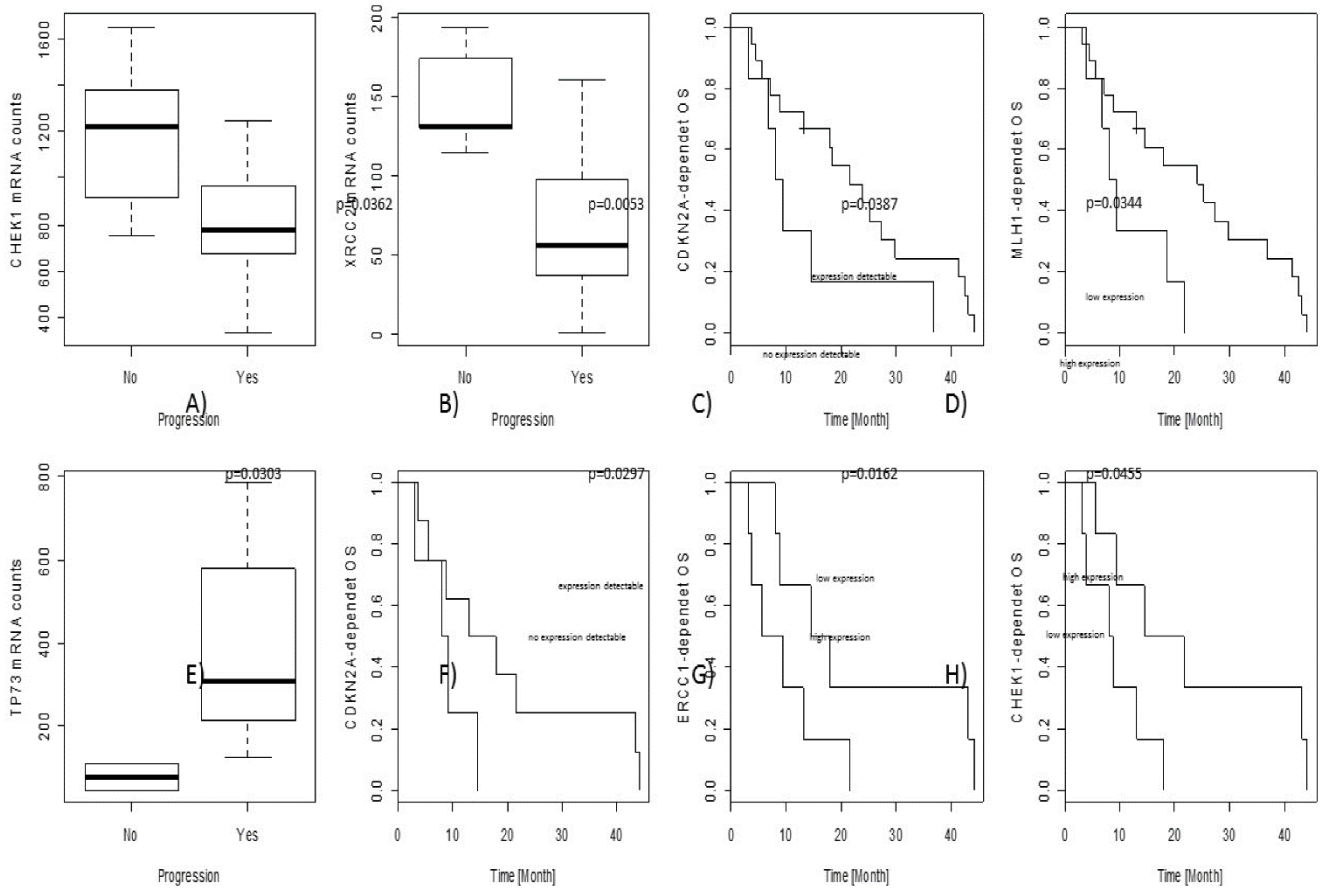

Figure 3. Prognostic and predictive results are illustrated in the figure. The upper line (A-D) show results from the overall collective, the lower line (E-H) results from the adjuvant cohort only. In particular, *CDKN2A*, *ERCC1* and *CHEK1* seem to be predictive markers for platin-response in the adjuvant-treated patients, gaining a real survival benefit.

Table 3. List of all significant p-values after Bonferroni-correction for multiple testing. For the adjuvant treated subgroup only, just predictive parameters as survival or progression were tested.

	Gene	Variable	Test-Method	p-Value	Likelihood ratio test	Wald test	Score (logrank) test	Z	rho
Overall Cohort	CDC25A	N-Status	Spearman's rank correlation rho	0,0358	-	-	-	-	-0,6351
	PARP1	N-Status	Spearman's rank correlation rho	0,0078	-	-	-	-	0,7506
	BRCA1	IMIG-Stage	Spearman's rank correlation rho	0,0078	-	-	-	-	0,7505
	TP73	IMIG-Stage	Spearman's rank correlation rho	0,0284	-	-	-	-	0,6561
	NTHL1	TNM-Stage	Spearman's rank correlation rho	0,0478	-	-	-	-	-0,6068
	XRCC3	TNM-Stage	Spearman's rank correlation rho	0,0026	-	-	-	-	0,8090
	CDK7	Age	Spearman's rank correlation rho	0,0450	-	-	-	-	-0,4148
	MSH2	Age	Spearman's rank correlation rho	0,0267	-	-	-	-	-0,4548
	OGG1	Age	Spearman's rank correlation rho	0,0148	-	-	-	-	-0,4957
	PRDX5	Age	Spearman's rank correlation rho	0,0301	-	-	-	-	-0,4461
	RBX1	Age	Spearman's rank correlation rho	0,0390	-	-	-	-	-0,4261
	XRCC1	Age	Spearman's rank correlation rho	0,0030	-	-	-	-	-0,5887
	H2AFX	Therapy	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0100	-	-	-	2,5403	-
	MYC	Therapy	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0205	-	-	-	-2,3094	-
	PCNA	Therapy	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0242	-	-	-	2,2517	-
	XRCC1	Therapy	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0387	-	-	-	2,0785	-
	CHEK1	Progression	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0362	-	-	-	-2,0969	-
	XRCC2	Progression	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0053	-	-	-	-2,6679	-
	-	PFS	Cox Proportional Hazard Model	-	-	-	-	-	-
	CDKN2A	OS	Cox Proportional Hazard Model	-	0,0387	0,0263	0,0214	-	-
	MLH1	OS	Cox Proportional Hazard Model	-	0,0344	0,0382	0,0401	-	-
Adjuvant Cohort	TP73	Progression	Exact Wilcoxon Mann-Whitney Rank Sum Test	0,0303	-	-	-	2,1483	-
	-	PFS	Cox Proportional Hazard Model	-	-	-	-	-	-
	CDKN2A	OS	Cox Proportional Hazard Model	-	0,0297	0,0433	0,0460	-	-
	CHEK1	OS	Cox Proportional Hazard Model	-	0,0162	0,0246	0,0283	-	-
	ERCC1	OS	Cox Proportional Hazard Model	-	0,0455	0,0479	0,0458		

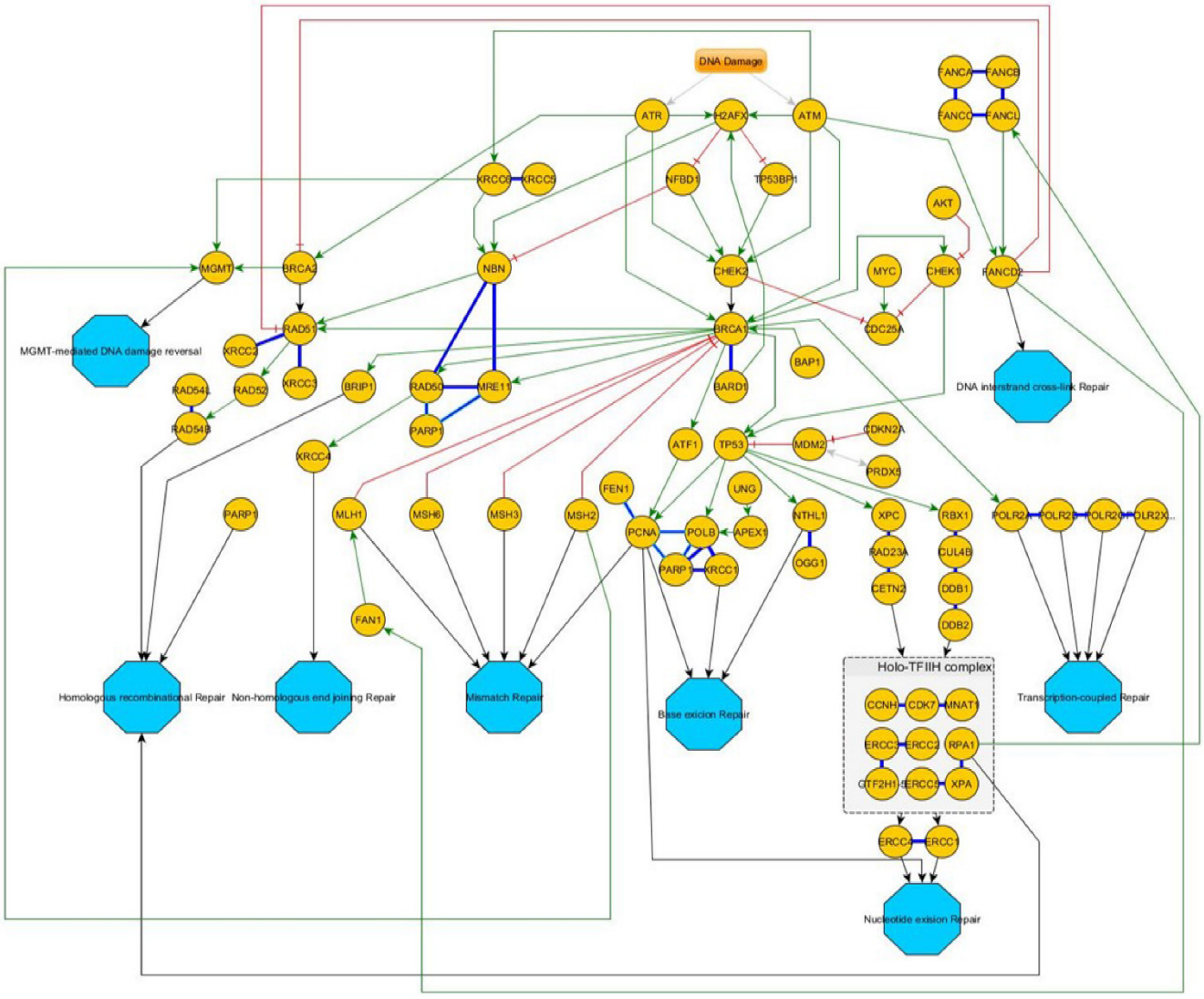

Figure 4. Overview of the DNA-damage response pathway-network. After an initial stress signal (DNA-damage), ATR and ATM transmit these signals downstream over CHEK1/2 to BRCA1/2 and then distribute to the different pathways. Green Arrows indicate activating signals; red cross-marks indicate inhibitory signals. Blue bold lines indicate complexes. If a protein is involved in different independent complexes, different shades of bold-blue lines indicate the different complexes. Turquoise octagons indicate the different pathway end-points.

Discussion

We present a study focussing on a mRNA screening for DNA-damage response associated targets aiming to understand the mechanisms of platinum-based therapy-induced apoptosis and to identify possible predictive markers.

An overview of the corresponding DNA-repair pathways is given in figure 4.

Besides pemetrexed, platinum-compounds are standard chemotherapeutic agents for the treatment of MPM [24]. Besides, Platinum-containing regimens have a greater activity than non-platinum containing combinations in MPM [56].

Several studies have investigated carboplatin and cisplatin as single-agent chemotherapy. Cisplatin resulted in a response rate of merely 14% and a median survival of below 8 months [13, 14]. Carboplatin resulted in similar response rates ranging from 6 to 16% [13, 14]. The reasons for the rather poor efficacy of platinum-compounds remain largely unknown.

DNA-Damage Recognition and TP53 Inhibitory Network

DNA double-strand breaks (DSBs) and DNA replication stress activate the ATM signalling pathways, which transduce the signal to downstream ATR/CHEK1 pathway [57, 58]. CHEK1 directly activates TP53 and thereby triggers TP53-induced apoptosis and senescence in response to DNA damage. Previous studies showed that cells lacking CHEK1 were more sensitive to gemcitabine in a TP53-independent manner [58, 59]. To date, the role of

the ATM/CHEK2 pathway in cells treated with platin-containing compounds is unknown [58]. Our results confirm this relationship. *CHEK1* was associated with both progression during therapy and OS in the adjuvant treated sub-cohort. Therefore, *CHEK1* gene expression seems to be a strong predictive marker for patients' response and outcome under platin-based chemotherapy. We recommend validation of this result in a larger prospective study to bring *CHEK1* to a presumable clinical application.

Of note, a direct inhibition of CHEK1 by small molecules already showed to promising results *in vitro* in breast cancer [60], *in vivo* in hepatic carcinoma [61] as well as in mesothelioma [62].

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 TP53 [63-66]. For instance, overexpression of MDM2 can lead to a loss of TP53 regulatory function in cancer cells by increased proteasomal degradation of TP53 [67-72]. Gene amplification can lead to increased MDM2 protein expression. However, many tumours present with high MDM2 protein levels without an increased gene copy number [73-77].

In previous studies, we have demonstrated that *MDM2* is overexpressed in approximately 20-30% of MPM and this finding was restricted to epitheloid MPM or the epithelioid components of biphasic MPM [78, 79]. Moreover, patients with *MDM2*-positive MPM presented with a significantly decreased OS compared to *MDM2*-negative MPM [78, 79]. The physiological inhibitor of MDM2 is P14/ARF and loss of P14/ARF activity may have a similar effect as loss of TP53 [80-83]. P14/ARF may control *TP53* transcription, represses TP53 degradation that is not MDM2-mediated and stimulates TP53 activity [84]. Additionally, loss of P14/ARF activity seems to occur in a reciprocal manner to *TP53* loss and seems to be typical for tumours that are *TP53* wild type [83]. In a previous study, we found *CDKN2A* (gene locus coding for *P14/ARF*) gene expression associated with OS and PFS in platinum-treated MPM patients [79]. In concordance, *CDKN2A* gene expression is significantly associated with OS in this cohort, and additionally in the adjuvant cohort only. This indicates that *CDKN2A* gene expression could be used as marker for platin-response prediction. Additionally, *TP73* shows an increasing expression with increasing IMIG-stages and associates significantly with progression in pleural mesothelioma.

Nucleotide Excision Repair (NER), Mismatch Repair (MMR) and Base Excision Repair (BER)

The impact of *ERCC1* expression on treatment response in MPM is discussed controversially. Recent studies have suggested that the expression levels of DNA-damage repair enzyme-related genes, including *ERCC1* mRNA, are associated with an overall survival benefit during cisplatin-based chemotherapy in MPM [12, 37, 51]. Yet, investigation of ERCC1 protein levels in a phase II clinical trial revealed a significant correlation between negative ERCC1 status and long PFS in a cohort of 54 MPM patients undergoing treatment with cisplatin and vinorelbine [21]. Our findings are in line with these earlier studies, as *ERCC1* gene expression level clearly impact the OS rate, but influence neither PFS nor progression.

During the recombinational repair processing of interstrand cross-links, MSH2 cooperates with several components of DNA damage repair pathways, including ERCC1 [24]. MutL homologue 1 (MLH1) protein is also a key component in the MMR pathway being involved in mismatch strand excision and subsequent repair. In a previous study, we showed that MLH1 protein levels were associated with PFS [37]. Additionally, in the recent study *MHL1* gene expression was found as a prominent marker for OS.

As DNA lesions occurring in mesothelioma are in majority single strand breaks (SSBs), the BER pathway plays a crucial role in survival of these cells [43, 44]. Several studies have investigated BER family members in tumorigenesis and pathophysiology of MPM [41, 62, 85-90]. In particular, *PARP1* was upregulated and activated in human mesothelial cells exposed to asbestos [87, 88]. PARP1 inhibition resulted in an accumulation of SSB lesions induced by direct asbestos exposure in human mesothelial cells [90]. Interestingly, beside its role in DNA repair, PARP1 has been found to induce apoptosis and cell death following asbestos exposure [87-89, 91]. In this study, the analysed tumour samples formed two distinct expression patterns with respect to *PARP1* gene expression levels. Interestingly, high *PARP1* expression associated with lymph node invasion.

Recombination Repair System

Homologue recombination (HR) and non-homologous end-joining (NHEJ) are of great importance, since DNA DSBs induce chromosomal deletions and translocations which are common in MPM [45, 92, 93]. Upregulation of various genes encoding HR protein members, including *RAD50*, *RAD54L*, *RAD21*, *BRCA1* and *BRCA2* could be detected by gene expression analysis in MPM [62, 94]. Our results indicate that a higher gene expression of *BRCA1* is highly associated with increasing clinical

malignancy (indicated by higher IMIG-stage). Furthermore, a reduced *XRCC2* gene expression, acting downstream of the MRN-complex [95], correlated with with progressive disease more often. Similarly, increased *XRCC3* gene expression, forming a complex together with *XRCC2*, was observed in tumours showing higher TNM-stage.

Cellular Response to Platin-Induced DNA-Damage

DNA damage, e.g. induced by platin-compounds, is first recognized by ATM and ATR. This leads to a subsequent phosphorylation of histone H2AFX, activating the different downstream cascades of cellular DNA damage response [96, 97]. Deregulation of H2AFX expression and modification has been reported to be associated with a variety of human malignancies [98-103]. Surprisingly, studies exploring *H2AFX* expression in MPM are still lacking. In this study, we observed an increase of expression induced by platinum-based chemotherapy, leading to the assumption that activation of *H2AFX* expression plays a crucial role for early DNA damage response. Furthermore, *PCNA* and *XRCC1*, forming a central complex together with the DNA polymerase beta (*POLB*) in BER [41], show the same increase during therapy, indicating the activation of *H2AFX* downstream targets during the accumulation of DNA SSBs and DSBs.

Conclusion

DNA damage response plays a crucial role in response to platin-based chemotherapeutic regimes. In particular, *CHEK1*, *XRCC2* and *TP73* are strongly associated with tumour progression. Therefore, we suggest that these markers are used along with staging and restaging. *MLH1*, *CHEK1*, *ERCC1* and most promising *CDKN2A* are prognostic markers for OS in MPM. Therefore, we suggest that these markers are at staging and upon diagnosis of MPM. *TP73*, *CDKN2A*, *CHEK1* and *ERCC1* seem to be also predictive markers in adjuvant treated MPMs. Those should be validated prospectively, hopefully finding their way into clinical and pathological practice, leading to an enhanced clinical management and improved clinical outcome.

Conflicts of interest

All authors state that they have no conflicts of interest to declare.

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