Melphalan-induced DNA damage *in vitro* as a predictor for clinical outcome in multiple myeloma

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ABSTRACT

Background and Objectives

As new therapeutic options for multiple myeloma (MM) emerge, identification of biological markers which could predict clinical response to standard treatment with high-dose melphalan (HDM) supported by autologous stem cell transplantation (ASCT) becomes more important.

Design and Methods

Melphalan-induced damage formation and repair of monoadducts and interstrand cross-links in the p53 gene were studied in peripheral blood mononuclear cells obtained from 32 patients prior to therapy. The same studies were performed in the peripheral blood cells of these patients immediately after subsequent HDM administration. Clinical response and time to progression were correlated with molecular end-points obtained *in vitro*.

Results

Values for all molecular end-points examined *in vitro* were highly correlated with the respective *in vivo* results within individual patients. All *in vitro* end-points indicative of increased DNA damage and slower repair capacity were predictive of a favorable response to HDM; the area under the curve of total adducts (AUC-TA) had the highest predictive ability. Using the cut-off value of 736 adducts/10^6 nucleotides x h for the AUC-TA, the positive predictive value for clinical response to HDM was 100%. Moreover, patients with an AUC-TA equal to or higher than this cut-off value had significantly longer times to progression than had patients with an AUC-TA lower than the cut-off value (hazard ratio 0.19; 95% confidence intervals 0.06 to 0.60).

Interpretation and Conclusions

An *in vitro* assay to quantify melphalan-induced p53-specific damage formation/repair can be used to select those patients with MM who are more likely to benefit from HDM supported by ASCT.

Key words: melphalan, multiple meyloma, prediction of clinical outcome, *in vitro*-induced adducts, p53-specific damage formation/repair.

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Multiple myeloma (MM) is a malignant plasma cell disorder accounting for about 10% of hematologic malignancies. This malignancy, although treatable, is considered incurable and accounts for 1% of all cancer deaths.[1] For several decades melphalan and corticosteroids were the main drugs for the treatment of MM. Subsequently, high-dose melphalan (HDM) supported by autologous stem cell transplantation (ASCT) was shown to improve progression-free and overall survival in some but not all studies.[2-4] Despite the activity of HDM in this disease, only 30% of patients achieve an immunofixation-negative complete response and the long-term disease-free survival is less than 10%. Over recent years several novel agents such as thalidomide, bortezomib and lenalidomide have shown activity not only in patients who are refractory to, or are relapsing after conventional chemotherapy, but also after HDM.[5-7] Furthermore, these novel agents when combined with steroids display significant activity in previously untreated MM patients and may also have a role after HDM.[8,9] Thus, as the treatment options for patients with MM are increasing, it is becoming important to identify a biological marker, that could select the patients more likely to benefit from HDM. Melphalan is a member of the nitrogen mustard class of chemotherapeutic agents and elicits its mechanism of action by the alkylation of DNA. It is capable of producing a number of different DNA adducts, the majority of which are monoadducts; a small proportion of these go on to form interstrand cross-links by reaction with the opposite DNA strand. The formation of cross-links between the two strands of DNA is considered to be a critical event, since there is clear evidence that their formation and subsequent persistence correlates with *in vitro* cytotoxicity.[10] Monoadducts are exclusively repaired by nucleotide excision repair (NER), while a number of multistep pathways, including NER, homologous and non-homologous recombination contribute to the repair of cross-links.[11-13]

Resistance to the nitrogen mustards in murine and human tumor cells has been reported to be secondary to (i) alterations in the transport of these agents,[14] (ii) cytoplasmic metabolism of the chloroethyl alkylating moiety to the inactive hydroxyethyl derivative via glutathione/glutathione-S-transferase,[15] (iii) overexpression of metallothionein, which confers resistance to cisplatin and cross-resistance to melphalan,[16] (iv) changes in apoptosis,[17] and (v) altered DNA repair capacity.[18] Although it has not been established which of these mechanisms are most relevant in the clinical setting, the importance of the role of DNA damage formation/repair in clinical resistance to agents such as the nitrogen mustards is becoming increasingly obvious. The formation and repair of interstrand cross-links was measured in plasma cells from melphalan-naïve and melphalan-treated patients, i.e., those who had relapsed after a melphalan-conditioned ASCT or oral melphalan therapy.[19] The formation of these lesions was very similar in both populations of patients and was unaffected by previous exposure to melphalan, suggesting that upstream resistance mechanisms such as drug transport and detoxification do not play a significant role in the development of melphalan resistance in MM.[19] Furthermore, a marked difference in the repair ability of melphalan-induced DNA damage was found between plasma cells from melphalan-naïve and melphalan-treated patients, suggesting that DNA repair may be an important mechanism by which melphalan resistance emerges after HDM therapy. The importance of understanding details of the interstrand cross-link repair pathway is also highlighted by the hypersensitivity to cross-links–inducing agents in the human genetic disease Fanconi anemia (FA) and in cells mutant for the breast cancer genes BRCA-1 and –2.[20-22] Moreover, enhanced the interstrand cross-link repair via the FA/BRCA pathway contributes to acquired drug resistance in melphalan-resistant myeloma cell lines, and disruption of this pathway reverses drug resistance.[23]

We have previously shown that measuring DNA adduct formation/repair in a readily accessible tissue such as peripheral blood mononuclear cells (PBMC) provides a non-invasive method for evaluating the effectiveness of melphalan in MM.[24] Importantly, we found that MM patients treated with HDM and supported by ASCT display wide variations in individual p53-specific DNA damage formation/repair during the first 24 hours after HDM administration, while increased DNA damage and slower repair capacity correlates with improved response and longer progression-free survival.[25] To further investigate the molecular mechanisms of the therapeutic action and drug resistance to melphalan, we measured the *in vivo* induction and repair of DNA damage in genes with dissimilar transcription levels.[13] Gene-specific repair was observed and the extent of the repair correlated with the level of transcription of the gene and the *looseness* of the chromatin structure. Furthermore, when DNA damage was measured in the two strands of the active genes, no strand bias was found, indicating that the global genome repair subpathway of NER may play a crucial role in the repair of these lesions. In the present study, studying a different patient group, we tested the hypothesis that quantification of p53-specific damage formation and repair induced in PBMC by *in vitro* melphalan treatment before therapy correlates with the respective data obtained *in vivo*, i.e. after therapeutic administration of HDM. Furthermore, we studied whether this *in vitro* assay is of value in predicting the clinical outcome after HDM.

Design and Methods

Patients

Between February 2004 and January 2006, 32 consecutive patients were included in this study. Patients were eligible for the *in vitro* and *in vivo* measurements if their physi-

cians considered that it was possible that they would benefit from HDM (200 mg/m^2) and ASCT, and if they had measurable serum and/or urine monoclonal protein at the time of HDM. The latter criterion was included in order to be able to correlate response after HDM with melphalan adduct formation/repair. All patients provided informed consent according to institutional guidelines. The patients' characteristics and disease features are shown in Table 1. All patients were staged according to the International Staging System (ISS) before the initiation of the first-line treatment and prior to HDM. Response and progression were assessed according to the European Bone Marrow Transplantation Group criteria.[26] Nineteen patients received HDM after responding to conventional chemotherapy (first remission), 11 patients received HDM after not having responded to front-line therapy (primary refractory) and in two patients HDM was administered during relapse of MM (relapsed). Thus, 19 patients were considered chemotherapy-sensitive and 13 patients were considered chemotherapy-resistant. None of the patients had previously received alkylating agent therapy (melphalan-naïve patients). Blood samples were obtained from the patients within 1 week prior to ASCT and at least 1 month after exposure to any anti-myeloma treatment. PBMC were isolated from freshly drawn peripheral blood and treated with 10 µg/mL melphalan for 1 h at 37°C.[24] In all experiments cell viability, assessed by trypan blue exclusion, was greater than 95%. After incubation, cell suspensions were cooled on ice and washed twice. For the time-course experiment, cells were resuspended in drug-free medium and incubated at 37°C for 2, 4, 8 and 24 h. Cells were harvested and stored at -70°C until the analysis of DNA adducts. Blood samples were also obtained 0, 2, 8 and 24 h after treatment of the same MM patients with HDM, DNA was extracted and analysis of DNA adducts was performed as described.[25]

Table 1. Characteristics, response to HDM and progression of patients with MM.

Characteristic	*N. of patients (% of total)*
Sex	
Female	14 (43.8%)
Male	18 (56.8%)
Age (years) Median (Range)	59 (23 to 71)
Myeloma type	
G	21 (65.7%)
A	7 (21.9%)
D	1 (3.1%)
Light chain only	3 (9.4%)
ISS at diagnosis	
I	15 (46.9%)
II	8 (25.0%)
III	9 (28.1%)
ISS prior to HDM	
I	25 (78.1%)
II	7 (21.9%)
III	–
Disease status	
First remission	19 (59.4%)
Primary refractory	11 (34.4%)
Relapse untreated	2 (6.3%)
Response to HDM	
Complete remission	9 (28.1%)
Partial remission	14 (43.8%)
Stable disease	1 (3.1%)
Progressive disease	8 (25.0%)
Progression	
No	17 (53.1%)
Yes	15 (46.9%)

Detection of DNA damage

The formation and repair of monoadducts, interstrand cross-links and total adducts (monoadducts and cross-links together) in the p53 gene were measured as described previously.[13,24,25] Briefly, for the measurement of N-alkylpurines (monoadducts and cross-links together), following DNA isolation from PBMC, genomic DNA was digested to completion with Hind III and DNA samples dissolved in sterile deionized H_2O were heated at 70°C for 30 min to depurinate N-alkylated bases. Apurinic sites were converted to single-strand breaks by the addition of NaOH for 30 min at 37°C, size fractionated using agarose gel electrophoresis, Southern blotted and hybridized with the p53-specific probe. For the detection of cross-links, following DNA isolation and restriction enzyme digestion as above, alkylations were not converted to strand breaks and DNA was denatured before gel electrophoresis and Southern blotting. The p53-specific probe was a 610-bp fragment of the human gene and was amplified using the following primer pair: forward, 5'-AGG-TTG-GCT-CTG-ACT-GTA-CC-3'; reverse, 5'-ATT-GTC-CTG-CTT-GCT-TAC-CTC-3'. Three types of molecular end-points were measured: (i) total amounts of each type of DNA damage over time, represented by the area under the curve (AUC) for DNA adducts during the whole experiment (0-24 h), (ii) the peak of each type of DNA adducts, assessed at 2 h for monoadducts as well as total adduct and at 8 h for cross-links, and (iii) the rate of DNA repair, defined as the percentage of decrease of DNA damage from the time of highest DNA damage to 24 h (% repair).

Statistical analysis

All analyses were performed using the STATA statistical software. Frequency distributions were used for descriptive purposes. *In vivo* and *in vitro* values for all molecular end-points are presented as medians with the associated ranges. The comparisons of median values were done with the Wilcoxon Rank sum test. Spearman's correlations coefficient was used to assess correlation of *in vivo* to *in vitro* parameters in the p53 gene. To assess the linear association between *in vivo* and *in vitro* results, linear regression of *in vivo* on *in vitro* parameters was performed. Logistic regression models were used to estimate the impact of *in vitro* parameters on tumor response. The ability to predict response to HDM was compared across the different *in vitro* parameters by calculating the respective receiver operating characteristic (ROC) areas. Specificity, sensitivity, and positive and negative predictive values were estimated for various cut-

off values of the *in vitro* parameters. The associations of the *in vitro* parameters of the p53 gene with time to progression were assessed with proportional hazards (Cox) models. In these models, progression was the focal outcome event whereas the primary variable was time from initiation of HDM until progression. Subjects whose disease had not progressed were considered censored as of the date of the analysis. Survival curves were estimated with the Kaplan-Meier method and compared with the log-rank test.

Results

Clinical outcome after HDM

Thirty-two consecutive MM patients were included in this study. Twenty-three patients (72%, responders) achieved a complete (n=9) or partial response (n=14). Nine patients (28%, non-responders) did not have tumor reduction after HDM. During the follow-up period (median months 15.4; range, 2.4 to 26 months), 15 patients (47%) experienced disease progression and six patients (19%) died.

Increased DNA damage induced by HDM in vivo is individual and associated with clinical outcome

We recently found that the levels of p53-specific damage formation and the individual rates of repair measured in peripheral blood from 26 HDM-treated MM patients varied up to 16-fold.[25] These results were confirmed here in an additional group of different patients. Using total DNA adducts, we found a 15-fold variation in the AUC among patients (range, 29.8-443.4 adducts/10^6 nucleotides x h), a 1.9-fold variation in the peak values (range, 9.9-23.4 adducts/10^6 nucleotides), and a 2.7-fold variation in the % repair of these adducts (range, 22.0-59.9%) (Table 2, columns 2 and 3). Similar results were also obtained for monoadducts and cross-links, indicating that melphalan-induced DNA damage *in vivo* is highly individual (Table 2, columns 2 and 3). Confirming our previous results,[24] significantly greater mean levels of DNA damage and slower mean rates of repair were found in patients who achieved tumor reduction relative to those in non-responding patients. As shown in Table 2 (columns 2 and 3), for all types of adducts, responders to HDM had higher mean peak and AUC values and lower mean rates of repair than non-responders. These differences between responders and non-responders were statistically significant for all DNA damage-related parameters and for all types of DNA adducts analyzed (Table 2, column 4). We also examined whether the individual levels of HDM-

Table 2. ***In vivo*** **and** ***in vitro*** **DNA damage-related parameters, and associated Spearman's correlation coefficients, by category of clinical response**

	Response to HDM						*Spearman's correlation coefficient‡*
	No n=9*	*Yes† n=23*	*p value¶*	*No* n=9*	*Yes† n=23*	*p value¶*	
DNA damage-related parameters	*In vivo*			*In vitro*			
Total adducts: Peak[1]	10.40 (9.94 to 13.80)	13.90 (11.30 to 23.40)	0.0009	40.60 (26.80 to 57.30)	60.70 (31.40 to 80.20)	0.0009	0.91
Total adducts: % repair	50.00 (33.20 to 58.90)	29.90 (22.00 to 59.90)	0.0043	75.20 (70.90 to 84.70)	63.30 (41.90 to 84.10)	0.0007	0.71
Total adducts: AUC[2]	134.24 (29.82 to 243.20)	243.50 (139.39 to 443.40)	0.0009	409.30 (259.40 to 735.80)	921.20 (262.20 to 1441.30)	0.0004	0.93
Cross-links: Peak[1]	0.95 (0.80 to 1.40)	1.5 (1.10 to 2.30)	0.0020	8.00 (5.00 to 15.20)	16.80 (4.80 to 28.20)	0.0021	0.77
Cross-links: % repair	50.00 (33.60 to 65.50)	31.50 (19.90 to 63.60)	0.0015	73.70 (33.70 to 79.20)	59.70 (29.50 to 79.10)	0.0309	0.53
Cross-links: AUC[2]	10.45 (8.80 to 22.60)	27.30 (12.10 to 45.30)	0.0005	126.80 (76.90 to 272.60)	289.70 (77.10 to 548.30)	0.0018	0.81
Monoadducts: Peak[1]	10.40 (9.94 to 13.80)	13.85 (11.30 to 22.00)	0.0029	36.80 (22.30 to 49.20)	49.60 (28.20 to 64.90)	0.0017	0.76
Monoadducts: % repair	50.00 (39.40 to 58.90)	35.40 (25.60 to 58.90)	0.0022	81.50 (76.30 to 90.50)	68.70 (53.40 to 89.90)	0.0011	0.60
Monoadducts: AUC[2]	121.48 (29.82 to 220.60)	219.30 (124.32 to 398.10)	0.0024	302.80 (181.40 to 535.00)	622.90 (194.80 to 932.40)	0.0006	0.86

**Patients with stable disease and progressive disease. †Patients with complete response and partial response. ‡Spearman's correlation coefficient between the in vivo and in vitro values of each of the DNA damage-related parameters. ¶From comparisons (Wilcoxon's rank sum test) between responders and non-responders for all the in vivo and in vitro DNA damage-related parameters [1]adducts/10^6nucleotides. [2]adducts/10^6nucleotides x h.*

induced p53-specific damage formation/repair correlate with progression-free survival (PFS). Patients were arbitrarily divided into two groups according to their individual values of each of the three biological end-points in the p53 gene (Kaplan-Meier curves), that is, patients having values higher or lower than the corresponding *in vivo* mean value of the given biological end-point were analyzed separately. In agreement with our previous study,[25] it was found that patients with greater DNA damage and/or a slower rate of repair in p53 had a longer PFS, with the AUC for total adducts showing the highest predictive ability (p=0.01) (*data not shown*). These results indicate that patients with greater *in vivo* AUC values for DNA adducts during the first 24 h after treatment have a better clinical outcome in the long term.

In order to assess the independent role of the AUC of total DNA adducts on PFS we performed multivariate Cox regression analysis. To increase the power of this analysis, we combined patients from the current study with those analyzed in our previous work,[25] and we assessed the impact of AUC of total *in vivo* induced DNA adducts on time to progression adjusting for the potential effect of age at study entry, type of induction chemotherapy (thalidomide-containing or not) and ISS stage. Patients with values equal to or higher than the respective median value of the AUC of total *in vivo*-induced DNA adducts had a statistically significant reduced rate of tumor progression of 66% compared with that of patients with values lower than the median. The adjusted hazard ratio of tumor progression was 0.34 (95% confidence interval 0.13 to 0.89, p-value 0.029).

In vitro DNA damage formation/repair is individual and correlates with the corresponding *in vivo* data

Following *in vitro* treatment of PBMC with melphalan, a substantial person-to-person variation was found, i.e. a 5.6-fold variation in the AUC for total adducts (AUC-TA; range, 259.40-1441.30 adducts/10^6nucleotides x h), a 3-fold variation in the peak values (range: 26.80-80.20 adducts/10^6 nucleotides), while a 2-fold variation was observed in % adduct repair (range, 41.90-84.70%) (Table 2, columns 5 and 6). Similar results were obtained for monoadducts and cross-links separately, indicating that melphalan-induced DNA damage is also individual *in vitro* (Table 2, columns 5 and 6).

The correlation coefficients between the *in vitro* and *in vivo* DNA adduct formation/repair are presented in the last column of Table 2. All correlations were positive and generally high, with values for the AUC-TA showing the highest correlation coefficient (0.93), indicating that the individual degree of *in vitro* DNA damage formation/repair in PBMC examined before administration of HDM reflects the respective results obtained *in vivo*. This was further confirmed using linear regression models for each of the parameters shown in Table 2, which yielded linear associations of the *in vivo* with the *in vitro* measured values in all cases (Figure 1). This association was strongest for the AUC-TA (R^2=0.83; Figure 1A).

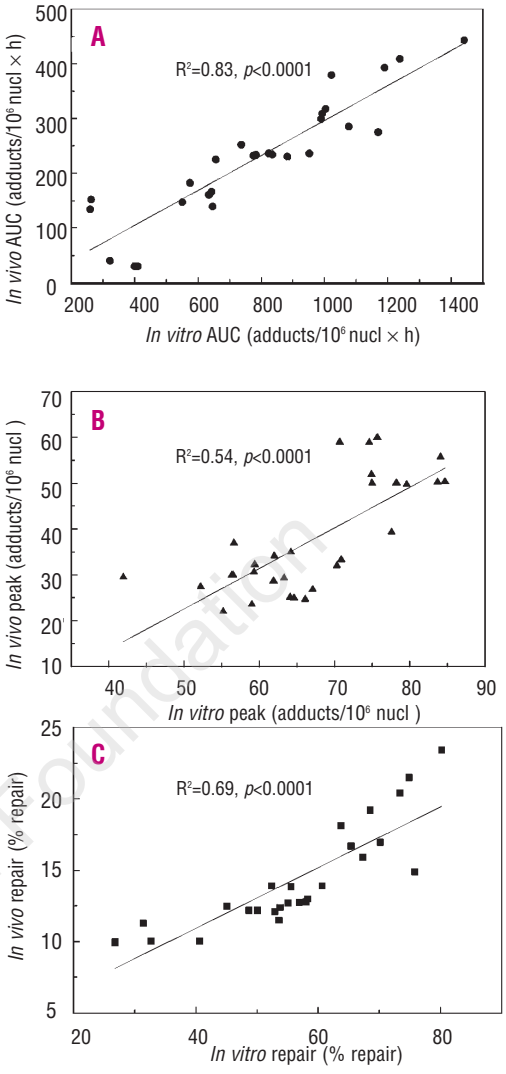

Figure 1. Correlation between *in vitro* and *in vivo* DNA damage formation/repair. Individual p53-specific damage AUC (A), peak (B) and % repair (C) following *in vivo* therapeutic treatment with HDM correlated with the corresponding *in vitro* data in the PBMC from MM patients.

The levels of DNA damage-related parameters measured in vitro are predictive of clinical outcome

As shown in Table 2 (columns 5 and 6), for all types of adducts, responders to HDM had higher *in vitro* mean peak and AUC values and lower mean rates of repair compared to non-responders. These differences were statistically significant for all types of adducts (Table 2, column 7). We also examined various other clinical prognostic parameters such as age, β2 microglobulin level, ISS stage, type of induction chemotherapy (thalidomide containing or not); none of these parameters correlated sig-

Table 3. Odds ratios of response to HDM associated with a 1-unit increase in the *in vitro*-induced DNA damage parameters.

In vitro parameters	*Response* *OR (95% CI)*	*p value*
Total adducts: Peak	1.145 (1.040 to 1.262)	0.006
Total adducts: % repair	0.798 (0.676 to 0.941)	0.007
Total adducts: AUC	1.008 (1.002 to 1.013)	0.006
Cross-links: Peak	1.387 (1.071 to 1.796)	0.013
Cross-links: % repair	0.933 (0.865 to 1.006)	0.073
Cross-links: AUC	1.017 (1.003 to 1.030)	0.014
Monoadducts: Peak	1.181 (1.048 to 1.330)	0.006
Monoadducts: % repair	0.807 (0.687 to 0.948)	0.009
Monoadducts: AUC	1.011 (1.004 to 1.019)	0.004

Table 4. Response to HDM by category of AUC of the *in vitro*-induced total DNA adducts in patients with MM.

Total adducts AUC (adducts/10^6 nucleotides x h)	*Response to HDM* *No* (number of patients)*	*Yes† (number of patients)*	*Total (number of patients*
<736‡§	9	5	14
≥736‡§	0	18	18
Total	9	23	32

**Patients with stable disease and progressive disease. †Patients with complete response and partial response. ‡The cut-off value of 736 adducts/10^6 nucleotides x h was selected based on an analysis of the ROC curve associated with the diagnostic ability of AUC of total adducts with respect to response to HDM. §Based on the cut-off value of 736 adducts/10^6 nucleotides x h, the sensitivity was 78.3%, the specificity was 100%, the positive predictive value was 100% and the negative predictive value was 64.3%.*

nificantly with the levels of DNA damage (*results not shown*). Furthermore, no difference in DNA damage was observed between patients in first remission and those with refractory/relapsed disease. Patients in first remission had similar outcomes, i.e. response to HDM therapy, PFS and overall survival compared to those with refractory/relapsed disease [response rate 76.5% (13/17) versus 63.6% (7/11), p=0.47; median PFS not reached versus 11 months, p=0.34; median overall survival not reached for both groups, p=0.38].

Table 3 shows the odds ratios for the change (increase or decrease) in the probability of responding to HDM for 1-unit increments for all types of adducts and molecular end-points measured following *in vitro* treatment. It can be seen that all parameters reflecting DNA damage were significantly associated with response to HDM, with the exception of percent repair of cross-links, which just failed to reach statistical significance. As expected, the association with clinical response was positive for peak and AUC values of DNA damage and negative for the rate of DNA repair. These associations remained statistically significant when disease status prior to transplant (i.e. first remission versus primary refractory disease), or β^2 microglobulin values were included in the models.

To assess which of the DNA damage-related parameters presented in Table 3 better predicts clinical response, we compared the associated ROC curves, which were estimated for all types of molecular end-points and all types of adducts. The estimated ROC area of AUC-TA was 0.91 (highest), indicating a strong ability of this parameter to predict clinical response to HDM. Based on the estimation of the ROC area of the AUC-TA, we assessed the sensitivity, specificity and the positive and negative predictive values for tumor response for various cut-offs. It was found that an AUC cut-off value of 736 adducts/10^6 nucleotides x h maximized sensitivity and specificity. As shown in Table 4, all patients who did not respond to HDM had values lower than this cut-off (specificity 100%), while all patients with AUC values equal to or greater than the cut-off achieved a complete or partial response (positive predictive value 100%). Some patients (21.7%) achieved tumor reduction although their values for AUC-TA determined *in vitro* were lower than the cut-off (sensitivity 78.3%).

We also compared time to progression across patients with values lower than, equal to or higher than 736 adducts/10^6nucleotides x h for the AUC of total *in vitro*-induced DNA adducts. As depicted in Figure 2, patients with values equal to, or higher than the indicated cut-off value had a longer time to progression than that of patients with lower values (p=0.002). The hazard ratio of tumor progression was 0.19 (95% confidence intervals 0.06 to 0.60), indicating a reduction of 81% in the rate of tumor progression for patients with values equal to or higher than 736 adducts/10^6 nucleotides x h compared to patients with lower values.

Discussion

Among several potentially predictive markers for patients' outcome after HDM, factors associated with tumor burden, such as β^2 microglobulin level, and intrinsic features of the malignant clone, such as cytogenetic abnormalities, have shown the strongest correlation with response and progression.[1] However, parameters associated with the specific mechanisms of action of HDM have not been thoroughly studied.

We recently reported that higher levels of melphalan-induced damage and slower DNA repair in the p53 gene of peripheral leukocytes were closely associated with a positive clinical outcome following HDM[25] and these results were confirmed here in a different group of patients. This observation implies firstly that individual response to HDM therapy is determined primarily by the overall DNA damage burden (initial damage formation and subsequent repair), rather than post-DNA damage responses of the tumor cells, and secondly that interindividual variation in the overall DNA damage burden in blood leukocytes parallels the treatment effect to the tumor tissue.

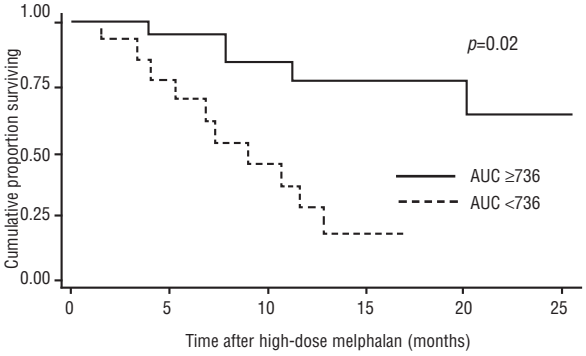

Figure 2. Relationship between the *in vitro* p53-specific damage formation/repair and progression-free survival. Patients are divided into two groups according to the cut-off value of the *in vitro* total adducts AUC (736 adducts/10^6 nucleotides x h).

Given that melphalan brings about its DNA damaging activity directly, i.e. without any intermediate metabolism, it was feasible to evaluate individual susceptibility to accumulate DNA damage by treating blood leukocytes with melphalan *in vitro*. Therefore, in the present study, we examined the hypothesis that formation/repair of DNA adducts in PBMC obtained prior to chemotherapy and treated *in vitro* with melphalan correlates with the corresponding parameters measured in the same patients during chemotherapy, as well as with clinical outcome following HDM. Indeed, a close correlation between DNA damage/repair *in vitro* and *in vivo* was observed, suggesting that no systemic drug detoxifying pathway significantly modulated the ability of melphalan to cause damage to blood leukocytes. Moreover, we found that all molecular end-points indicative of increased DNA damage and slower repair following *in vitro* treatment were predictive of a favorable response to HDM, with the AUC for total adducts (which reflects the overall DNA damage burden resulting from initial damage formation and DNA repair) showing the highest predictive ability. Notably, a strong correlation between the cytotoxicity of melphalan and the total AUC for DNA interstrand cross-links was found in a human melanoma cell line (RPMI 8322), indicating that both the initial induction as well as the rate of removal of DNA adducts are important for the cytotoxic effects of bifunctional alkylating agents.[27] Assuming that the cytotoxicity of the drug is caused by the inactivation of DNA as a template for DNA synthesis by the presence of DNA damage, it is not yet known what amount of DNA adducts is required to kill normal or neoplastic human cells. Indeed, it is not known whether cell lethality is simply a question of the quantity of DNA adducts or whether a subfraction of these lesions, by virtue of a specific strategic location in the genome and/or by causing a structural perturbation in the chromatin structure, cause cell death. Whichever is the case, the AUC for DNA adducts induced by melphalan is likely to reflect the proportion of cells that attempt DNA synthesis while carrying a lethal amount of DNA damage.

Using a cut-off value of AUC for *in vitro*-induced total adducts of 736 adducts/10^6 nucleotides x h, all patients with values equal to, or higher than this value were found to achieve a complete or partial response after HDM treatment (positive predictive value 100%) and to have a longer time to progression relative to patients with values lower than this cut-off (p=0.002). The effect of the AUC (in an analysis of *in vivo*-induced total adducts) remained statistically significant even when age, type of induction chemotherapy and ISS stage were further adjusted for. This strongly points to a high level of DNA damage being the dominant factor determining response to therapy. On the other hand, five out of 14 patients with an AUC below the threshold had a complete or partial response (sensitivity 78.3%). There are various possible explanations for this, e.g. DNA damage in tumor cells may have been greater than implied by the levels found in blood leukocytes, or at relatively low levels of DNA damage, cellular responses other than DNA repair may be important in determining individual response to therapy. Further studies to clarify these issues may help to improve sensitivity.

The Southern blot methodology[28] was successfully used for the measurement of gene-specific damage formation and repair in the present study. Although this method can be used as a routine predictive test for many patients, even more simple assays, suitable for routine clinical use, such as quantitative polymerase chain reaction and the single cell gel electrophoresis (comet) assay are currently validated in our laboratory.[19,29]

To conclude, we found that the extent of p53-specific damage formation/repair in PBMC from MM patients following *in vitro* exposure to melphalan correlates with the respective results obtained *in vivo*, i.e. outcome after treatment with HDM, and is of value in predicting clinical response and PFS. Thus, measurement of the individual levels of DNA damage induced *in vitro* after treatment with melphalan of PBMC collected before chemotherapy may help in the selection of patients more likely to benefit from HDM.

Authors' contributions

MAD, VLS, AA, CB, AP, IB, ET, SAK, PPS: conception and design of the study, analysis and interpretation of data; drafting the manuscript; final approval of the version to be published.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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