The Hematology Journal (2004) 5, 47–54
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www.nature.com/thj

Characterisation of *TP53* abnormalities in chronic lymphocytic leukaemia

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Abnormalities of *TP53* in chronic lymphocytic leukaemia (CLL) correlate with aggressive disease and transformation. We studied 115 patients with CLL including 90 untreated, 25 with heavily pretreated/refractory CLL using fluorescent *in situ* hybridisation (FISH) to detect allelic loss at chromosome 17p and flow cytometry (FC) to test p53 protein overexpression. A total of 17 cases were identified with *TP53* deletion and/or protein expression. Both tests correlated in 10 of 17 patients; in six, one or the other abnormality was detected and in one case, with a deletion, flow cytometry failed. Material for direct DNA sequencing was available in 14 of 17 cases. Mutations were found in seven cases. Five of 14 patients with allelic loss and seven of 13 expressing p53 protein had a mutation. These were single-base substitutions and were located in exons 5, 7 or 8. Mutations were not found in 13 of 14 other cases without deletions by FISH or protein expression. The incidence of p53 abnormalities in this series was 15%, with a significant difference between untreated patients (7%) and the pretreated/refractory group (50%; $P<0.01$). Abnormal p53 was predicted for shorter survival, regardless of the method used. We confirm that p53 abnormalities are more common in refractory CLL and that mutations occur at the known hot spots. Testing for *TP53* deletions by FISH and protein expression by FC is an effective and simple way of screening patients who are likely to have aggressive disease. DNA sequencing adds little to these methods in identifying the population at risk.
The Hematology Journal (2004) **5,** 47–54. doi:10.1038/sj.thj.6200325

Keywords: *TP53*; P53; CLL; therapy

Introduction

Several chromosomal abnormalities, among them 17p deletion or a dysfunctional p53, have been shown to correlate with worse prognosis in chronic lymphocytic leukaemia (CLL),[1,2] the most common leukaemia in adults. p53, a 53 kD protein, first described in 1979, has 393 amino acids encoded on the short arm of chromosome 17p13.1 and is highly conserved across animal species.[3] *TP53* is the gene most frequently altered in human cancer.[4] The protein is composed of four identical chains bound together to form a four-'armed' structure at the tip of which is an activation domain, which binds to transcription activation proteins and the regulatory protein mdm2. The expression of p53 is induced in response to DNA damage when it can initiate two processes to prevent uncontrolled growth. p53 can halt cell division arresting the cell at the G1 check point of the cycle or it can initiate apoptosis, eliminating the damaged cell. The loss or inactivation of both alleles of *TP53* results in loss of the G1/S check point and mechanisms for apoptosis induction. This leads to the deregulation of cell proliferation and therefore increased frequency of neoplasia.[5,6]

p53 abnormalities in CLL have been shown to be associated with advanced stage, excess of prolymphocytes,[7] higher incidence of transformation and poor response to treatment with purine analogues such as fludarabine and alkylating agents.[1,8,9]

It is not clear as to which method of detecting p53 abnormalities has the greatest impact on clinical outcome in CLL. Döhner *et al.*[9] found that *TP53* deletions predict poor outcome and Cordone *et al.*[1] found abnormal p53 protein expression by immunocytochemistry to be a marker of bad prognosis. The presence of protein accumulation suggests that *TP53* may be mutated, as overexpression is caused by a failure of mdm2, which neutralises p53 to bind to the mutated protein[10–12] or as suggested by others by lack of mdm2 in these cells as only wild-type *TP53* can act as a

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Received 7 February 2003; accepted 24 June 2003

transcription factor for mdm2.[13] Our previous experience using a combination of loss of heterozygosity, abnormal protein expression and mutational analysis showed that p53 abnormalities correlated with poor prognosis in CLL and CLL/PL.[7]

We have investigated a large number of patients, including previously untreated patients and pretreated/refractory patients with aggressive disease for p53 abnormalities by flow cytometry (FC) and fluorescent *in situ* hybridisation (FISH). Patients with an abnormality by either method had subsequent DNA sequencing of the *TP53* gene from exons 4 to 9, which contain 90% of the described mutations in CLL.[14] This would also enable us to determine the frequency, type and exonic locations of *TP53* mutations. To assess the sensitivity and specificity of the methods used, we also tested for mutation samples from patients who had no detectable abnormality by FISH or FC.

Methods

Peripheral blood mononuclear (MN) cells from 115 patients were studied. This included 88 from untreated patients entered into the CLL4 trial (Binet stages progressive A, B and C) , and 27 patients from our hospital, of which 25 had pretreated/refractory CLL including one with Richter's transformation and one who transformed subsequently. The remaining two patients had familial CLL and had not required treatment.

FC was performed on separated MN cells from samples less than 72 h old. MN cells were fixed in cold 2% paraformaldehyde for 30 min at +4°C and permeabilised with ice-cold 80% ethanol added dropwise while vortexing. At this stage, cells were stored at −20°C for up to 2 weeks. Staining consisted of a 15-min incubation with 5 μl of a monoclonal antibody recognising amino acids 11–25 of both wild-type and mutant p53 (clone DO1, Novocastra, Newcastle upon Tyne, UK) in 50 μl 2% AB serum. Following two washes in phosphate-buffered saline (PBS)-azide cells were incubated with FITC-conjugated goat anti-mouse IgG $F(ab)_2$ (1 : 25) (ICN Biomedicals, Inc., Basingstoke, UK). An isotypic control was included for each sample. Cells were analysed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, California, USA) using Cell Quest software for data acquisition and analysis. p53 expression was determined from the value of the mean fluorescence intensity ratio between the test sample and the isotypic control. Cutoff levels were established testing MN cells from 10 normal healthy individuals. The average of the mean fluorescence intensity ratio of all 10 normal individuals +2.5 s.d. was taken as a positive for p53 overexpression. In each experiment, we used the CEM (T-ALL) cell line known to have biallelic *TP53* mutations and a high expression of p53 protein.

FISH analysis was performed using standard methods on MN cells, washed twice in Hank's balanced salt solution, fixed in methanol : glacial acetic acid (3 : 1) and stored at −20°C until needed. When fresh cells were not available, frozen low-density cells were retrieved from liquid nitrogen and prepared in the same way. Slides were made by placing 15 μl of cell suspension onto a microscope slide and dried overnight at room temperature. Hybridisation was performed as follows: slides were incubated with RNAse A (0.1 mg/ml, Sigma) at 37°C for 1 h and washed in 2 × SSC at room temperature for 5 min. This was followed by digestion in pepsin (0.1 mg/ml, Sigma) at 37°C for 10 min and washed with PBS at room temperature for 5 min and in 2 × SSC at 37°C for 30 min. Slides were then dehydrated in 70, 90 and 100% ethanol series (3 min in each) and dried. Denaturation took place on a dry-heat block at 72°C using 100 μl of denaturing solution (70% formamide, 2 × SSC and 0.05 M sodium phosphate buffer, pH 7.0) under a 22 × 50 mm coverslip. Slides were then quickly rinsed in 2 × SSC, dehydrated and dried. We used a *TP53* locus-specific probe (LSI p53 Spectrum Orange, Vysis, Richmond, UK) in combination with a probe specific for the chromosome 17 centromere (CEP17 Spectrum Green, Vysis) to distinguish the possibility of monosomy 17 causing loss of hybridisation signal for the *TP53* probe. Probes were prepared and denatured according to the manufacturer's instructions. Hybridisation was carried out overnight at 37°C. Posthybridisation washes consisted of three washes in 1 × SSC at 45°C followed by three washes in 0.1 × SSC at 60°C and one wash in 4 × SSC/Triton X-100 at room temperature, dehydrated in ethanol series, dried and mounted with a Vectashield mounting medium with DAPI (Vector Labs, Peterborough, UK).

Slides were assessed under a fluorescent microscope (Zeiss, Oberkochen, Germany) equipped with dual and triple band pass filters. A total of 200 nuclei were scored per patient's sample. Cells from 10 healthy donors prepared as above were used as normal controls. In total, 500 nuclei were scored per control slide.

Direct sequencing was conducted in 14 samples with deletion and/or protein expression, and 14 with neither abnormality, all obtained at the same time as those used for FISH and FC experiments. In three cases with p53 abnormalities by FISH or FC, no DNA was available. Sequencing of exons 4–9 of *TP53* was performed by polymerase chain reaction (PCR) amplification of exonic sequences from genomic DNA followed by fluorescent automated cycle sequencing of both DNA strands.

For *PCR amplification*, high molecular weight DNA was extracted from cryopreserved cells by standard methods. The quality of DNA was assessed by electrophoresis following digestion with *Eco*RI. PCR was performed in a total volume of 25 μl using 0.5 μl of genomic DNA (1 mg/ml), 2.5 μl 10 × Thermoprime Plus Taq Buffer, 1.5 μl $MgCl_2$ (25 mM), 1 μl dNTP (10 mM), 0.5 μl Thermoprime Plus Taq polymerase (1 U/μl) (all reagents from ABgene, Epsom, UK), 1 μl of each primer (5 μM) and water. The cycling programme comprised 35 cycles of denaturation (94°C; 1 min), annealing (exons, 4 and 10: 55°C, exons 5, 6, 9: 61°C, exon 7: 62°C, exon 8: 59°C; 1 min) and extension (72°C, 2 min). The sequences of the primers were as follows:

exon 4: forward 5′GACCTGGTCCTCTGACTGCT3′, reverse 5′GCATTGAAGTCTCATGGAAG3′, exon 5: forward 5′ACTTGTGCCCTGACTTTCAACT3′, reverse 5′CAATCAGTGAGGAATCAGAGGC3′, exon 6: forward 5′TCAGATAGCCGATGGTGAGCAG3′, reverse 5′GCCACTGACAACCACCCTTA3′, exon 7: forward 5′AGGCGCACTGGCCTCATCTT3′, reverse 5′GAAATCGGTAAGAGGTGGGC3′ exon 8: forward 5′GGAGTAGATGGAGCCTGGTTT3′, reverse GGTGATAAAAGTGAATCTGAGGC3′, exon 9: forward 5′GGAGACCAAGGGTGCAGTTAT3′, reverse 5′GTTAGTTAGCTACAACCAGGAGCC3′

Sequencing reaction

The same primers as the ones used for PCR were used for the sequencing.. Sequencing was performed using 4 μl of reaction premix (dRhodamine Terminator Ready Reaction Kit; ABI, Perkin Elmer, Warrington, UK), 4 μl of primer and 4 μl of PCR product. The following cycling conditions were applied: denaturation (96°C; 30 s), annealing (50°C; 15 s) and extension (60°C; 60 s) for 25 cycles. Excess of dye terminator was removed by precipitation of DNA in 100 μl 80% ethanol and 6 μl sodium acetate (3 M, pH 5.2) for 10 min on ice. All PCR products were sequenced in both directions. All mutations were resequenced from a different PCR product. The obtained DNA sequences were analysed using Sequence Analysis software (version 3.0) (ABI) and aligned and compared to published *TP53* sequence using Sequence Navigator software (ABI). Point mutations were compared with the IARC database (http://www.iarc.fr/p53/) to determine whether they were polymorphisms or true mutations.[6]

Statistical methods

Using SPSS statistical software package (SPSS Inc. Chicago, Illinois, USA), lifetable curves were calculated using the Kaplan–Meier method and analysis of the effect of prognostic factors on survival was undertaken using Cox's regression, hazard ratios satisfying $P<0.05$ being considered statistically significant.[15,16]

Results

Samples from the 115 patients samples were studied using FC and FISH (Table 1). A total of 17 patients (15%) were found to have abnormal p53; 14 had a deletion by FISH and 13 had abnormal protein expression by FC (Table 1). There was a correlation between FISH and FC in 10 out of 17 patients. Three with *TP53* deletions by FISH had no protein expression by FC, while p53 was overexpressed by FC was positive in three patients without deletion by FISH (Figures 1 and 2). FC was not informative in one patient with a deletion by FISH due to poor cell viability. All the other samples were negative by both methods.

Table 1 Results with three methods for p53 abnormalities

				Direct sequencing			
Case no.	*Diagnosis*	*% deletion by FISH*[a]	*Protein expression (FC)*	*Exon*	*Codon*	*Mutation/polymorphism*	*Amino-acid substitution*
Patients with p53 expression only							
1	Untreated CLL	<5	Positive	4	72	CGC to CCC (polymorphism)	Arg to Pro
2	Untreated CLL	<5	Positive	8	278	CCT to GCT	Pro to Ala
3[b]	Untreated CLL	<5	Positive	7	248	CCG to CAG	Arg to Glu
Patients with deletions only (FISH)							
4	Untreated CLL	14	Negative			No mutation	
5	Untreated CLL	47	Fail			No mutation	
6	Refractory CLL	87	Negative			No DNA available	
7	Refractory CLL	14	Negative	4	72	CGC to CCC (polymorphism)	Arg to Pro
Patients with both FISH deletion and p53 expression (FC)							
8[b]	Untreated CLL	74	Positive	5	143	CGT to CAT	Val to Met
9	Refractory CLL	75	Positive	5	175	CGC to CAC	Arg to His
10	Richter syndrome	50	Positive	5	176	TGC to TTC	Cys to Phe
11	Refractory CLL	50	Positive	8	277	TGT to TTT	Cys to Phe
12	Refractory CLL	65	Positive	8	273	CGT to CAT	Arg to His
13	Refractory CLL	60	Positive	4	72	CGC to CCC (polymorphism)	Arg to Pro
14	Refractory CLL	75	Positive			No DNA available	
15	Refractory CLL	63	Positive			No mutation	
16	Refractory CLL	16	Positive			No DNA available	
17	Refractory CLL	20	Positive			No mutation	
Patients with normal screening (FISH and FC)							
18–30	Untreated CLL	<5	Negative			No mutation	
31	Refractory CLL	<5	Negative			Frame shift mutation	

[a] >5% deletion by FISH defined as abnormal.
[b] Subsequent nonresponder to first-line treatment.

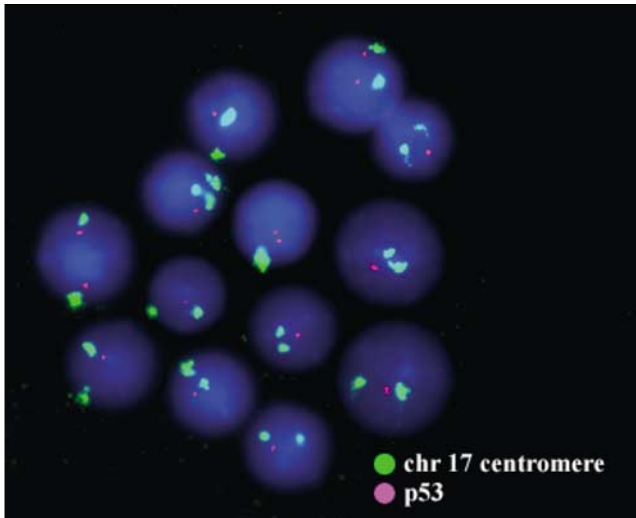

Figure 1 Hemizygous p53 deletion by FISH analysis.

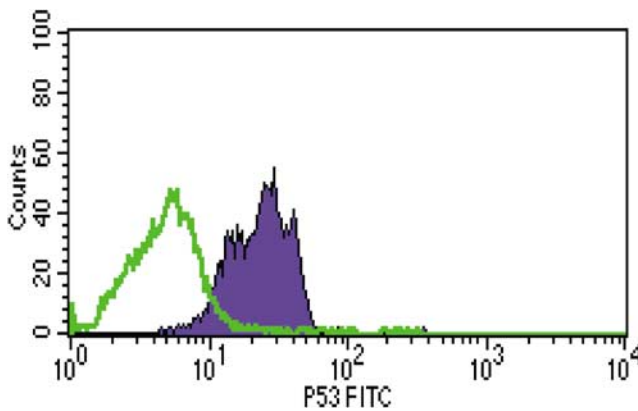

Figure 2 p53 protein expression demonstrated by FC.

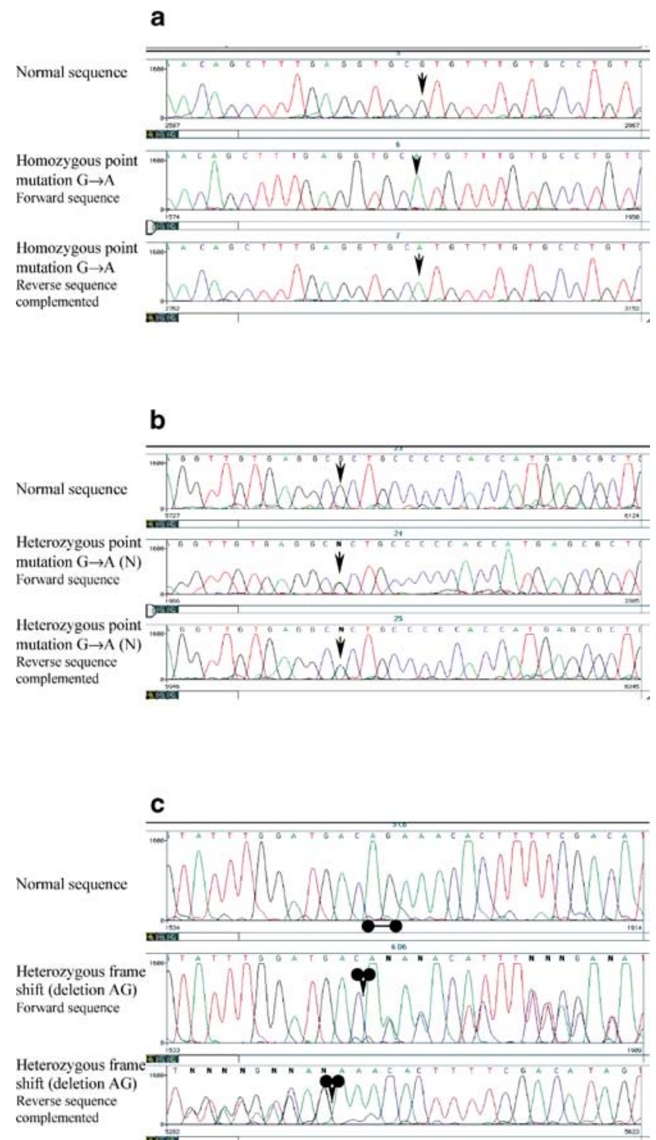

Figure 3 Mutations of p53 gene: (a and b) point mutations; (c) frame shift.

DNA was available for sequencing in 14 out of these 17 cases. Mutations in exons 4–9 were found in seven (Table 1) which were single-base substitutions and were located in exon 5 (three cases), exon 8 (three cases) and exon 7 (one case). Of the seven cases with single point mutation, all showed overexpression of the protein by FC and five of the seven showed monoallelic loss. The remaining seven cases had no mutations detected (No.s 1, 4, 5, 7, 13, 15, 17), of which three had a polymorphism in exon 4 (No.s 1, 7, 13). DNA was also sequenced in 14 cases (case no. 18–31), where no abnormal p53 was detected by either screening method. No mutations were found in 13 samples from untreated patients, but a frame shift mutation was found in one of these patients with refractory CLL (Table 1; Figure 3).

Specificity and sensitivity of FC and FISH in detecting *TP53* mutations

Using DNA sequencing as a 'gold standard' for detecting mutations of *TP53*, we calculated the specificity and sensitivity of FC and FISH in the 28 patients who had available DNA for sequencing. FC produced a sensitivity of 87.5% and a specificity of 80%. This compared favourably with FISH that gave a specificity of 83% and a sensitivity of 73%.

However; as it is generally accepted that sequencing will detect only mutations in more than 20% of cells in the sample and two of our patients had $<20\%$ deletion by FISH, thus these may be 'false negatives'.

Response to treatment

The incidence of p53 abnormalities in previously untreated patients was 6/88 (7%), which is similar to the described frequency in patients investigated at presentation in a recent publication by Oscier *et al.* [17] These untreated, yet progressive, patients were randomised to receive chlorambucil, fludarabine or

fludarabine in combination with cyclophosphamide as per the UK MRC CLL4 trial.

In refractory/pretreated CLL, the incidence of p53 abnormality was significantly higher and found in 12/24 (50 %), $P < 0.01$. The group with pretreated CLL had a median of three previous treatments including chlorambucil, fludarabine and CHOP (cyclophosphamide, vincristine, doxorubicin and prednisolone). Two-thirds of them were refractory to fludarabine. Of the 24 cases, including the patient with Richter's syndrome and one who went on to develop Richter's transformation, 12 patients had p53 abnormalities.

In all, 13 out of 24 (54%) with treated CLL are dead, and 10 of the 13 (77%) had detectable p53 abnormalities. In the previously untreated group of 88 patients, 10 (11%) are dead of which one-third had p53 abnormalities.

Survival was taken from date of diagnosis to last follow-up or date of death. The median follow-up was 30 months in this series. Survival analysis showed that the presence of a p53 abnormality (by any method) predicted for shorter survival (Figure 4). In fact, the presence of a deletion by FISH and/or protein expression by FC with no detectable mutation in exons 4–9 was sufficient to confer worse survival (Figure 5). Patients without abnormalities had a median survival of 268 (standard error 115) months and those with abnormal FISH or FC had a median survival of 64 (58) months. Patients with a detectable mutation had a median survival of 77 (13) months. Cox's regression analysis showed that the hazard ratios relative to the group with no abnormality were 3.9 (95% CI: 1.4–11.1) and 6.0 (95% CI: 2.2–16.7), respectively. Although the hazard ratio for the latter group was higher, it was not possible to demonstrate a significant difference in survival between patients with a detectable mutation and those with abnormal FISH or FC ($P = 0.42$).

Discussion

Abnormalities of the *TP53* gene, whether there are detected by protein expression,[1] FISH[9] or direct DNA sequencing,[18] all confer worse prognosis and drug resistance in CLL. However, it is not evident from the current literature whether all these tests need to be performed and how they correlate in the same population. Over the years, an increasing number of laboratory methods have been established to ascertain an abnormal *TP53* gene in human cancer.[19] Furthermore, it has been recently suggested that it is important to investigate abnormalities of the whole p53 mechanism, rather than the gene encoding for p53 alone, as other chromosomal abnormalities can decrease the p53 function. For example, trisomy 12[20] may lead to overexpression of mdm2, the inhibitory protein of p53. In addition, the ataxia telangectasia-mutated (ATM) protein can activate p53 by phosphorylation; thus ATM mutations may also have a detrimental effect on the normal function of p5321 as demonstrated by Lin *et al*.[22] in CLL.

Our study analysed the clinical impact and sensitivity of three methods to detect p53 abnormalities in 115 CLL patients, of which 90 were previously untreated and 25 heavily pretreated or had refractory disease. Using all three methods, we found 17 patients with p53 abnormalities (15%). The correlation between FC and FISH methods was seen in 10 of 17 cases. DNA sequencing detected one further patient with a frame shift mutation that had normal FISH and FC.

Sequencing of the *TP53* gene was performed on 28 patients, 14 who were abnormal by initial screening tests and 14 with apparently normal *TP53*. Out of 14, seven (50%) patients with abnormal screening had detectable mutations and one out of 14 (7%) with normal screening had a detectable mutation, suggesting that a combination of the two screening is a reliable method to detect *TP53* mutations ($P < 0.05$). FC was the most rapid

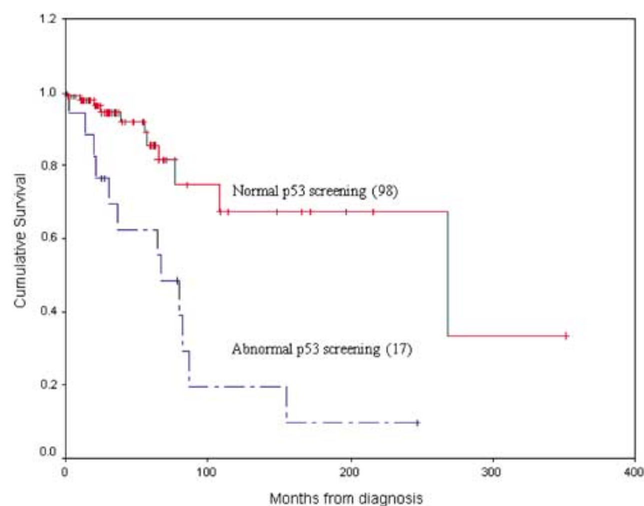

Figure 4 Survival by p53 status in two major groups: normal or abnormal by any screening (FISH/FC) method. The difference is statistically significant ($P = 0.0003$).

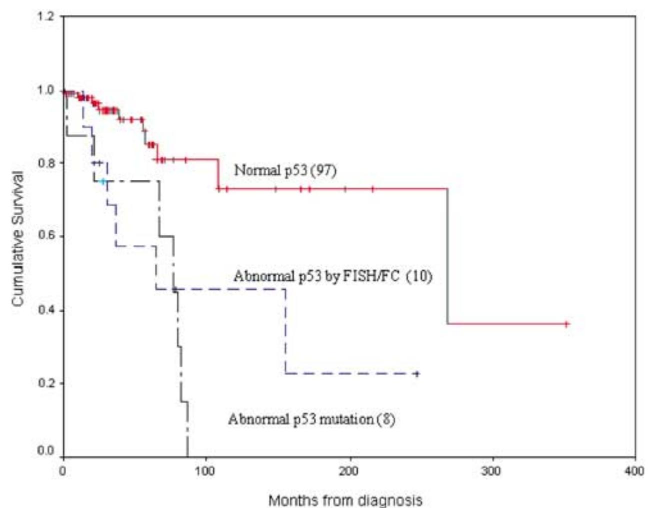

Figure 5 Survival according to p53 status in three groups ($P = 0.0003$). Cox's regression analysis shows no significant difference between abnormal FISH/FC with/without detectable *TP53* mutation on DNA sequencing.

method to determine abnormalities of p53, yet requires facilities to maintain a cell line for a positive control. However, this method should be amenable to most laboratories and is the least expensive with regard to equipment time and training.

We found that FC was more specific and sensitive than FISH in detecting mutations of *TP53* using DNA sequencing as our gold standard. Out of three patients, two with abnormal FC and yet with no obvious deletion by FISH had a mutation detected on sequencing. As these patients were tested relatively early in their disease course, our findings suggest that *TP53* mutation and protein expression may precede deletion in CLL, as demonstrated in colorectal and breast tumours.[23,24]

In our series, three patients with abnormal p53 expression showed no mutations on sequencing from exons 4 to 9. Overexpression of p53 suggests that the gene may be mutated, as mdm2, which neutralises p53, is unable to bind to a mutated protein and there is subsequent protein accumulation.[10–12] Another possibility is that mdm2 is absent in these cells as only wild-type p53 can act as a transcription factor for the mdm2 gene.[13] It is possible that these three patients had a mutation outside exons 4–9. Alternatively, p53 overexpression can also be caused by alterations in other members of the p53 pathway, extrinsic to *TP53* itself, causing stabilisation of the protein not due to mutation. Cases with protein expression with no apparent mutations have been previously described.[7,25–28]

FISH detects loss of genetic material from the 17p locus of *TP53*. Döhner *et al.*[9] found *TP53* deletion to be the strongest predictor for survival despite only finding mutations by SSCP in 64% of patients with deletion. We found FISH to have similar, but lower, specificity and sensitivity to FC in detecting *TP53* mutation using DNA sequencing as a gold standard. A possible explanation as to why seven of the patients here with *TP53* abnormalities by FISH had normal *TP53* sequence is either that mutation was present in a proportion of cells below the sensitivity of the technique or that other genes on 17p that may control p53 function were deleted. Deletion and mutation can be separate in time and thus, in some cases, deletion precedes the onset of mutation as shown in CML[29] and SLVL.[30]

Of the seven patients with abnormal screening tests, found to have mutations on sequencing, one had a point mutation in codon 273 (Figure 3). This is the most commonly mutated site within *TP53* with mutations resulting in defective DNA binding and loss of the ability of *TP53* to act as a transcription factor.[31] The other mutations were found at codons 143, 175, 176, 248, 277 and 278 located in the highly conserved regions of the *TP53* gene and occurred in two of the five 'hot spot' regions commonly mutated in *TP53*.[32]

Direct sequencing showed a polymorphism in exon 4 involving codon 72 changing CGC→CCC (arginine to proline) in three cases: one with abnormal p53 expression the only screening abnormality, one with a deletion and one with abnormal expression and a deletion. There are, however, no reports of abnormal p53 protein expression or deletions in individuals with this variant.[33] In one patient, a 2 bp deletion at codon 209 causing a frame shift and leading to a stop codon five amino acids on from the site of mutation. This patient had no deletion and no protein expression. The lack of protein expression is most probably caused by the stop codon, which triggers a protein terminated within the DNA-binding domain in exon 6. Although the antibody used recognises the N-terminus of the protein, the protein product of this mutation does not oligomerise and must be folding in a way that renders it unrecognisable for the antibody.

p53 abnormalities in CLL are felt to be late-acquired events in the disease process.[34] Therefore, one would expect a much greater proportion of p53 abnormalities in the pretreated/refractory CLL group. The current study confirms this is as only 7% abnormalities were found in the previously untreated group, whereas 50% of the pretreated group had abnormalities detected. Survival curves show poor survival of patients with abnormal p53 regardless of the technique used. Five patients with deletion by FISH, yet no mutation, had aggressive disease behaviour, perhaps due to haploinsufficiency of *TP53*[35] or loss of other tumour suppressor genes on 17p as has been suggested in malignant gliomas by Frankel *et al.*[24] We feel therefore that routine DNA sequencing is unlikely to add to combined FISH and FC screening when endeavouring to segregate a CLL population with worse prognosis due to p53 abnormalities.

A number of authors have reported the detection of new clones with p53 abnormalities on sequential studies, which changes the behaviour of the disease or in some cases associated with transformation to high-grade lymphoma.[2,18,36] A recent study by Barnabas *et al.*[37] found p53 mutations in 47% of CLL cases and suggested that mutations did not predict clinical outcome. Our study involves a larger group of patients including 88, previously untreated (but requiring treatment) patients entered into a clinical trial. Our findings are in agreement with previous studies in that p53 is associated with poor response to therapy and more aggressive disease course.[1,2,8,9] The incidence of 15% is also in agreement with the frequency of p53 abnormalities in CLL reported by previous authors.[18,38,39]

In conclusion, our findings confirm that p53 is associated with more aggressive disease, shortened survival and resistance to treatment. The mutations we found occur at the known hot spots. A combination of testing for deletion of *TP53* by FISH and protein expression by FC is an effective method for screening patients for p53 abnormalities who are more likely to follow a more aggressive disease pattern.

Acknowledgements
PT was supported by a clinical fellowship from the Leukaemia Research Fund and AMGW was supported by a grant from the Arbib Foundation. We are grateful to participants in the LRF CLL4 trial for supplying samples from their patients.

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