EUROPEAN JOURNAL OF CANCER 47 (2011) 326–332

available at www.sciencedirect.com

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Induction of senescence markers after neo-adjuvant chemotherapy of malignant pleural mesothelioma and association with clinical outcome: An exploratory analysis

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ARTICLE INFO

Article history:
Received 5 May 2010
Accepted 28 September 2010
Available online 29 October 2010

Keywords:
Malignant pleural mesothelioma,
DNA-damaging chemotherapy
Accelerated senescence
Senescence markers
PAI-1
Apoptosis
Clinical outcome

ABSTRACT

The aim of this study was to assess the induction of senescence markers versus apoptosis pathways in malignant pleural mesothelioma (MPM) tumour samples before and after neo-adjuvant platinum-based chemotherapy and to investigate their relationship with clinical outcome.

Specific senescence pathways were assessed by quantifying the expression of p21 and plasminogen activator inhibitor-1 (PAI-1) for the p21–p53 pathway, IGFBP7 for the IGF pathway and ALDH1A3 for the IFN pathway. p21 and PAI-1 expression were also assessed by immunohistochemistry. In addition, beta-galactosidase activity staining at pH 6.0 was performed. Apoptosis was determined by TUNEL assay. Clinical outcome was assessed by modified RECIST criteria, progression-free and overall survival.

In a training set ($n = 9$ patients) paired comparison demonstrated a significant increase in p21 ($p < 0.05$), PAI-1 ($p < 0.01$) and apoptosis ($p < 0.01$) after neo-adjuvant chemotherapy. The patients with the highest increase in PAI-1 had stable disease, whilst patients with little change in senescence markers accompanied by a high increase in apoptosis had an objective response after chemotherapy. The hypothesis that stable disease might be associated with an increase in senescence markers was confirmed in a tissue microarray ($n = 26$ patients) using p21 and PAI-1 immunohistochemistry as readouts. For patients where survival and time to progression data were available, increased PAI-1 levels were significantly associated with a worst outcome.

Our results demonstrate induction of senescence markers by neo-adjuvant chemotherapy in a proportion of patients with MPM and its potential association with a poor outcome.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.09.044

1. Introduction

Cisplatin-based combination therapy has become the preferred chemotherapy option for the treatment of malignant pleural mesothelioma (MPM).[1] For potentially operable disease, combined modality therapy including extrapleural pneumonectomy is an option.[2] We and others have examined the use of neoadjuvant therapy followed by extrapleural pneumectomy, initially with cisplatin and gemcitabine and more recently with cisplatin and pemetrexed.[3] In a proportion of our patients tumour tissue at the time of diagnosis and after chemotherapy is available. This allows us to directly explore the effect of chemotherapy on the tumour.

It is widely accepted that DNA damage following chemotherapy engages a signal amplification cascade known as DNA-damage response which can have two outcomes: induction of apoptosis or induction of cellular senescence, also called accelerated senescence. The latter is a state of stable cell cycle arrest with active metabolism. In contrast to apoptosis where the cytotoxic signals converge in a common mechanism, senescence is typically a delayed stress response involving multiple effectors mechanisms including epigenetic regulation,[4] DNA damage response,[5,6] senescence-associated secretion phenotype[7–10] and autophagy.[11] The relative contribution of these effectors varies depending on the trigger and cell type. In the context of cancer therapy it is not yet clear whether senescence is indicative of an irreversible growth arrest, since some studies have shown an escape from senescence.[12,13] Also, it was recently demonstrated that senescent cells can secrete mitogenic factors.[8]

The aim of this study was to assess the extent of activation of senescence and apoptosis pathways in MPM tumour samples before and after neo-adjuvant platinum-based chemotherapy and to explore their relationship with clinical outcome.

2. Material and methods

2.1. Patient population and clinical response assessment

The tissue samples were obtained from patients treated for MPM at the Department of Medical Oncology and the Department of Thoracic Surgery at the University Hospital of Zurich during the time period from May 1999 until the 31st January 2009. The study was approved by the Zurich University Hospital ethic committee and a written informed consent was obtained from all patients. Neoadjuvant chemotherapy consisted of three cycles of cisplatin 80 mg/m^2 on day 1 and gemcitabine 1000 mg/m^2 on days 1, 8 and 15 administered every 28 d or, since March 2003, of cisplatin 80 mg/m^2 on day 1 and pemetrexed 500 mg/m^2 on day 1 administered every 21 d with vitamin supplementation. Response to chemotherapy was assessed by CT scan after completion of the third cycle of chemotherapy. Clinical response was evaluated according to modified RECIST criteria.[14] Surgery was performed within 6 weeks after completion of the last administration of chemotherapy. Follow-up was performed in our outpatient clinics.

2.2. Tissue samples

Tumour specimens obtained for diagnostic purpose before chemotherapy and at the time of resection after chemotherapy were immediately processed for total RNA extraction using Qiagen RNAeasy®. In addition, parts of tumour specimens were embedded in OCT and immediately frozen or fixed in paraformaldehyde for paraffin embedding. Six normal pleura samples were received from six patients undergoing mesothelioma unrelated thoracic surgery.

2.3. RT-PCR and analysis of markers

From the extracted RNA, reverse transcription was performed on 400–500 ng RNA (Qiagen QuantiTect® Reverse Transcription protocol). To investigate the quantitative expression of the different genes, cDNA was amplified by the SYBR-Green PCR assay and products were detected on a 7900HT Fast real-Time PCR system (SDS, ABI/Perkin Elmer). Histone RNA was used to standardise the total amount of cDNA. Primers for the different markers are listed in supplementary Table 1. Specificity of PCR was checked by analysing the melting curve and agarose electrophoresis. Relative mRNA levels were determined by comparing the PCR cycle thresholds between cDNA of a specific gene and histone ($^{\Delta}$Ct).

In order to assess that clinical samples contained at least 50% tumour content a preliminary study was performed comparing different genes expression in normal pleura and tumours. Hierarchical clustering (http://discover.nci.nih.gov/cimminer/index.jsp) was performed using euclidian distance metric on $^{\Delta}$Ct raw data. As a second quality control, tumour content assessed on H&E stained sections was correlated with the quantitative expression of MPM markers calretinin,[15] podoplanin[16] and mesothelin.[17] The comparison of morphological versus MPM markers was used to set up a threshold defining samples containing at least 50% tumour cells. First, a score was calculated as an average of centred $^{\Delta}$Ct calretinine + $^{\Delta}$Ct podoplanine + $^{\Delta}$Ct mesothelin for each sample. When this score was compared to morphology we found that a score of >10% relative to histones expression corresponded to more than 50% of tumour cells. Therefore only samples satisfying this criterion were further analysed.

2.4. Senescence associated-β-gal activity and apoptotic cell detection

Cryosections were stained for β-galactosidase activity as described by Dimri et al.[18] Briefly, cells were fixed with 0.5% glutaraldehyde in PBS and washed twice in PBS. Staining was performed by o/n incubation in 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$. Positive control consisted of samples incubated in the same reaction mixture at pH 4, whilst negative controls were at pH 7.5.

Tumour apoptosis of paraffin-embedded tissues was detected by TUNEL assay. Deparaffinized sections were incubated with terminal deoxynucleotidyl transferase and TMR red-dUTP (Roche Applied Science), according to the manufac-

turer's instructions. The slides were treated with Prolong Gold antifade reagent with DAPI (Invitrogen), then checked for fluorescence under Leica widefield microscope. A minimum of 200 cells were evaluated whenever possible. Ten random high power fields were analysed. The apoptotic index was recorded as the number of positive cells observed per number of cells evaluated.

2.5. TMA construction and immunohistochemistry

A set of three tissue microarrays (TMA) with double pre-chemotherapy and quadruple post-chemotherapy punches per patient (total $n = 156$, core diameter 600 micrometre) was prepared with a custom-made, semi-automatic tissue arrayer as previously described.[19] De-paraffinized sections were stained on either a Ventana (Ventana Medical Systems, Tucson, AZ, United States) or a Bond automat (Visio Biosystems, Melbourne, Australia), using the following primary antibodies: polyclonal anti-p21 (1:50, clone SC-397, SCBT, Santa Cruz, CA, United States) and mouse monoclonal anti-PAI-1 (1:40, clone TJA6, Novocastra, United Kingdom). Detection was performed with respective secondary antibodies with Ultraview Amp (Ventana) or refine-DAB (Vision Biosystems). The intensity level of immunoreactivity was scored 0 (negative), 1 (mild), 2 (moderate) and 3 (strong). The frequency of positive cells was scored 0 (no stained cells), 0.1 (1–10%), 0.5 (11–50%) or 1 (51–100%). The product of intensity and frequency was called individual H-score ($I \times F$, range 0–3) for a particular core. The sum of the 2 (pre) or the 4 (post) cores was called global H-score (range 0–6 and 0–12, respectively). Finally, the global H-score was normalised by subdividing its value for two and for four in the pre- and postchemotherapy cores respectively.

2.6. Statistical analysis

Statistical analysis was performed using paired t test and Mann–Whitney test. The Kaplan–Meier method and log rank tests were used for correlation of overall survival and time to progression with the p21 and PAI-1 score changes.

3. Results

3.1. Clinical characteristics of study patients

We identified 31 patients with tumour material available before and after chemotherapy. There were 29 males and 2 females, with a median age of 59 (range 44–72). The histotype was epithelioid in 21 patients (68%), biphasic in 7 (23%) and sarcomatoid MPM in 3 (10%).

Clinical stage according to TNM-classification[20] was I in 15 patients (49%), II in 5 (16%), and III in 11 (35%). Neoadjuvant chemotherapy was administered to all patients and consisted of three cycles of cisplatin and gemcitabine in 5 (16%) or three cycles of cisplatin and pemetrexed in 26 patients (84%). 30 patients underwent thoracotomy, which was explorative in three, pleurectomy/decortication in 6 and extrapleural pneumonectomy in 21 patients. From one patient tumour material was obtained by lymph node biopsy via mediastinoscopy after chemotherapy. He did not undergo thoracotomy.

3.2. Clustering of training set by senescence pathway gene expression profiling

An initial gene expression characterisation (Fig. 1) demonstrated that samples from the same patient at the two time points clustering together tightly, with the exception of samples containing less than 50% of tumour that were discarded for further analysis. The ratio of senescence markers (p21, PAI-1, ALDH1A3, IGFBP7[21]) measured after versus before chemotherapy were evaluated in nine patients where RNA from 'bonafide' tumour samples was available. The expression of p21 and PAI-1 after chemotherapy was significantly increased ($p < 0.05$ and <0.01, respectively, paired t test), (Fig. 2A), whilst there were no changes in ALDH1A3 and IGFBP7. Furthermore, the increase of PAI-1 was significant ($p < 0.05$, paired t test) in patients with stable disease, but not in patients with partial response (Fig. 2B). These data were corroborated at the protein level for p21 and PAI-1 by immunohistochemistry (Fig. 2C) confirming, at least for p21, previous studies showing strict correlation between p21 protein and mRNA expression.[22] In addition we observed an increase in senescence-associated β-galactosidase activity (Fig. 2D). Taken together, these results indicate that in some patients there is a strong induction of accelerated senescence after platinum-based chemotherapy.

3.3. Relationship between senescence markers, apoptosis and clinical response

The induction of apoptosis triggered by the DNA-damaging drugs would be the desired outcome of chemotherapy. The latter could be investigated by TUNEL assay, a method based on the in situ labelling of nuclear DNA breaks through the binding of terminal deoxynucleotidyl transferase (TdT) to 3′-OH ends of DNA in 6 of the 9 patients. Apoptosis could be detected in 1% (range 0–4) and 8% (range 1.5–15) of the cells before and after chemotherapy, respectively (Fig. 3A and B), representing a significant ($p < 0.01$, Mann–Whitney test) increase. Looking at individual results of these six patients, objective radiological responses were only seen in patients with induction of apoptosis and no change in PAI-1 senescence marker.

3.4. Determination of senescence markers by immunohistochemical analysis of TMA and clinical response

For 26 patients paired TMA material was available from before and after chemotherapy and was used to examine p21 and PAI-1 protein expression. This approach was necessary since mRNA was not available for all patients at the two time points. A linear relationship between p21 mRNA and IHC staining was observed ($r^2 = 0.71$, $p < 0.001$), whilst for PAI-1 the relationship between mRNA and immunohistochemistry was significant ($p < 0.05$) but less stringent ($r^2 = 0.33$) as it has been previously described for secreted proteins.[23] The best relationship between mRNA and PAI-1 IHC was found when staining of stromal cells was taken into account; hence PAI-1 score corresponded to the sum of tumour and stromal cells protein expression.

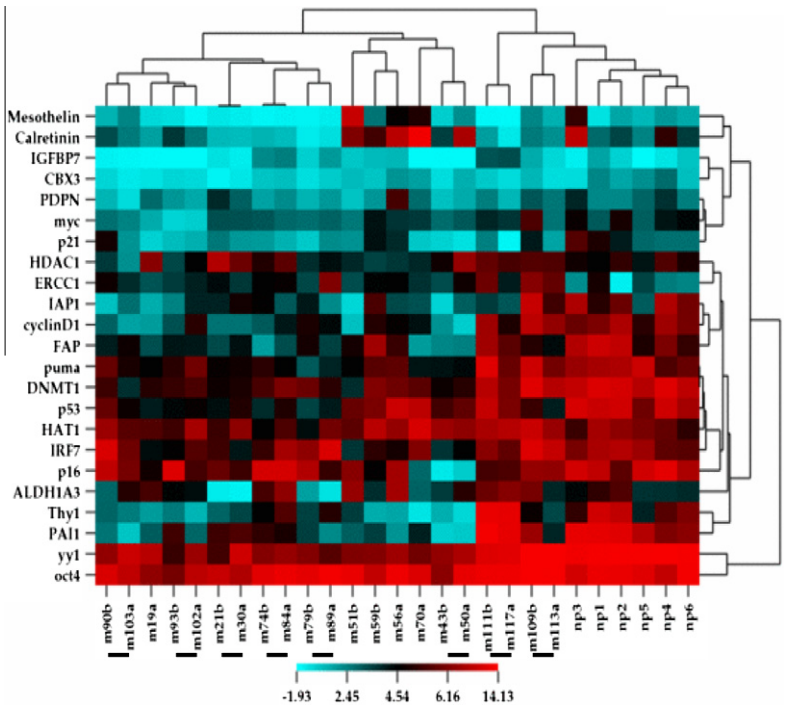

Fig. 1 – Gene expression characterisation used to classify by unsupervised clustering tumour and normal pleura samples. Analysed genes include MPM markers (mesothelin, calretinin and podoplanin), genes involved in apoptosis (puma, IAP1, p53, cyclinD1), senescence (p21, p16, PAI-1, ALDH1A3, IGFBP7, IRF7), embryonic stem cell-like core activated in human cancers and fibroblast activation protein (FAP). Matrix of relative gene expression values is shown as heat map. Heat map is a grid of rectangles with colours that indicate the value of the matrix elements, where high expression is blue and low expression is red. Rows of each heat map correspond to genes, whilst columns correspond to either samples before (indicated by 'b') or after (indicated by 'a') chemotherapy or normal pleura (indicate as 'np'). Paired samples from the same patient before and after chemotherapy are underlined.

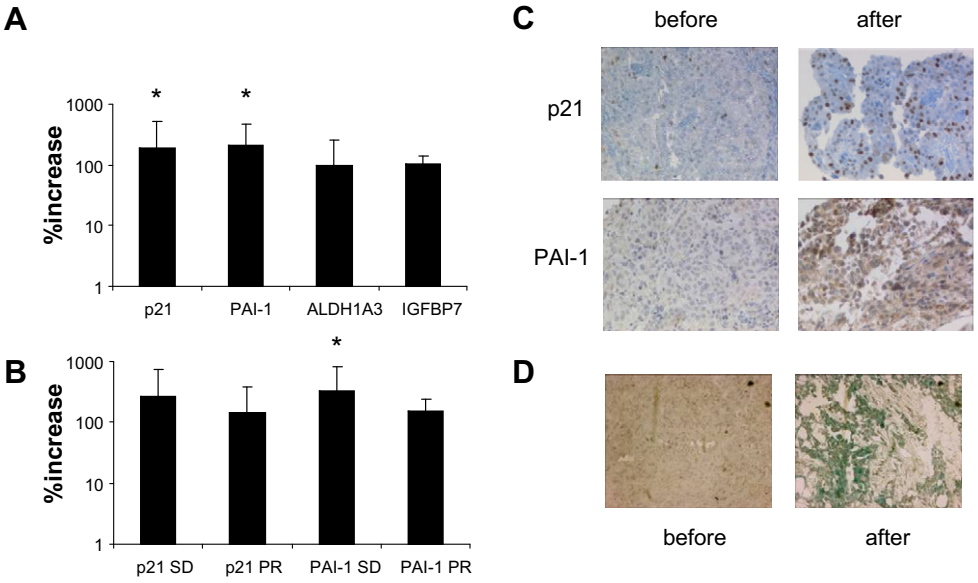

Fig. 2 – Increase in p21 and PAI-1 expression after chemotherapy. (A) The expression of p21 ($p < 0.05$) and PAI-1 ($p < 0.01$) but not ALDH1A3 or IGFBP7 was significantly increased after chemotherapy ($n = 9$). (B) PAI-1 increase was statistically significant ($p < 0.05$, $n = 4$) in stable disease (SD) but not partial response (PR, $n = 5$) patients. (C) Immunostaining of p21 and PAI-1 in a representative patient. (D) Senescence-associated beta-galactosidase staining before and after chemotherapy. Images are at same magnification (40×).

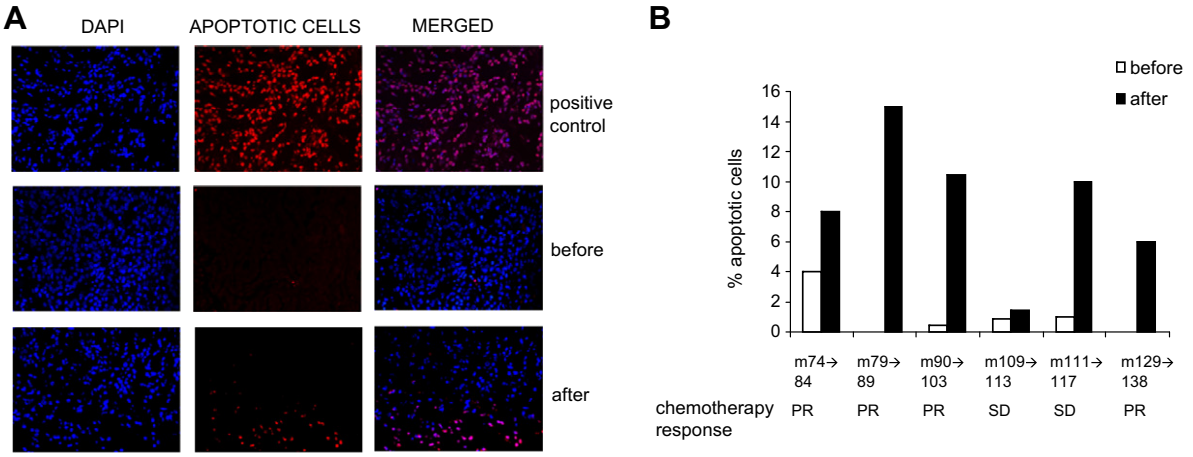

Fig. 3 – Modification of apoptosis after chemotherapy. (A) Apoptotic cells were detected by the TUNEL assay. Positive control corresponded to DNAse treated sample. Images are at same magnification (40×). (B) Quantification of the percentage of apoptotic cells before and after chemotherapy (significant increase after chemotherapy, $n = 6$ $p = 0.006$) in partial response (PR) or stable disease (SD) patients.

Data on response assessment were available for 23 patients; 14 were classified as stable disease and 9 as partial response according to modified RECIST criteria. Patients with stable disease tended to have more often an increase of PAI-1 expression as compared to patients with objective response (Fig. 4) whilst for p21 no trend was evident.

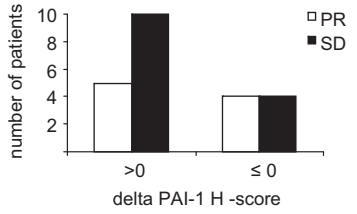

Fig. 4 – Increase of PAI-1 immunostaining (delta score >0) is more frequent in patients with stable disease (SD) compared to patients with partial response (PR).

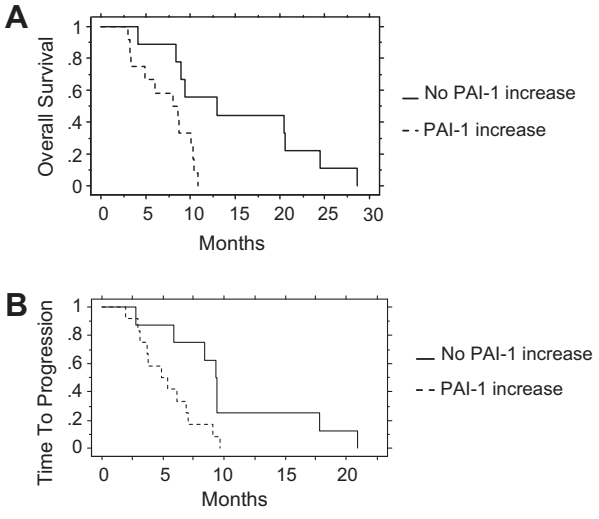

Fig. 5 – Overall survival (A, $p = 0.0098$, $n = 21$) and time-to-progression (B, $p = 0.022$, $n = 20$) are inversely correlated with increase in PAI-1 immunostaining.

In an exploratory analysis we looked for a possible association of increase of PAI-1 expression with overall survival and progression-free survival. As shown in Fig. 5 there was a significant association of increased PAI-1 expression with both poor survival and shorter progression-free survival ($p = 0.0098$ and $p = 0.022$, respectively).

4. Discussion

Despite recent advances in the chemotherapy of MPM, an objective response is reached in less than 50% of patients and the response duration is limited. As shown by our study platinum-based chemotherapy of MPM not only induces apoptosis but also senescence.

The *in vivo* role of senescence in treatment outcome was investigated first in an experimental model of non-Hodgkin's lymphomas where apoptosis was blocked by overexpression of Bcl-2. In this model, cyclophosphamide-induced senescence programme contributed to treatment outcome, since mice harbouring tumours capable of senescence but not apoptosis, although surviving less well compared to mice capable of apoptosis, had a substantially better post-therapy prognosis than those harbouring tumours with defects in both processes.[24]

Experimental evidence has already suggested that senescence might be responsible for poor chemotherapy response in mesothelioma.[25–27] Compared to tumours undergoing apoptosis, there are several problems with tumours becoming senescent. The first more obvious one is that the tumour arrests and does not regress. Second, a cell-cycle-targeting chemotherapy will loose efficiency in growth arrested cells. Third, subpopulations of chemotherapy-induced senescent cancer cells have already been shown capable of escape leading to relapse.[13,28] In one of these studies, tumour cells escaping senescence had re-acquired cyclin-dependent kinase CDC2/CDK1 expression which had been lost upon senescence, and

escape from senescence could be blocked using CDC2/CDK1 inhibitors[13,28] indicating that strategies can be found to avoid tumour relapse. Fourth, senescent tumour cells are metabolically active and can secrete cytokines and other paracrine factors which feed and stimulate the growth of nearby cells.[29] This could have some implications in treatment outcome, especially in terms of time to progression.

In an exploratory analysis we have identified increased PAI-1 as being associated with lack of objective response. PAI-1 is a secreted protein, component of the plasminogen activation system, that has been investigated in several cancers and elevated levels of PAI-1 are correlated with shortened overall and/or disease-free survival in renal, ovarian and breast cancer.[30] Although expression of PAI-1 has been observed in mesothelial cells *in vitro* after exposure to TGFβ[31] it is the first time that PAI-1 expression is investigated in malignant pleural mesothelioma tissues *in vivo*. In our study the relative change after chemotherapy was determinant for the response, suggesting that it plays a role in tumour evolution. PAI-1 is considered as a senescence marker,[21,32] is essential for p53-induced senescence[7] and has tumour promoter functions.[33] Since too few frozen samples were available, a definitive association between increase in PAI-1 levels and induction of senescence-associated β-galactosidase activity could not be determined in our study and it will be necessary to clarify this issue in further studies.

In conclusion, both apoptosis and senescence can be observed after chemotherapy in malignant pleural mesothelioma and increased expression of senescence marker PAI-1 was associated with lack of response to chemotherapy. A better understanding of mechanisms controlling the balance between the two processes may benefit the patients by identifying factors that should be targeted.

Conflict of interest statement

None declared.

Acknowledgements

We thank Drs. Stefan Ambs and Monika Hegi for critical reading of the ms and Ubiratan Moura and Susanne Dettwiler for skillfull assistance. Grant support: Oncosuisse, the Zurich Krebsliga and the Stiftung für angewandte Krebsforschung Zürich to RAS and EFB.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.09.044.

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