Research Paper

Heterogeneity of p53-pathway Protein Expression in Chemosensitive Chronic Lymphocytic Leukemia: A Pilot Study

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Abstract

The presence of p53-pathway dysfunction in chronic lymphocytic leukemia (CLL) can be used to identify patients with chemotherapy-refractory disease. Therapeutic responses are known to vary between patients with chemosensitive CLL and may relate to differences in p53-pathway activity. We hypothesized that the magnitude or type of p53-pathway protein expression is heterogeneous in patients with chemosensitive disease and could associate with white cell responses. In this pilot study, changes in p53 and its transcriptional targets, p21/waf1 and MDM2 were analyzed by immunoblotting and densitometry in CLL cells from 10 patients immediately prior to the start of chemotherapy, and after culture for 24 hours (h) with fludarabine (n=7) or chlorambucil (n=3). The i*n vitro* response was also compared to that *in vivo* in circulating cells pre-treatment, and at 24h and 96h of chemotherapy. Disease responses were evident in all patients after the first treatment-cycle. Significant p53 induction was observed in CLL cells treated *in vitro* and *in vivo*. Greater heterogeneity in the expression-intensity was observed *in vivo* ($\sigma2=45.15$) than *in vitro* ($\sigma2=1.33$) and the results failed to correlate ($r^2=0.18$, p=0.22). p21/waf1 and MDM2 expression-profiles were also dissimilar *in vitro* and *in vivo*. Higher *in vivo* (but not *in vitro*) responses associated with changes in white cell count (p=0.026). Thus, heterogeneity of p53-pathway activity exists in chemosensitive CLL; in unselected patients, *in vivo* changes do not correlate with those *in vitro*, but may associate with post-treatment white cell responses.

Key words: p53, CLL, *in vivo*, *in vitro*.

Introduction

A number of pre-treatment variables are capable of determining prognosis in chronic lymphocytic leukemia (CLL).[1,2] Of these, the identification of *TP53* mutations is associated with a poor response to chemotherapy[3,4] since DNA damage responses are impaired in the presence of mutant *TP53*. According-

ly, abnormal p53-pathway protein expression has been observed in leukemic cells with mutant *TP53* following *in vitro* radiation.[5-7] In these radiation-based assays, it is also possible to identify p53-pathway dysfunction predictive of poorer outcomes, despite the presence of wild-type *TP53*.[7-9] Comparative *in vivo* and *in vitro* assays using chemotherapy (instead of radiation) as the genotoxic stimulus have yielded similar results, further indicating differences in p53-pathway activity between patients with chemosensitive and refractory disease.[10-12] Thus, the identification of reduced p53-pathway activity due to a variety of genetic aberrations including *TP53* or *ATM* mutations or *p21/waf1* polymorphisms is predictive of aggressive disease.[3-12]

Most CLL patients however have chemosensitive disease that is responsive to alkylating agents and nucleoside analogue drugs,[13,14] with the addition of the anti-CD20 monoclonal antibody Rituximab further improving outcomes.[15] The speed and depth of responses following standard chemotherapeutic regimens are known to vary amongst patients,[14] but underlying mechanisms have not been investigated. Given the central role of wild-type p53 in CLL responses, and the observations that the cellular outcome of p53 activation may relate to the strength of induction,[16,17] variation in the magnitude of p53-pathway activation could be responsible for heterogeneity of disease responses. Alternatively, there is evidence for differential induction of pro- or anti-apoptotic downstream targets of p53 under different circumstances, that may also influence the overall response to therapy.[18-20] Therefore, in this pilot study, we have investigated whether it is possible to detect variation in the induction of p53 and its downstream proteins in patients with chemosensitive CLL. Simultaneously, we have investigated whether protein expression following exposure to genotoxic chemotherapy *in vitro* and *in vivo* correlate, and associates with immediate white cell responses.

Patients and Methods

Patient selection

Ten patients (median age 73 years, range 56-79) starting their first cycle of oral treatment with fludarabine ($24mg/m^2$/day) and cyclophosphamide ($150mg/m^2$/day) for a total of 5 days (n=7), or chlorambucil (10mg/day) for 7 days (n=3) for symptomatic progressive CLL were included in the study. Six patients had received no previous therapy. The study was approved by the Tayside Committee on Medical Research Ethics and written informed consent was obtained from all patients.

CLL cell separation and processing

Peripheral blood mononuclear cells (MNC) were isolated by density gradient centrifugation (Ficoll-Paque™ Plus) at three time points: immediately prior to therapy (baseline) and at 24 and 96 hours after start of treatment. These time-points were arbitrarily selected, but have been validated in studies on the temporal course of p53-dependent gene-expression in CLL cells following treatment with fludarabine.[10-12] The enrichment for neoplastic cells was confirmed by the co-expression of CD5 and CD19 in >92% of immunolabelled cells by flow-cytometry (FACSscan) (not shown). Standard protocols were used to extract genomic DNA (BioRobot EZ1, Qiagen); RNA (Tissue Mini Kit, Qiagen) and proteins using buffer containing 1%Tergitol-type NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitors. An aliquot of MNC was also cyropreserved.

TP53 sequence analysis

Exons 5-9 of *TP53* gene (common 'hot-spot' for mutations affecting DNA-binding domain) were amplified by PCR using genomic DNA extracted from cells prior to therapy. The purified products were directly sequenced using the ABI PRISM® BigDye™ Terminators V 3.0 sequencing kit and run on an ABI 3130 genetic analyzer (Applied Biosystems).

Fluorescence *in situ* hybridization

Interphase flourescent *in situ* hybridisation (FISH) studies were carried out on CLL MNC using the CEP17/*TP53* (17p13.1) and *ATM* (11q22.3) probes (Vysis). Samples were treated with a hypotonic solution (50% potassium chloride) and fixed in methanol/acetic acid (3:1) solution. Fixed material was dropped onto clean slides, dehydrated and denatured for 5 minutes in 70% formamide/saline sodium citrate solution at 72 °C. CEP17/*TP53* and *ATM* probes were denatured at 72°C for 5 minutes and hybridization was performed at 37°C overnight. Slides were washed at 72°C for 2 minutes in 0.4x saline-sodium citrate (SSC)/0.3% Nonidet P40 (NP40) and at room temperature for 1 minute in 2xSSC/0.1% NP40 and mounted in DAPI/Antifade (Vysis). Samples were evaluated using an Olympus BX60 (Olympus UK Ltd, Southend-on-Sea, UK) epifluorescence microscope equipped with the appropriate filter sets. Images were taken with a charge-coupled device camera using the CytoVysion image analysis and capture system (Leica Microsystems, UK). At least 100 cells were scored by two independent scorers (blinded to the identity of the specimen) against a normal control sample.

Cell culture

Cryopreserved MNC (2×10^6 cells/ml) obtained prior to therapy were cultured in RPMI/10% fetal calf serum at 37°C for 24 hours in the presence of the same drug(s) used to treat individual patients. Trypan-Blue exclusion assays suggested that greater than 99% of cells were viable prior to culture. In our hands, studies comparing viability of freshly isolated and cryopreserved cells in paired cultures using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or cleaved caspase-3 expression have indicated equivalent levels of apoptosis, not influenced by cryopreservation (r=0.98; p=0.003). In addition, where possible, expression profiles of p53-pathway proteins from cultures established using freshly isolated or cryopreserved cultured CLL cells were compared and found to be identical, in keeping with the previously described concordance of biological phenomena in fresh and cryopreserved CLL cells.[10,21]

Cells were incubated with 1μg/ml 9-μ-D-arabinosyl-2-fluoroadenine monophosphate (Sigma) or 15μM chlorambucil. Negative controls consisted of cells in medium containing 0.1% DMSO. Cellular protein was extracted after culture.

Intracellular protein expression

Changes in p53-pathway proteins were studied by immunoblotting. The following antibodies were used: anti-p53 (DO1, 1:100), anti-p21/waf1 (clone 118; 1:50) and anti-MDM2 (2A10, 1:50). Anti-p53, p21 and MDM2 antibodies were gifted by Dr B Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Republic. Proteins from normal blood MNC cultured with cisplatin and phytohemagglutinin (PHA), and untreated MNC probed with antibodies on the same membrane served as positive and negative controls respectively. To control for protein loading, each membrane was re-probed with a mouse monoclonal antibody to β-actin (AC-74; Sigma).

Immunoreactive bands for p53 were quantified by densitometry in a linear range using calibrated office scanner and ImageJ software, and the ratio of the immunoreactive p53 protein band and β-actin was calculated. Levels of p53 at 24 and 96 hours were expressed as a fraction of pre-treatment values or controls (for *in vitro* studies) on the same membrane and expressed as the fold-change. Repeat analysis of protein extracts from cells collected before and during treatment, and following cell culture was performed to ensure reproducibility of results.

CD44 cell-surface expression

The expression of CD44, a cellular adhesion molecule expressed at high levels in CLL cells in patients with advanced disease[22] is known to be down-regulated by p53 via binding to a noncanonical p53-binding sequence in the *CD44* promoter.[23] Potential changes in CD44 expression, reflecting functional p53-pathway responses were studied by flow-cytometry following chemotherapy. In brief, 1×10^6 freshly isolated or cultured CLL cells were stained with murine anti-human antibodies labeled with CD19 (FITC) and CD44 (PE) (BD Pharmingen, San Jose, USA). Negative controls were incubated with mouse IgG1 (BD Pharmingen, San Jose, USA). Fluorescence was measured by flow-cytometry using a Becton-Dickinson FACScan immediately after staining

Statistical analysis

The arithmetic mean was used to measure the central tendency of data and the dispersion of values around the mean was expressed as the standard deviation (s.d.) in analysis of raw data, i.e. mean±s.d. The significance of difference between means was tested using Student's t-test or Wilcoxon Two Sample Test (depending on the variances within data). All p-values were two-tailed and statistical-significance was set at the level of p<0.05.

Results

Analysis of *TP53* sequence and ATM

Molecular analysis revealed the presence of mutant *TP53* (exons 5-9) in one patient (c.736A>G: p.Met246Val mutation in codon 246) and del(17p) was identified by FISH in 20% of circulating cells in this patient. In another patient, monoallelic deletion of *ATM* was identified in 117/120 cells.

Expression of p53 *in vitro* and *in vivo* (Figure 1A)

All data on *in vitro* p53-pathway activity reported here are from 24 hour assays since longer periods of culture resulted in reduced cell recovery and weaker expression of β-actin making accurate interpretation of p53-expression intensity unreliable. Consistent p53 induction was observed at 24 hours of culture with fludarabine or chlorambucil (Figures 1A and B), indicating integrity of signaling mechanisms that stabilize p53 in all patients. *In vivo*, a mean 2-fold increase over baseline levels was observed at 24 hours (24h-p53); by 96 hours, the mean-intensity (96h-p53) had increased to 7.8-fold over baseline levels (p=0.003), with the variance ($\sigma2=45.15$) indicating greater heterogeneity in the strength of p53-induction than *in vitro* ($\sigma2=1.33$, Figure 1A).

A

B

C
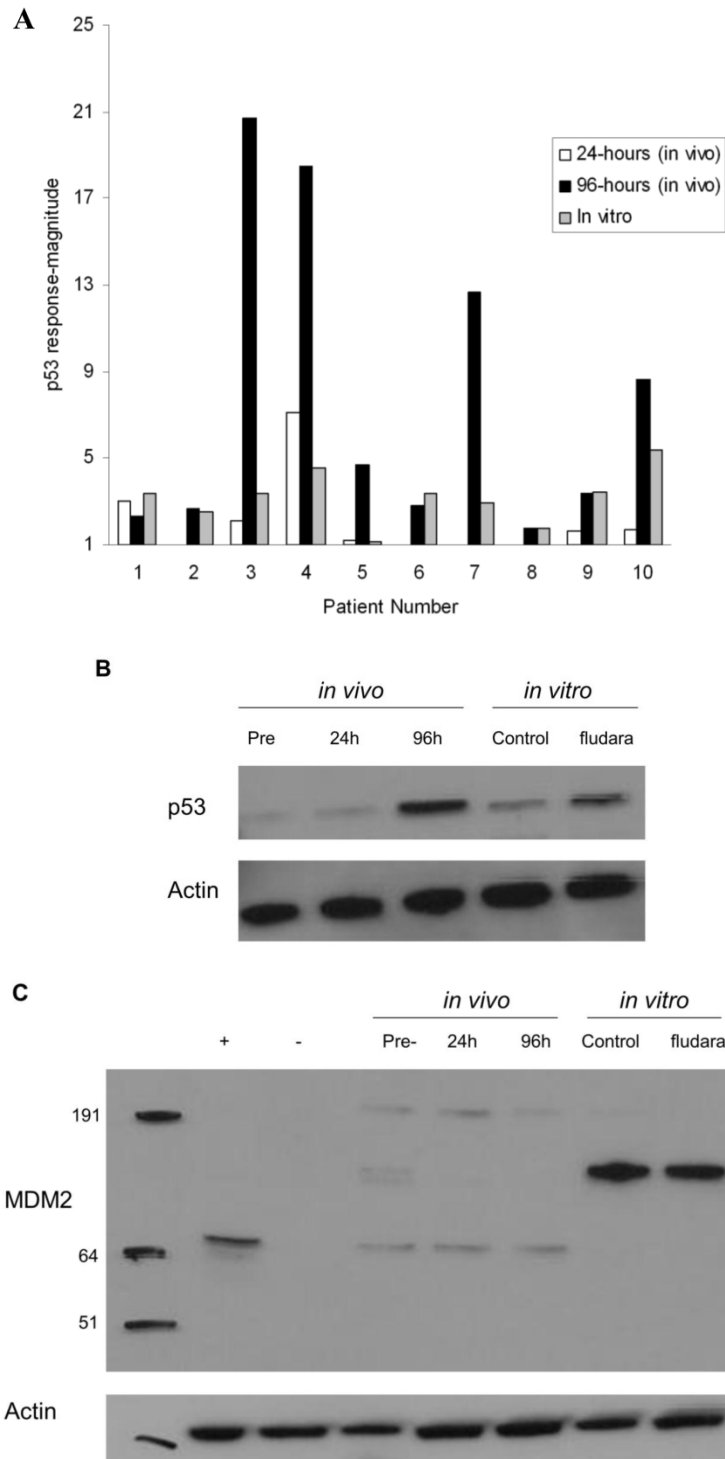
Figure 1. Changes in p53 pathway-protein expression in CLL cells following chemotherapy *in vivo* and *in vitro*. p53 levels in circulating CLL cells at 24 hours (24 hours in vivo) and 96 hours (96 hours in vivo) following the start of chemotherapy were quantified and expressed as the fold-change over pre-treatment values (see Patients and Methods for details). In Figure 1 A, the *in vivo* results from individual patients can be compared with the corresponding p53 induction in cells cultured with chemotherapy, expressed as the fold-difference in expression over untreated cells (in vitro). In Figure 1B, the gel image from Patient 7 is used to exemplify the heterogeneity in the magnitude of p53 response *in vivo* and *in vitro*. Here, *in vitro* assays with fludarabine accurately captured trends of *in vivo* p53 expression (fludara) over untreated cultures (Control), but the response-magnitude was dissimilar. Figure 1C shows the expression of MDM2 to be discordant *in vivo* and *in vitro* (Figure 1C) and different from the expression profile in normal peripheral blood mononuclear cells (MNC) treated with cisplatin (+). No MDM2 was detected in freshly isolated normal MNC (-).

When the *in vitro* and *in vivo* expression intensities were compared, the response in cultured cells was greater than the 24h-p53 (p=0.049), but lower than 96h-p53 (p=0.055). In the entire cohort, no correlation was observed between the p53-induction magnitude assayed *in vitro* and *in vivo* (r^2=0.18, p=0.22), with discordant results evident in 6 at 24 hours and 5 patients at 96 hours (Figure 1A, and exemplified in Figure 1B). In particular, the p53-expression intensity *in vivo* and *in vitro* appeared to be dissimilar in 5 out of 7 patients on fludarabine and cyclophosphamide.

Expression of p53-transcriptional target proteins

When downstream protein targets of p53 were analyzed, 4 patients expressed p21/waf1 *in vitro*. No expression of p21/waf1 was detected *in vivo* at 24 hours, but was detectable in 4 patients at 96 hours. *In vitro* p21/waf1 was identified in only one of these patients. Patients with detectable p21/waf1 *in vivo* had a trend towards a higher p53 response-magnitude (12.6±8-fold increase over baseline) compared to patients with no p21/waf1 induction (4.6±4-fold p=0.07), although this association was not evident *in vitro*. Dissimilarities of MDM2 expression were also observed *in vitro* and *in vivo* (Figure 1C). Complex patterns of MDM2 expression between 40 kDa to 191 kDa were observed *in vivo* but not in cultured cells, suggesting that post-transcriptional or post-translational modifications *in vitro* could be different to those occurring *in vivo*. Despite p53 induction in all 10 patients, MDM2 expression remained unchanged in 7 patients *in vitro* and *in vivo*, with an increase observed in only 3 patients.

In contrast to the discordant expression of p21/waf1 and MDM2 *in vitro* and *in vivo*, the expression of CD44 (100%) and the mean fluorescence intensity of CD44 expression remained stable *in vitro* and at 24 and 96 hours despite significant induction of p53.

Post-chemotherapy responses

Following the first cycle of chemotherapy, all patients had chemosensitive disease with 4 partial (PR) and 6 complete remissions (CR).[24] The responses include CR in the patient with del(17p) suggesting a low burden of *TP53* mutant cells at the time of therapy. PR in the patient with monoallelic loss of chromosome 11q (*ATM*) suggested likely preservation of p53-pathway signaling through the residual wild-type *ATM* allele.[8] Accordingly, induction of p53 following chemotherapy was evident in both these patients.

Changes in WCC relative to p53-pathway protein expression

No association was observed between WCC at 24 hours (WCC-24h) or 96 hours (WCC-96h) and the p53 induction-intensity *in vitro* or at 24 hours *in vivo*. When the 96-hour p53 response-magnitude *in vivo* was compared to changes in WCC using two-step cluster analysis and Akaike's information criterion (SPSS), two clusters based on WCC-96h were identified (Figure 2A). The mean WCC-96h in Cluster 1 (89±7.3% of pre-treatment WCC, n=5) was higher than in Cluster 2 (48±9.3%, p<0.0001). Significantly, in Cluster 1, the mean *in vivo* p53 response-magnitude at 96 hours (3±0.9-fold over baseline levels) was lower than in Cluster 2, where the p53 expression-magnitude registered a mean increase of 12±7.6-fold (p=0.026, Figure 2B). The WCC-28d in Cluster 2 was significantly lower than patients in Cluster 1 (4.6±2.5% and 28.2±17.3% of pre-treatment values respectively, p=0.016). The induction of p21/waf1 *in vitro* did not associate with WCC-28d, but the expression at 96 hours *in vivo* associated with lower WCC-28d (3.2± 3.9%, n=4) compared to patients with p53 but no p21/waf1 induction (25± 18%, n=6 p=0.036, Figure 2C).

Table 1. Pre-treatment characteristics of patients treated with chlorambucil (CLB) or fludarabine and cyclophosphamide (FC). Unique numbers can be used to identify individual patients in Figure 1. Clinical and hematological parameters stipulated by IWCLL were used to define partial (PR) or complete remissions (CR) at the end of the first cycle of chemotherapy.

ID	Gender	Current treatment	Previous treatment	Interval from previous therapy (months)	Response
1.	Male	CLB	None	NA	PR
2.	Male	CLB	CLB	19	PR
3.	Male	FC	CLB	0.5	CR
4.	Female	FC	None	NA	CR
5.	Female	FC	CLB, FC	18	PR
6.	Female	FC	None	NA	CR
7.	Male	FC	None	NA	CR
8.	Male*	FC	CLB	2	CR
9.	Male**	CLB	None	NA	PR
10.	Female	FC	None	NA	CR

NA: not applicable.

*patient with del(17p) in 20% of cells and c.736A>G: p.Met246Val mutation in codon 246 of *TP53*.

**patient with monoallellic deletion of *ATM*.

A

B

C
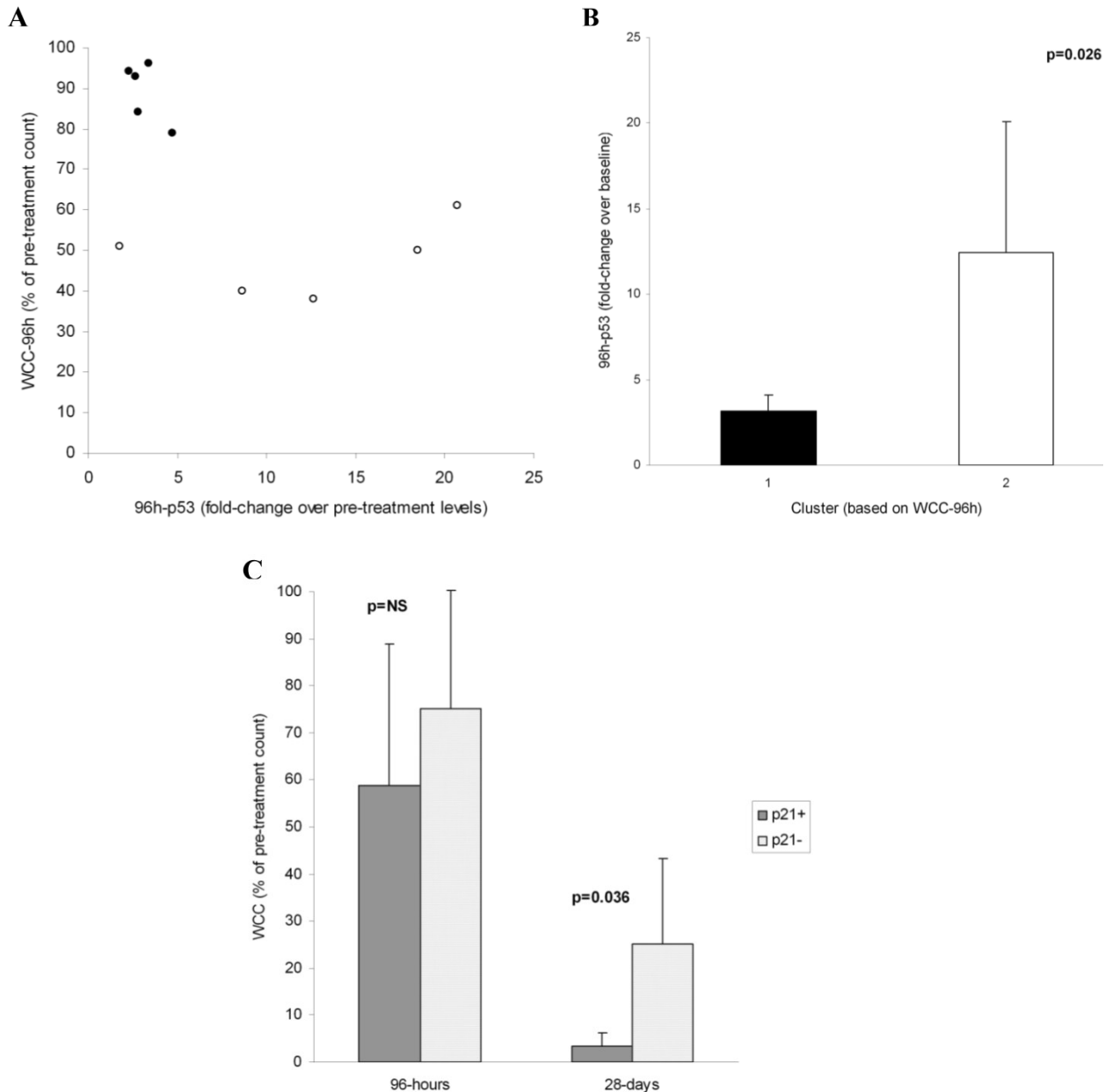
Figure 2. Changes in white cell counts (WCC) relative to p53-pathway protein expression. The induction-intensity of p53 (relative to baseline values) at 96 hours (96h-p53) was compared with corresponding WCC at 96 hours (WCC-96h). Two-step cluster analysis identified two clusters based on WCC-96h (Figure 2A): Cluster 1 (•) had a higher WCC, and the mean increase in 96h-p53 here was 4-fold lower than in Cluster 2 (○) (p=0.026, Figure 2B). Co-expression of p21/waf1 with p53 at 96 hours *in vivo* associated with lower WCC-28d (3.2± 3.9%, n=4) than in patients without p21/waf1 induction (25± 18%, n=6, p=0.036) (Figure 2C), but did not impact on the WCC at 96 hours.

Discussion

The inability to activate p53-pathway function in CLL cells following exposure to genotoxic agents *in vitro* reliably identifies patients with genetic aberrations resulting in a severely compromised p53 pathway and disease that is aggressive or poorly responsive to chemotherapy.[3-12] However, few studies have investigated whether quantifiable heterogeneity of p53-pathway function exists in patients responding to chemotherapy. In some cellular models, activation of p53 can lead to distinct outcomes of either growth-arrest or apoptosis through the differential induction of distinct down-stream targets (for a review, see refs. 25 and 26). Evidence that the decision between growth arrest and apoptosis could be dependent on the levels of induced p53[16,17] suggests that potential variation in the magnitude of the p53 re-

sponse to genotoxic stimuli could account for different levels of leukemic control in chemosensitive disease. Alternatively, there are suggestions that activated p53 may preferentially activate apoptotic or growth arrest programs depending on cellular contexts including cell types and genetic background.[16,17,19,26] In contrast, human follicular lymphoma cells undergo apoptosis following therapeutic irradiation, despite equivalent induction of apoptotic or growth arrest programs *in vivo*.[27] Thus, the ability to identify differences in p53 stabilization levels and functional activation of specific downstream targets in CLL would enable a correlation with clinical responses and potentially, the development of dose-adapted strategies.

Here, by directly comparing p53-pathway activity *in vitro* and *in vivo* in patients responding to chlorambucil or combination therapy with fludarabine and cyclophosphamide, we have demonstrated significant induction of p53 in these patients, in accordance with previous studies.[10-12] However, while assays identify heterogeneity of response-magnitude *in vitro* and *in vivo*, the results do not correlate, particularly in patients receiving fludarabine and cyclophosphamide. Similar discordance in expression of p21/waf1 and MDM2 expression was also observed. These results contrast with the similarity of p53-downstream target induction observed at the transcription level in cultured cells and from patients receiving fludarabine,[10-12] and may be related to differences in post-transcriptional regulation *in vitro* and *in vivo*, or differences in assay sensitivity. In particular, MDM2 expression profiles varied widely between patients, with variable dominance of 75-kDa, 90kDa or higher molecular weight proteins in different patients, as reported previously.[28]

In contrast to p21/waf1 and MDM2, the proportion of cells expressing CD44 and the mean fluorescence intensity of expression remained identical *in vitro* and *in vivo*, despite significant expression of p53. Although not commonly studied in p53-pathway function assays in CLL, CD44, the antigenic moniker for a protean ensemble of cell-surface transmembrane glycoproteins is known to promote tumour migration and adhesion,[29] and is known to be expressed on CLL cells.[22] Since the expression of CD44 is reportedly repressed by p53 in solid organ malignancies,[23] we had expected down-regulation of CD44 on CLL cells following chemotherapy and investigated its potential as a measurable marker of p53-pathway activity. The stability of surface CD44 observed even after significant p53-induction in CLL cells suggests that the regulatory relationship between p53 and CD44 may differ in circulating CLL cells compared to other malignancies.

It is important that the clinical correlates of our study are interpreted cautiously. The failure of the *in vitro* p53-expression-magnitude to associate with early (96 hour) and late (28 day) WCC responses could reflect the inadequacy of current culture techniques to simulate the *in vivo* microenvironment and migration in CLL.[12] p53 expression *in vivo* at 96 hours associated with early WCC responses and patients co-expressing p21/waf1 *in vivo* (but not *in vitro*) experienced a greater reduction in WCC at 28 days, in agreement with previous studies[9] indicating the clinical significant of p21/waf1 induction in *ex vivo* genotoxic assays of p53-pathway function. However, given the size of our patient cohort, and the higher p53-response magnitude in patients co-expressing p21/waf1, it is not possible to establish at present whether p21/waf1 induction is an independent predictor of white cell responses.

In conclusion, our pilot study has indicated that it is feasible to identify variation in p53-pathway activity amongst unselected patients with chemosensitive CLL *ex vivo*, but assay-results do not correlate with the heterogeneity of protein expression observed *in vivo*. Possible differences between the strength of the genotoxic stimulus *in vitro* and *in vivo* in individual patients, and potential selection bias during sampling of circulating cells means that developing chemotherapy-based *in vitro* culture systems to prospectively predict the magnitude of *in vivo* p53-stabilization is likely to be challenging. A larger study on uniformly treated patients is now required to confirm the importance of p53-p21/waf1 activity *in vivo* to immediate and longer-term disease responses.

Abbreviations

ATM: *Ataxia Telangiectasia Mutant*; CLL: Chronic Lymphocytic Leukemia; CR: Complete remission; p21/waf1: cyclin-dependent kinase inhibitor 1; d: day; FISH: Fluorescence *in situ* hybridization; h: Hour; MNC: Mononuclear cells; MDM2: Murine double minute; NP40: Nonidet P40; PR: Partial remission; SSC: saline-sodium citrate; *TP53*: *Tumour Protein 53*; WCC: White cell count.

Acknowledgements

Conflict of Interest

All authors have no conflicts of interest.

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