Development and validation of a PCR-based assay for the selection of patients more likely to benefit from therapeutic treatment with alkylating drugs

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Previous studies have indicated that the levels of DNA damage induced in peripheral blood mononuclear cells by the alkylating drugs melphalan, cisplatin and carboplatin can serve as useful biomarkers predictive of the therapeutic response of cancer patients to these drugs.

WHAT THIS STUDY ADDS

- In the present study we developed a quantitative PCR-based assay, for the measurement of DNA damage. The advantages of this methodology are based on:
 - its far greater sensitivity (about 250 times) than the traditional Southern blot-based method (the detection limit is ~10–20 lesions/10⁶ nucleotides from the equivalent DNA of ~8000 cells),
 - its simplicity and speed (results obtained within ~8h),
 - its excellent reproducibility, with a coefficient of variance of 10–15% for different DNA preparations from similarly treated cells,
 - its requirement for only minute amounts of material, and
 - the avoidance of radioisotope labeling.
- Moreover, emphasis was given to translate basic research findings into clinical practice through the validation of this assay for prediction of clinical outcome in multiple myeloma patients.

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AIM

In order to develop and validate a simple, sensitive and rapid method for the quantitation of alkylating drug-induced DNA damage.

METHODS

HepG2 cells and blood samples were treated with alkylating drugs (melphalan, cisplatin, carboplatin). Gene-specific damage was examined using Southern blot and a multiplex long quantitative PCR (QPCR) carried out in a 7 kb fragment (part of the p53 gene) and a 0.5 kb fragment (part of the IFN-β1 sequence; internal standard).

RESULTS

The extent of PCR amplification of a p53 fragment was inversely proportional to the treatment concentrations of all anticancer drugs examined, indicating a dose-related inhibition by the DNA adducts formed. Parallel analysis of the same samples using both Southern blot and QPCR showed that the DNA adducts measured by QPCR corresponded to the interstrand cross-links in the case of melphalan, and to total drug-induced lesions in the case of the platinum drugs. The detection limit was ~10–20 lesions/10⁶ nucleotides using DNA from ~8000 cells. The method is about 250 times more sensitive than the Southern blot-based method and the reproducibility is excellent, with an intraday coefficient of variance (CV) of 5–9% and an interday CV of 4–12%. Application of the QPCR assay to *ex vivo* melphalan-treated peripheral blood mononuclear cells from multiple myeloma patients, showed that the positive predictive value of this assay for clinical response to melphalan therapy was 92.9%.

CONCLUSION

The PCR-based assay developed in this study can be used for the selection of cancer patients more likely to benefit from therapeutic treatment with alkylating drugs.

Introduction

DNA repair plays an essential role in the protection of cells following exposure to genotoxic agents, including DNA alkylating agents used in cancer chemotherapy. Previous investigations have found substantial variation in the levels of DNA damage and its repair in cancer patients treated with alkylating drugs, including widely used nitrogen mustards and platinum drugs [1–3]. In many cases [1–6], although not always [7], the levels of drug-induced DNA damage have been found to correlate well (inversely) with patient therapeutic response, suggesting that they may serve as clinically useful biomarkers. In particular, we have found significantly slower repair (and correspondingly higher accumulated levels) of DNA damage in peripheral blood mononuclear cells (PBMCs) induced by the nitrogen mustard melphalan in multiple myeloma (MM) patients who responded better (with tumour reduction and longer progression-free survival) to this drug [3, 8]. Based on this finding, we went on to show that the levels of DNA damage in PBMCs from MM patients following *ex vivo* treatment with melphalan (i) correlated closely with those found after therapeutic (*in vivo*) treatment of patients and (ii) could distinguish between patients with different degrees of therapeutic response, thus providing the basis for pre-screening and selection of those patients more likely to benefit from treatment [6].

Alkylating agents are the oldest class of anticancer drugs and are still commonly used [9]. They include monofunctional methylating agents (e.g. temozolomide, procarbazine, dacarbazine), bifunctional alkylating agents such as nitrogen mustards (e.g. melphalan, cyclophosphamide) or chloroethylating agents (e.g. carmustine, fotemustine) as well as platinum-based drugs (e.g. cis- and carboplatin). Melphalan {4-[bis(2-chloroethyl)amino]-L-phenylalanine} combined with autologous stem cell transplantation (ASCT) is currently the standard treatment of patients with MM, an incurable but treatable plasma cell malignancy which is the second most commonly diagnosed haematological malignancy [10, 11]. Melphalan reacts with DNA, producing mostly N-alkylpurine monoadducts, a small proportion of which goes on to form interstrand cross-links (ICL) [12–14]. Platinum drugs are used extensively in the treatment of ovarian, cervical, head and neck as well as non-small-cell lung cancer [15]. The most common platinum-based compounds in clinical use are cisplatin [cis-diamminedichloroplatinum(II)] and carboplatin [cis-diammine(cyclobutane-1,1-dicarboxylato)-platinum(II)]. The DNA lesions generated by these compounds include intra- and inter-strand cross-links and monofunctional adducts [16]. Nucleotide excision repair is the main mechanism for removing melphalan- and platinum-induced intrastrand cross-links and monofunctional adducts, whereas homologous recombination appears to play a more prominent role in the repair of interstrand cross-links.

In our previous studies with melphalan, mentioned above, DNA adduct levels were measured in specific genomic regions using a method based on Southern blot analysis [3, 6, 8]. This method involves the generation of restriction fragments of genomic DNA containing the region of interest, conversion of DNA adducts contained within them into DNA strand breaks and quantitation of the intact fragments remaining thereafter. Using this methodology, in addition to the correlations with clinical response described above, we also observed substantial variation in the rate of DNA repair (and the corresponding levels of DNA damage) between different genomic regions, whose clinical significance is yet to be fully evaluated [17]. Although very sensitive, Southern blot analysis is complex and time-consuming. In addition, it requires radioactive (^{32}P) isotope labelling as well as large amounts of high quality genomic DNA and depends on the presence of suitable restriction enzyme recognition sites within the region of interest. Finally, in order to use the method for assaying DNA damage induced by different types of agents, the availability of methodology for converting DNA adducts into strand breaks (e.g. lesion-specific, incision-generating repair enzymes) is also a prerequisite. Therefore, a more rapid and convenient methodology of wider applicability, suitable for application in a clinical context, is highly desirable.

The ability of certain types of bulky DNA lesions (including lesions produced by common chemotherapeutic alkylating agents) to block DNA replication by Taq polymerase forms the basis of an alternative, quantitative PCR (QPCR-block) assay for measuring the levels of such DNA adducts [18]. Although theoretically a single Taq-blocking lesion should be sufficient to prevent completion of DNA synthesis along a given DNA fragment, the sensitivity of such an assay depends on the ratio of adducts per length of the PCR fragment and in practice requires relatively long fragments for high sensitivity. Recent advances in PCR allow the generation of PCR products in the range of 6–24 kb, thus opening the way to the development of sensitive methods to measure DNA damage at specific loci [19]. This methodology has been previously employed to study the repair of u.v.-induced photoproducts in different genomic regions, while its general utility has been demonstrated by measuring the production of cisplatin-DNA adducts [19–24].

We now present the development and clinical application of a simple and rapid (results obtained within ~8 h) QPCR-block method for measuring the levels of DNA damage induced in PBMCs by three anticancer drugs, melphalan, cisplatin and carboplatin. Application of this assay to *ex vivo*-treated human blood samples from MM patients taken prior to treatment with melphalan showed significantly greater damage in the p53 gene locus in responders relative to non-responders, demonstrating the utility of this methodology for the selection of patients more likely to benefit from therapeutic treatment.

Methods

Patients

Blood samples were obtained from 22 consecutive MM patients on the day of stem cell mobilization and at least 1 month after any anti-myeloma treatment. Fifteen patients (responders) achieved a complete ($n=6$) or partial ($n=9$) response. Seven patients (non-responders) did not have tumour reduction after high dose melphalan treatment. Blood samples from eight healthy donors were also used. The study has been subject to Institutional Body Review ('Alexandra' Hospital) and all subjects provided informed consent according to the Declaration of Helsinki. Response and progression were assessed according to the European Bone Marrow Transplantation Group criteria [25]. None of the patients had previously received alkylating agent therapy.

Cytotoxicity assay

Following drug treatment, viable cells (HepG2, PBMCs) were counted by trypan blue dye-exclusion [26]. Briefly, following incubation with the appropriate concentrations of the drug, cells were washed and resuspended in complete medium. An equal volume of 0.4% trypan blue reagent was added to the cell suspension and the percentage of viable cells was evaluated. Assays were performed in triplicate.

Cell treatment

In the case of HepG2, cells were treated with melphalan ($0-1000 \mu\text{g ml}^{-1}$) for 1 h at 37°C . In some experiments, cells were treated with the monofunctional derivative of melphalan (monohydroxymelphalan) for 1 h at 37°C . In platinum drug experiments, a range of concentrations of cisplatin ($0-50 \mu\text{g ml}^{-1}$) for 3 h or carboplatin ($0-300 \mu\text{g ml}^{-1}$) for 24 h were used. Following incubation, the cells were pelleted, washed twice with ice-cold sterile PBS and frozen for later DNA preparations.

Turning to the treatment of human samples, fresh whole blood samples were treated with various doses of melphalan ($0-600 \mu\text{g ml}^{-1}$) for 1 h at 37°C , PBMCs were isolated using standard methods [27], DNA was extracted and analysis of DNA adducts was performed using the QPCR-block assay. In other experiments, PBMCs were isolated from freshly drawn peripheral blood and subsequently treated with melphalan ($0-600 \mu\text{g ml}^{-1}$ for 1 h), cisplatin ($0-150 \mu\text{g ml}^{-1}$ for 3 h) or carboplatin ($0-1400 \mu\text{g ml}^{-1}$ for 24 h). Following incubation, the cells were pelleted, washed twice with ice-cold sterile PBS and frozen for later DNA preparations.

Preparation of genomic DNA

High molecular weight DNA was isolated using the Gentra DNA isolation kit (Qiagen, Chatsworth, CA), as described by the manufacturer.

Southern blot analysis

The measurement of melphalan-induced DNA damage (monoadducts and interstrand cross-links) was performed as described previously [8]. For the measurement of platinum-induced ICL, the DNA was digested with HindIII for 1 h at 37°C , size-fractionated by gel electrophoresis, Southern blotted, hybridized with appropriate probes and the intensity of relevant signals measured by densitometry [8]. For the measurement of platinum-induced total adducts, DNA was digested with the same restriction enzyme and treated with ABC excinuclease at 37°C for 15 min as described previously [28]. The DNA was subsequently subjected to gel electrophoresis and Southern blotted followed by measurement of signal intensity. Due to the fact that ABC excinuclease incised the damaged DNA with only about 30% efficiency in both gene sequences, the number of frequency of total adducts was multiplied by a factor of 2.5–3.3 [28].

Multiplex QPCR assay

Measurement of DNA adducts using multiplex QPCR was performed by an adaptation of a previously reported method [29]. As target sequence for the measurement of DNA damage we used a 7-kb fragment of the p53 gene, while a short (500 bp) fragment of the IFNb1 gene served as internal control. All amplification reactions were carried out in a volume of 50 μl . Each reaction consisted of 100 ng of high molecular weight DNA, primers to amplify the p53 gene fragment ($0.4 \mu\text{M}$ each) and the IFNb1 sequence fragment ($0.4 \mu\text{M}$ each) and deoxynucleotides (0.1 mM each; Invitrogen). Also, 2.5 units of Taq DNA polymerase (DyNAzyme EXT DNA polymerase, Finnzymes) and the supplied buffer were used for amplification. The primer nucleotide sequences were as follows: for the p53 gene, from exon 4 to exon 11, sense $5'-\text{TGAGGACCTGGTCCTC TGAC}-3'$ and antisense $5'-\text{TGACGCACACCTATTGCAAG}-3'$, for the IFNb1 sequence, sense $5'-\text{ATGAGCTACAACCTTGCT TGGA}-3'$ and antisense $5'-\text{TCAGTTTCGGAGGTAACCTGT}-3'$. The cycling profile for the p53 gene and IFNb1 sequence in order to amplify together was an initial denaturation at 95°C for 4 min, 30 cycles at 94°C for 30 s, at 65°C for 30 s, at 67°C for 7 min and a final extension for 10 min at 72°C . The QPCR assay was performed in a MJ Research PTC-200 Peltier thermal cycler. Then, 5 μl of PCR products were electrophoresed in $0.5 \times$ Tris-borate-EDTA buffer on a 1.5% agarose gel, and the gel was stained with ethidium bromide and visualized under u.v. illumination for quantitative analysis.

Lysate QPCR

One million cells per dose or time point were pelleted, washed and lysed by being resuspended in 500 μl of K-buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 0.5% Tween-20, 100 $\mu\text{g ml}^{-1}$ proteinase K, 40 $\mu\text{g ml}^{-1}$ RNase A and 0.2 U ml^{-1} RNaseT1), followed by incubation at 55°C

for 1 h and then heating at 94°C for 10 min to inactivate proteinase K, as previously described [30]. Cell lysates thus prepared were stored at –20°C until use. Multiplex QPCR using these lysates was carried out as described above with the following minor modifications: primers to amplify the 7-kb fragment of the p53 gene were at 1 µM each (instead of 0.4 µM), primers to amplify the 500-bp fragment of the IFNβ1 sequence were at 0.2 µM each (instead of 0.4 µM) and 3 units (instead of 2.5 units) of Taq DNA polymerase were used. A typical 50 µl reaction mixture contained 4 µl of cell lysate, primers, deoxynucleotides, Taq DNA polymerase and the supplied buffer used for amplification. The cycling profile for the p53 gene and IFNβ1 sequence was the same as with the multiplex QPCR assay described above.

To quantify the amount of DNA damage using the multiplex QPCR or lysate QPCR assays, the amounts of PCR products were measured with Image Quant 5.2 software. The amount of amplified product of the target p53 gene was normalized relative to the co-amplified template concentration control (IFNβ1 sequence). This normalized value of each treated sample was divided by the normalized value of the amplified product from control (not damaged) DNA to give the fraction of non-damaged templates at a given dose of DNA damaging agent (the zero class). Based on the assumption that the lesions were distributed randomly, the Poisson equation was used to calculate the lesion frequency per strand: $S = -\ln(Ad/A0)$, where S = lesion frequency/strand, $A0$ = absorbance units produced from a given amount of non-damaged DNA template and Ad = absorbance unit produced from a given amount of damaged DNA template (damaged by a particular dose of drug), so that $Ad/A0$ = fraction of non-damaged template at a given dose.

Preparation of monohydroxymelphalan

Preparation of the monofunctional derivative of melphalan was performed as previously described [31]. Briefly, melphalan (Sigma) was partially hydrolyzed by incubation in HCl and reaction products were separated by chromatography using a C18 Sep-pak cartridge (Vac 35 cm³, 10g capacity, from Waters, Millipore Corp).

Statistics

Spearman's correlation coefficient was used to assess correlation between cisplatin- and carboplatin-induced adduct levels in the p53 gene. To assess the linear association between the cisplatin- and the carboplatin-induced adduct levels, linear regression analysis was performed. The ability of melphalan-induced adduct levels in the p53 gene to predict clinical response to melphalan therapy was assessed by calculating the respective receiver operating characteristic (ROC) area. Specificity and sensitivity were estimated for different cut-off values of melphalan-induced adduct levels. The Student's *t*-test as well as the

paired *t*-test were also used to compare mean values of different groups. Statistical significance was assumed when $P < 0.05$.

Results

Cytotoxicity

The inhibition of the growth of HepG2 and PBMC cells following exposure to melphalan or platinum drugs (cisplatin and carboplatin) was examined using the trypan blue dye-exclusion method. Both HepG2 and PBMC cell viabilities were found to be over 95% at all drug doses used and at all time points analyzed.

Development and optimization of the multiple QPCR assay

Initial experiments were carried out using 10–500 ng of non-damaged DNA during 20–35 cycles of PCR. As already stated, the target sequence for analysis of DNA damage was a relatively long (7-kb) fragment of the p53 gene, while a short fragment (500 bp) of the IFNβ1 sequence, which owing to its small size was expected to remain largely free of DNA damage, served as an internal control for PCR efficiency. The amplified products were separated by electrophoresis and the gel was stained with ethidium bromide followed by densitometric analysis. The amplification signal increased linearly for both the p53 and the IFNβ1 sequences, during 25–32 cycles of amplification (Figure 1A, C). Beyond 35 cycles the amplification of both fragments was no longer proportional to the amount of input template concentration. Moreover, for both fragments, a linear increase of the amplification signal was found when the amount of the DNA template was 25–200 ng (Figure 1B, D). Based on these results, subsequent experiments were performed using 100 ng DNA and 30 cycles of amplification.

Subsequently, the sensitivity and reproducibility of this multiplex long QPCR assay was examined in relation to the measurement of melphalan-induced DNA damage. Thus, melphalan damage formation was measured after treatment of HepG2 cells with various doses of melphalan (0–600 µg ml^{–1}) for 1 h at 37°C. The fraction of fragments bearing one or more damaged nucleotides was reflected in a reduction in the amount of amplified product using the QPCR (Figure 1G). Notably, the adduct levels measured at the end of the 1 h treatment with melphalan, represent the residual amounts of adducts present in DNA at the time of sampling and which were not repaired within the limited time period since the beginning of the treatment.

In order to clarify the type of melphalan adducts (total adducts, monoadducts, interstrand cross-links) measured by QPCR, DNA damage in the p53 gene of HepG2 cells was measured by both multiplex QPCR and Southern blot analysis. Comparison of the results obtained by the two methods showed that the adduct levels measured by

Figure 1

Development and optimization of the multiple QPCR assay. The range of cycles (A, C) and the initial concentration of DNA (B, D) that could provide quantitative amplification for each target during PCR. The amplified products were then separated by electrophoresis and the gels were stained with ethidium bromide (C, D) followed by densitometric analysis. Representative autoradiograms showing melphalan-induced total adducts (E) and interstrand cross-links (F) following Southern blot analysis. The intensity of relevant signals was measured by densitometry. DS, double-stranded DNA; SS, single-stranded DNA. (G) Following exposure of HepG2 cells to various doses of melphalan, the PCR-amplified products were separated by electrophoresis and the gel was stained with ethidium bromide followed by densitometric analysis. The data shown are based on two independent experiments with at least three analyses each. 7-kb fragment (●); 0.5-kb fragment (▲)

QPCR were close to those of interstrand cross-links levels measured by Southern blot analysis and much lower than those of monoadducts, indicating that the melphalan adducts measured by QPCR correspond to ICL (Figures 1E, F, 2A).

In order to elucidate further the type of adducts measured by QPCR, a monofunctional derivative of melphalan

(mono-hydroxy-melphalan) was prepared by partial hydrolysis of melphalan and purification by reversed phase chromatography. Following treatment of HepG2 cells with this agent, measurement of DNA damage using both QPCR and Southern blot confirmed that only monoadducts, and no ICL, were induced by the mono-hydroxy-melphalan (Figure 2B). Moreover, no adducts were detected using

Figure 2

Measurement of DNA damage in the p53 gene of HepG2 cells. Parallel analyses of the same samples using multiplex QPCR, lysate QPCR and Southern blot following treatment with various doses of (A) melphalan, (B) mono-hydroxy-melphalan, (C) cisplatin and (D) carboplatin. Experiments were performed in triplicate. (A) monoadducts (●); ICL (△); QPCR (▼); (B) monoadducts/Southern blot (●); ICL/Southern blot (▲); adducts/QPCR (▼); (C) QPCR (○); total adducts (southern) (▲); ICL (southern) (▼); lysate QPCR (□); (D) total adducts (southern) (▲); ICL (southern) (▼); QPCR (○); lysate QPCR (□)

the QPCR assay, confirming that this method detects only ICL.

Following the establishment of the multiplex QPCR assay for measuring melphalan-induced DNA damage as described above, the ability of the same assay to measure the levels of DNA damage induced by platinum drugs (known to induce intra- and inter-strand crosslinks as well as a small proportion of monoadducts) was evaluated. Thus, platinum-induced damage formation was measured after treatment of HepG2 cells with various doses of cisplatin (0–50 μg ml⁻¹) for 3 h at 37°C (Figure 2C) or 0–296 μg ml⁻¹ carboplatin for 24 h at 37°C (Figure 2D). A linear, dose-dependent increase in the amplification signal was obtained, indicating that this QPCR-block method can be used for the quantitative measurement of DNA damage induced by cisplatin and carboplatin. In accordance with previous studies [32], carboplatin was found to be less

reactive than cisplatin since higher concentrations of carboplatin are required to achieve the same amounts of DNA adducts (Figure 2C,D). Moreover, the type of platinum adducts measured by the QPCR assay was determined by analyzing the DNA damage induced in the p53 gene of HepG2 cells treated with cisplatin or carboplatin using both the Southern blot and the QPCR assay. These studies showed that the adduct levels measured by QPCR were almost identical to the total adduct levels measured by Southern blot, indicating that the QPCR-based assay measures the sum of DNA lesions induced by platinum drugs (i.e. monoadducts, intra- and inter-strand cross-links) (Figure 2C, D).

Once quantitative conditions were established, the intraday as well as the interday coefficient of variation (CV) were determined. The intraday CV was estimated by performing five repeated analyses of the same quality control

samples (three samples) in the same day and was found to be 5–9%. Also, the interday CV was assessed over 3 consecutive days with the same sets of samples and was found to be 4–12%.

Lysate QPCR

In addition to the QPCR assay described above, a simplified version of the assay was developed and validated which can facilitate its application in clinical settings, named lysate QPCR. It is based on the same principles as the multiplex QPCR assay described above, but involves no isolation or quantitation of DNA. Instead, isolated cells are lysed into the appropriate buffer using proteinase K and the resulting lysates were analyzed directly using the QPCR assay.

Initial experiments were designed to determine the sensitivity and reproducibility of the lysate QPCR assay. Thus, non-treated HepG2 cells were lysed and various quantities (1–10 μ l) of cell lysate were titrated using QPCR. The amplification signal was found to be linearly related to the amount of template between 1 and 4 μ l, and reached a plateau when more than 4 μ l of cell lysate were used (data not shown). Based on these results, subsequent experiments were performed using 4 μ l of lysate and 30 cycles of amplification. Parallel analyses of the same samples using multiplex QPCR and lysate QPCR gave almost identical adduct levels, indicating that the lysate-based multiplex QPCR assay can substitute the multiplex QPCR described above (Figure 2C, D).

Application of the QPCR assay to blood samples treated ex vivo with melphalan and its use to predict patient response

In our previous studies, Southern blot analysis was successfully used for the measurement of locus-specific damage in the context of evaluating its usefulness in predicting individual response to therapy [3, 6]. Although this method can be used as a routine predictive test, there is a need for a simpler and more rapid assay, suitable for routine clinical use, such as QPCR. In order to evaluate the utility of QPCR for this purpose, samples of whole blood were collected from eight healthy donors (four males/four females), as well as from 22 patients (15 males/seven females) with MM undergoing HDM with ASCT as part of their first line therapy. The blood samples were collected from these patients prior to melphalan treatment, on the day of stem cell mobilization. Following therapeutic treatment, 15 of these patients (responders) achieved myeloma reduction with six achieving complete response (CR) and nine partial response (PR). Seven patients (non-responders) did not have tumour reduction following treatment with melphalan.

Two independent experiments were performed on these blood samples. In the first experiment, blood samples were collected, PBMCs were isolated, treated with melphalan (400, 500 and 600 μ g ml^{-1}) for 1 h at 37°C and subsequently analyzed by the lysate QPCR. Then, the

DNA adduct levels were adjusted to 1 mg ml^{-1} melphalan and from each individual the mean value was calculated. In accordance with our previous studies using Southern blot analysis [3, 6], the levels of DNA damage thus measured varied up to 12-fold, a variation which probably reflects mainly inter-individual differences in DNA repair. Significantly greater p53-specific damage was found in the responders group compared with that of non-responders [mean value 176.8 ± 67.3 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan), range 41.0 to 273.0 for responders and 65.1 ± 39.4 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan), range 22.0 to 135.0 for non-responders, $P = 0.004$] (Figure 3A). Adduct levels in healthy donors appear to be slightly higher than those of responders ($P = 0.561$), while they are significantly higher than those of non-responders ($P = 0.008$).

In the other experiment, fresh whole blood samples were treated with various doses of melphalan (400, 500 and 600 μ g ml^{-1}) for 1 h at 37°C, PBMCs were isolated and analysis of DNA adducts was performed using the lysate QPCR assay. The DNA adduct levels were adjusted to 1 mg ml^{-1} melphalan and from each individual the mean value was calculated. Again, greater p53-specific damage was found in the responders group compared with that of non-responders, although the difference did not reach statistical significance [mean value 110.8 ± 66.5 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan), range 35.0 to 242.0 for responders and 47.0 ± 2.1 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan), range 45.0 to 49.0 for non-responders, $P = 0.141$] (Figure 3B). Also, healthy donors showed higher levels of DNA damage [mean value 160.5 ± 70.0 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan), range 102.0–245.0] than responders ($P = 0.246$) and non-responders ($P = 0.041$).

No correlation was found between PBMC-treated and whole blood-treated data from the same individuals (Figure 3C). Interestingly, the levels of DNA damage following treatment of isolated PBMCs with melphalan are higher ($P = 0.0475$) than those obtained after treatment of the whole blood with this drug [mean value 165.4 ± 80.8 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan) for PBMC-treated and 112.3 ± 68.4 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan) for whole blood treatment]. The dotted line in the Figure 3C represents the theoretical line for a perfect correlation between the two sets of data. Most of the points are on or under the dotted line, confirming that the levels of DNA damage measured following treatment of isolated PBMCs with melphalan are higher than those obtained after treatment of the whole blood with this drug.

Based on the ROC analysis, we estimated a cut-off value for the number of adducts, in order to distinguish between patients responders and non-responders to melphalan therapy and assessed the sensitivity, specificity and the positive and negative predictive values for tumour response for this cut-off. In the PBMC-treated samples,

Figure 3

Application of the lysate QPCR assay to blood samples following *ex vivo* melphalan treatment. Box plots showing statistical distribution of DNA damage levels after whole blood (A) and isolated PBMC (B) treatment with various doses of melphalan. The horizontal line within the box represents the mean value and the vertical lines extending above and below the box indicate maximum and minimum values, respectively. (C) Correlation between PBMC-treated and whole blood-treated DNA damage levels (adjusted to 1 mg ml^{-1}) from the same individuals. The dotted line represents the theoretical line for a perfect correlation between the two sets of data. Assays were performed in triplicate

it was found that a cut-off value of 101 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan) maximized sensitivity and specificity. That is, six out of seven patients who did not respond to melphalan therapy had values lower than this cut-off (specificity 85.71%), while 13 out of 14 patients with values equal to or greater than the cut-off achieved a complete or partial response (positive predictive value 92.9%). Some patients (13.33%) achieved tumour reduction although their DNA adduct levels were lower than the cut-off (sensitivity 86.67%). As for the whole blood-treated samples, the cut-off value of 56 (adducts/ 10^6 nucleotides)/(mg ml^{-1} melphalan) maximized sensitivity and specificity. That is, all patients who did not respond to melphalan therapy had values lower than this cut-off (specificity 100%), and all patients with values equal to or greater than the cut-off achieved a complete or partial response (positive predictive value 100%). Some patients (11.11%) achieved tumour reduction although their DNA adduct levels were lower than the cut-off (sensitivity 88.89%).

Application of the QPCR assay to blood samples treated *ex vivo* with platinum drugs

Turning to the platinum drugs, isolated PBMCs from 10 healthy volunteers (25–45-year-old women) were treated with cisplatin (50, 100, $150 \mu\text{g ml}^{-1}$) for 3 h or carboplatin (1000, 1200, $1400 \mu\text{g ml}^{-1}$) for 24 h. Following drug treatment, DNA damage levels were measured using the lysate QPCR assay. The DNA adduct levels were adjusted to $100 \mu\text{g ml}^{-1}$ platinum drug and from each individual the mean value was calculated. A dose-dependent increase in the drug-induced DNA damage was observed in all subjects and with both drugs (Figure 4A, B). The Spearman correlation coefficient between the cisplatin- and carboplatin-induced adduct levels in samples from the same individuals was calculated. The correlation was positive and high (correlation coefficient, 0.855, P value 0.0016). This was further confirmed using a linear regression model, which yielded linear association of the cisplatin- and carboplatin-induced DNA adduct levels (Figure 4D, $r^2 = 0.68$, $P = 0.0033$), indicating that the individual levels of cisplatin DNA damage in PBMCs reflect the corresponding data obtained following exposure to carboplatin. Moreover, carboplatin was found to be less reactive than cisplatin, since ~ 12.5 -fold higher concentrations of carboplatin are required to achieve the same levels of DNA adducts [mean value 117.8 ± 50.7 (adducts/ 10^6 nucleotides)/($100 \mu\text{g ml}^{-1}$), range 53.2–207.4 for cisplatin and 9.0 ± 3.3 (adducts/ 10^6 nucleotides)/($100 \mu\text{g ml}^{-1}$), range 4.5–15.0] (Figure 4D).

Discussion

The optimization of the use of existing chemotherapeutic agents is highly desirable for therapeutic and cost-effectiveness reasons as well as for providing a rational

Figure 4

Application of the lysate QPCR assay to blood samples following *ex vivo* platinum drugs treatment. PBMCs taken from healthy volunteers were *ex vivo* treated with various doses of (A) cisplatin or (B) carboplatin and DNA damage levels were measured using lysate QPCR assay. (C) Box plots showing statistical distribution of DNA damage levels (adjusted to $100 \mu\text{g ml}^{-1}$) after cisplatin or carboplatin treatment. The horizontal line within the box represents the mean value and the vertical lines extending above and below the box indicate maximum and minimum values, respectively. (D) Correlation between cisplatin and carboplatin data in PBMCs from the same individuals. Assays were performed in triplicate. (A) $50 \mu\text{g ml}^{-1}$ (■); $100 \mu\text{g ml}^{-1}$ (□); $150 \mu\text{g ml}^{-1}$ (▨); (B) $1000 \mu\text{g ml}^{-1}$ (■); $1200 \mu\text{g ml}^{-1}$ (□); $1400 \mu\text{g ml}^{-1}$ (▨)

basis for further drug development. The design of chemotherapeutic protocols is still based on empirical observations such as the overall response rate in a given group of patients. On the other hand, evidence suggests that inter-individual heterogeneity in the expression of DNA repair pathways may be exploited to design individualized therapeutic protocols in a rational way based on scientific data. Multiple studies have aimed to evaluate the impact of repair capacity in the context of cancer patients' response to chemotherapy with alkylating drugs. Although reduced DNA repair capacity is undesirable from a cancer risk perspective, reduced tumoural DNA repair capacity can be exploited therapeutically with DNA-damaging therapeutics. That is, following treatment with DNA alkylating drugs, reduced DNA repair efficiency can result in the accumulation of increased amounts of DNA damage in tumour cells and the induction of cell death, which is the desired goal in

cancer chemotherapy. By understanding the relationship between DNA damage/repair on one hand and chemotherapeutic response/drug resistance on the other, better selectivity of therapy may be achieved. Our previous studies have shown a clear correlation between the ability of PBMCs taken from MM patients to repair melphalan-induced DNA damage and patient response to therapeutic treatment with the same drug [3, 6]. Those studies made use of a Southern blot-based assay to measure repair of DNA lesions in specific chromosomal loci. That assay requires large amounts of DNA and radioisotope labeling, depends on the presence of suitable restriction enzyme recognition sites within the region of interest and is time-consuming, characteristics which complicate its use in clinical settings.

On the other hand, quantitative PCR can serve as a tool for measuring the levels, at specific loci, of DNA damage

which blocks DNA polymerase [33]. Previous studies have shown that QPCR can be used for the measurement of DNA damage induced by various genotoxic agents. Thus, following treatment of cells with cisplatin, ultraviolet light (UV), nitrogen and quinacrine mustards as well as with hydrogen peroxide, quantitation of DNA damage formation and repair could be achieved using QPCR [19–24]. In order to enhance the sensitivity of the assay sufficiently for it to be useful for the analysis of clinical samples, the present study made use of a long QPCR assay which amplifies a 7-kb fragment containing exons 4 to 11 of the target p53 gene to measure DNA damage produced *ex vivo* by pharmacologically relevant doses of the drugs of interest. Furthermore, in order to overcome concerns regarding potential variations in the efficiency of amplification in different DNA preparations, co-amplification of a small DNA fragment acting as a control was included in a multiplex PCR assay. In the current protocol, a 500-bp fragment part of the IFNb1 gene was co-amplified with the main fragments under investigation.

The success of the QPCR assay for the quantitation of DNA damage relies on amplification yields being directly proportional to the starting amount of DNA template, a condition most easily met by keeping the PCR in the exponential phase. Thus, in the present study, experiments were designed to determine the initial DNA concentration as well as the range of cycles that could provide quantitative amplification for each target during PCR. Based on these results, PCR assays were performed using 100 ng DNA and 30 cycles of amplification.

Using the above conditions, we showed that QPCR could be used to quantitate the levels of DNA damage in DNA isolated from cells (HepG2, human PBMCs) treated with melphalan, cisplatin and carboplatin. As an improvement of this assay, we also developed and validated a simple and rapid method of template preparation which avoids DNA isolation. Thus, instead of using extensive purification and quantification of the DNA, cells were lysed in the presence of proteinase K at 55°C for 1 h, heated to 94°C for 10 min to inactivate the enzyme and the resulting homogeneous lysate analyzed directly by the QPCR assay. Since the preparation of the cell lysate was performed in a single tube and without any extraction, the preparation was quantitative and it was not necessary to measure the DNA concentration of each sample before PCR. The starting cell number was carefully controlled (~8000 cells/assay) so that the quantity of DNA used for each PCR would be the same. Experiments confirmed the reproducibility of the method.

Generally, the limit of detection for the QPCR assay corresponds to about 10–15% decrease in amplification. Assuming a random distribution of DNA damage, using a Poisson equation a 10% decrease in amplification of a 7-kb fragment is estimated to correspond to an average lesion frequency of 15 lesions/10⁶nucleotides. Since only 100 ng of purified genomic DNA is required for each PCR reaction,

this QPCR assay is about 250 times more sensitive than that based on Southern blot analysis [8]. The detection limit was ~10–20 lesions/10⁶nucleotides using DNA from ~8000 cells. This detection level, in combination with the ability to examine damage and repair in low numbers of cells, makes the assay a useful tool for measuring DNA damage during chemotherapy.

In order to understand which types of DNA adducts are detected and quantified by the newly developed assay, the QPCR methodology was applied, in parallel with the Southern blot analysis, for the analysis of DNA damage induced by melphalan, cisplatin and carboplatin. Melphalan-induced adduct levels measured by QPCR were found to be very similar to the ICL levels measured by Southern blot analysis, indicating that blockage of the PCR reaction is caused by this type of lesion. This was confirmed by using a monofunctional melphalan derivative, expected to generate DNA monoadducts but no ICL, when no adducts were detected using QPCR. As for the platinum-based drugs, we found that QPCR measures the sum of platinum-induced adducts (i.e. intra- and inter-strand cross-links and monoadducts).

Using the traditional Southern blot analysis, we have previously found that the levels of p53-specific damage formation/repair in PBMCs from MM patients following *ex vivo* exposure to melphalan correlate with the clinical outcome after therapeutic treatment with melphalan [6]. In the present report, studying a different patient group, we found that following treatment of isolated PBMCs with melphalan, the p53-specific damage measured by QPCR methodology showed a statistically significant difference ($P = 0.004$) between responders and non-responders (Figure 4A), confirming the clinical potential of this simple and rapid (results obtained within ~8 h) methodology in the selection of patients more likely to benefit from therapeutic treatment. Similar results were obtained using whole blood treatment, although the results are less clear and did not reach statistical significance. Higher levels of DNA damage were obtained following treatment of isolated PBMCs than following treatment of the whole blood, probably due to melphalan inactivation due to binding to plasma proteins. Serum albumin is the major binding protein, while α_1 -acid glycoprotein appears to account for about 20% of the plasma protein binding [34]. Moreover, no correlation was found between PBMC-treated and whole blood-treated results from the same individuals, probably due to different amounts of these binding proteins in different patients.

Using a cut-off value of the *ex vivo*-induced adducts of 101 (adducts/10⁶nucleotides)/(mg ml⁻¹ melphalan) for PBMCs [(56 (adducts/10⁶nucleotides)/(mg ml⁻¹ melphalan) for whole blood], 13 out of 14 patients for PBMCs (all patients for whole blood) with values equal to, or higher than this value were found to achieve a complete or partial response after high dose melphalan treatment (positive predictive value 92.9% for PBMCs and 100% for whole

blood). In accordance with our previous results [6], these data strongly point to a high level of DNA damage being the dominant factor determining response to therapy. On the other hand, two out of eight patients for PBMCs (one out of four for whole blood) with DNA damage levels below the threshold had a complete or partial response (sensitivity 86.67% for PBMCs and 88.89% for whole blood). There are various possible explanations for this, e.g. DNA damage in tumour cells may have been greater than implied by the levels found in PBMCs, 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.

Because the QPCR assay described here is based on the ability of melphalan-DNA adducts to block PCR amplification, it has the potential to be applied to other kinds of bulky DNA damage induced by chemotherapeutic alkylating drugs. Among the most important drugs of this kind are cisplatin and carboplatin which are widely employed in the treatment of ovarian, cervical, head and neck as well as non-small-cell lung cancers [15]. For this reason, in the presently reported study the potential of the same methodology to measure DNA damage and repair in relation to these drugs was evaluated. We showed that both versions of the assay described (QPCR and lysate QPCR) can be used to quantitate the levels of total DNA damage induced by these two drugs. The utility of such measurements in relation to patient response to such treatment has been suggested in previous studies [5] and remains to be further explored using the currently reported methodology.

In conclusion, the multiplex QPCR assay presented here can be used as a convenient, rapid and accurate method for the quantitation of DNA damage induced by melphalan, cisplatin and carboplatin, requiring only minute amounts of material (PBMCs) and yielding results within a few hours. In combination with the association between DNA damage thus measured and patient response, this assay can serve as a tool for prediction of patient response to therapy and outcome. This can lead to a shift in clinical oncology based on alkylating drugs, away from the currently practiced, population-based approaches towards personalized medicine.

Competing Interests

There are no competing interests to declare.

REFERENCES

- 1 Fichtinger-Schepman AM, van der Velde-Visser SD, van Dijk-Knijnenburg HC. Kinetics of the formation and removal of cisplatin-DNA adducts in blood cells and tumor tissue of cancer patients receiving chemotherapy: comparison with *in vitro* adduct formation. *Cancer Res* 1990; 50: 7887–94.
- 2 Parker RJ, Gill I, Tarone R. Platinum-DNA damage in leukocyte DNA of patients receiving carboplatin and cisplatin chemotherapy, measured by atomic absorption spectrometry. *Carcinogenesis* 1991; 12: 1253–8.
- 3 Dimopoulos MA, Souliotis VL, Anagnostopoulos A, Papadimitriou C, Sfikakis PP. Extent of damage and repair in the p53 tumor-suppressor gene after treatment of myeloma patients with high-dose melphalan and autologous blood stem-cell transplantation is individualized and may predict clinical outcome. *J Clin Oncol* 2005; 23: 4381–9.
- 4 Reed E, Ozols RF, Tarone R. Platinum-DNA adducts in leukocyte DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. *Proc Natl Acad Sci USA* 1987; 84: 5024–8.
- 5 Reed E, Ostchega Y, Steinberg SM, Yuspa SH, Young RC, Ozols RF, Poirier MC. Evaluation of platinum-DNA adduct levels relative to known prognostic variables in a cohort of ovarian cancer patients. *Cancer Res* 1990; 50: 2256–60.
- 6 Dimopoulos MA, Souliotis VL, Anagnostopoulos A, Bamia C, Pouli A, Baltadakis I, Terpos E, Kyrtopoulos SA, Sfikakis PP. Melphalan-induced DNA damage *in vitro* as a predictor for clinical outcome in multiple myeloma. *Haematologica* 2007; 92: 1505–12.
- 7 Fisch MJ, Howard KL, Einhorn LH. Relationship between platinum-DNA adducts in leukocytes of patients with advanced germ cell cancer and survival. *Clin Cancer Res* 1996; 2: 1063–6.
- 8 Souliotis VL, Dimopoulos MA, Sfikakis PP. Gene-specific formation and repair of DNA monoadducts and interstrand cross-links after therapeutic exposure to nitrogen mustards. *Clin Cancer Res* 2003; 9: 4465–74.
- 9 Chaney SG, Sancar A. DNA repair: enzymatic mechanisms and relevance to drug response. *J Natl Cancer Inst* 1996; 88: 1346–60.
- 10 Barlogie B, Kyle RA, Anderson KC, Greipp PR, Lazarus HM, Hurd DD. Standard chemotherapy compared with high-dose chemoradiotherapy: final results of Phase III US Intergroup Trial S9321 for Multiple Myeloma. *J Clin Oncol* 2006; 24: 929–36.
- 11 Child JA, Morgan GJ, Davies EE, Bell SE, Hawkins K. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. *N Engl J Med* 2003; 348: 1875–83.
- 12 Edler M, Jakubowski N, Linscheid M. Quantitative determination of melphalan DNA adducts using HPLC inductively coupled mass spectrometry. *J Mass Spectrom* 2006; 41: 507–16.
- 13 Balcome S, Park S, Quirk Dorr DR, Hafner L, Phillips L, Tretyakova N. Adenine-containing DNA-DNA cross-links of antitumor nitrogen mustards. *Chem Res Toxicol* 2004; 17: 950–62.
- 14 Hansson J, Lewensohn R, Ringborg U, Nilsson B. Formation and removal of DNA cross-links induced by melphalan and

- nitrogen mustard in relation to drug induced cytotoxicity in human melanoma cells. *Cancer Res* 1987; 47: 2631–7.
- 15** Desoize B, Madoulet C. Particular aspects of platinum compounds used at present in cancer treatment. *Crit Rev Oncol Hematol* 2002; 42: 317–25.
- 16** Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov* 2005; 4: 307–20.
- 17** Episkopou H, Kyrtopoulos SA, Sfrikakis PP, Dimopoulos MA, Souliotis VL. The repair of melphalan-induced DNA adducts in the transcribed strand of active genes is subject to a strong polarity effect. *Mutat Res* 2011; 714: 78–87.
- 18** Grimaldi KA, McGurk CJ, McHugh PJ, Hartley JA. PCR-based methods for detecting DNA damage and its repair at the sub-gene and single nucleotide levels in cells. *Mol Biotechnol* 2002; 20: 181–96.
- 19** Van Houten B, Cheng S, Chen Y. Measuring gene-specific nucleotide excision repair in human cells using quantitative amplification of long targets from nanogram quantities of DNA. *Mutat Res* 2000; 460: 81–94.
- 20** Jennerwein MM, Eastman A. A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. *Nucleic Acids Res* 1991; 19: 6209–14.
- 21** Kalinowski DP, Illenye S, Van Houten B. Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. *Nucleic Acids Res* 1992; 20: 3485–94.
- 22** Grimaldi KA, Bingham JP, Souhami RL, Hartley JA. DNA damage by anticancer agents and its repair: mapping in cells at the subgene level with quantitative polymerase chain reaction. *Anal Biochem* 1994; 222: 236–42.
- 23** Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci USA* 1997; 94: 514–9.
- 24** Chen X, Cullinane C, Gray PJ, Phillips DR. DNA damage by nitrogen mustard in a gene containing multiple Sp1-binding sites. *Mutat Res* 1999; 445: 45–54.
- 25** Blade J, Samson D, Reece D, Apperley J, Bjorkstrand B, Gahrton G. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. *Br J Haematol* 1998; 102: 1115–23.
- 26** Frgala T, Kalous O, Proffitt RT, Reynolds CP. A fluorescence microplate cytotoxicity assay with a 4-log dynamic range that identifies synergistic drug combinations. *Mol Cancer Ther* 2007; 6: 886–97.
- 27** Sfrikakis PP, Souliotis VL, Akbar AN, Katsilambros N, Hoffbrand V, Panayiotidis P. Regulation of bcl-2 and fas expression in primary activation of human peripheral lymphocytes is not sensitive to dexamethasone or cyclosporin-A. *Hum Immunol* 1996; 50: 121–6.
- 28** Thomas D, Morton AG, Bohr VA, Sankar A. General method for quantifying base adducts in specific mammalian genes. *Proc Natl Acad Sci USA* 1988; 85: 3723–7.
- 29** Wang Y-C, Lee P-J, Shih C-M, Chen H-Y, Lee C-C, Chang Y-Y. Damage formation and repair efficiency in the p53 gene of cell lines and blood lymphocytes assayed by multiplex long quantitative polymerase chain reaction. *Anal Biochem* 2003; 319: 206–15.
- 30** Oshita F, Eastman A. Gene-specific damage produced by cisplatin, ormaplatin and UV light in human cells as assayed by the polymerase chain reaction. *Oncol Res* 1993; 5: 111–8.
- 31** Tilby MJ, McCartney H, Gould KA, O'Hare CC, Hartley JA, Hall AG, Golding BT, Lawley PD. A monofunctional derivative of melphalan: preparation, DNA alkylation products, and determination of the specificity of monoclonal antibodies that recognize melphalan-DNA adducts. *Chem Res Toxicol* 1998; 11: 1162–8.
- 32** Husain K, Whitworth C, Rybak LP. Time response of carboplatin-induced nephrotoxicity in rats. *Pharmacol Res* 2004; 50: 291–300.
- 33** Bingham JP, Hartley JA, Souhami RL, Grimaldi KA. Strand-specific measurement of cisplatin-induced DNA damage and repair using quantitative PCR. *Nucleic Acids Res* 1996; 24: 987–9.
- 34** Reece PA, Hill HS, Green RM, Morris RG, Dale BM, Kotasek D, Sage RE. Renal clearance and protein binding of melphalan in patients with cancer. *Cancer Chemother Pharmacol* 1988; 22: 348–52.