J Hematopathol (2010) 3:61–68
DOI 10.1007/s12308-010-0068-2

ORIGINAL ARTICLE

p53 protein overexpression in bone marrow biopsies from chronic lymphocytic leukaemia is associated with *TP53* deletion and resistance to fludarabine

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Received: 30 July 2010 / Accepted: 14 September 2010 / Published online: 5 October 2010
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Abstract Abnormalities of the *TP53* gene in chronic lymphocytic leukaemia (CLL) are associated with large cell transformation, short survival and resistance to purine analogue therapy. Deletion of one allele and somatic mutation of the remaining allele have been described as the main mechanism of *TP53* inactivation in CLL, but its relationship with p53 protein expression remains unclear. We studied 103 CLL patients using fluorescence in situ hybridisation (FISH) to detect allelic loss at chromosome 17p and immunohistochemistry (IHC) to test for p53 protein overexpression. *TP53* deletion (≥10% of cells) was found in 21 cases (20.4%) and no deletion in 82 (79.6%). By IHC, 16 cases (15.5%) showed p53 protein expression and 87 (84.5%) were negative. There was a good correlation between FISH and IHC in 86 cases (83.5%; $p<0.001$) and these comprised ten cases positive for both assays and 76 negative cases. The remaining 17 cases had discrepant results: 11 cases showed *TP53* deletion and were p53 negative, and six cases had strong expression of protein and no *TP53* deletion (FISH). Seventy-two patients (70%) received fludarabine. The majority (86%) of patients without abnormalities of *TP53* responded to fludarabine and only eight cases were resistant. Within the rest, all patients positive with both methods were refractory, 60% of cases with overexpression without deletion and 40% of cases with deletion without protein overexpression were non-responders to fludarabine. Our findings indicate that IHC is a simple method and provides useful complementary information to FISH analysis for the evaluation of *TP53* dysfunction. Both methods can be carried routinely to identify patients with a high chance to be resistant to fludarabine containing regimens ($p=0.0003$).

Keywords Chronic lymphocytic leukaemia · *TP53* · FISH · Immunohistochemistry

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Introduction

B-cell chronic lymphocytic leukaemia (CLL) is characterised by a highly variable clinical course [1]. A number of biological markers such as mutational status of the immunoglobulin heavy chain variable gene (IGVH) [2, 3], CD38 and ZAP-70 expression [4–8] and cytogenetics, particularly *TP53* and *ATM* abnormalities, have been shown to have prognostic impact to predict the disease course in patients with early stages and/or response to treatment in those with progressive disease [9–11].

The *TP53* tumour suppressor gene, located on chromosome 17p13.1, encodes a nuclear 53-kDa phosphoprotein that plays a key role in response to cellular stress conditions by inducing

the transcription of genes controlling cell cycle arrest and induction of apoptotic cell death. *TP53* is one of the most commonly mutated genes in human cancers. In lymphoid malignancies, the frequency of *TP53* abnormalities is low compared to solid tumours and varies with histological subtype and aggressiveness of the tumour [12]. In CLL, allelic loss of 17p and/or somatic mutation of the remaining *TP53* allele have been described as the main mechanism of *TP53* inactivation [13]. Mutant p53 cells lose the ability to bind p53-binding sites and/or changes the global conformation of the heterodimeric protein complex, leading to partial or complete loss of p53 protein functions [14]. Several studies have shown that wild-type p53 function is required for the cell to undergo apoptosis induced by genotoxic damage, and thus, tumours expressing mutant or deleted *TP53* are likely to be chemoresistant to a wide range of anticancer drugs. Higher levels of p53 protein seem to result from a longer half-life of the mutated protein, arising from conformational changes that stabilise the protein. This abnormal long half-life of the protein allows its detection in the nucleus of the cells by immunohistochemical (IHC) techniques.

It is not clear whether expression of the non-functional aberrant p53 protein correlates with a mutated or deleted *TP53* gene [15]. Although early studies suggested that this is the case, others have documented a lack of correlation and suggested that the *TP53* pathway may also be disrupted in wild-type p53 tumours by other mechanisms like *MDM2* amplification or mutations of the gene encoding *ATM* [16]. Although an association between protein overexpression and poor survival or non-response to therapy has been reported in CLL [5, 17–19], it still remains unclear which assay is the best to use routinely to detect *TP53* dysfunction and predict drug resistance.

We have investigated the presence of *TP53* deletion (fluorescence in situ hybridisation (FISH)) and p53 protein expression (IHC) to establish the extent of correlation between these two assays, and their impact in terms of resistance to treatment with fludarabine.

Materials and methods

Patients and specimens

Blood and bone marrow samples from 103 CLL patients (22 at diagnosis and 81 at follow up) investigated and followed at the Royal Marsden Hospital were analysed. This series did not include patients entered into the UK CLL-4 study. The diagnosis of CLL was based on morphology and immunophenotype according to the World Health Organisation classification [20]. All patients had immunophenotypic CLL scores [21] greater than three. FISH analysis was carried out on isolated peripheral blood mononuclear cells and expression of p53 was performed by IHC in bone marrow biopsies. Expression of CD38, ZAP-70 and IgVH mutations were performed as previously described [22]. Informed consent was obtained from all patients. Cases were drawn from the RMH CLL database and were selected those where FISH and IHC were investigated at the same time point.

Fluorescence in situ hybridisation

Peripheral blood lymphocytes were separated by density gradient centrifugation, treated with hypotonic solution (KCl) and fixed with methanol-acetic acid. FISH analysis was performed using standard methods as previously described [19]. A *TP53* locus specific probe (LSI p53 and CEP 17, Vysis, Downers Grove, IL, USA) in combination with a probe specific for chromosome 17 centromere (CEP17, Spectrum Green, Vysis) were used. Cells from ten healthy donors were used as control specimens. A total of 200 nuclei were scored per patient's sample by two individuals. A threshold of ≥10% deleted cells was considered to be clinically significant according to the results of the LRF CLL-4 trial [23].

Immunohistochemistry

Staining was performed on deparaffinized 3 μm sections of routinely processed paraffined embedded tissue following heat-induced antigen retrieval using a monoclonal antibody to p53 protein, (clone NCL-p53-BP, Novocastra, Laboratories, Newcastle upon Tyne, UK) at a 1:50 dilution. Peroxidase enzyme staining with diaminobenzidine was used to visualise the p53 protein-positive cells. A case was considered positive when there was a strong nuclear staining in at least greater than 10% of cells [24]. Intensity of staining was considered to be more relevant than number of positive cells; those cases with few or weakly p53 positive cells with the staining confined to the proliferation centres were considered negative.

Statistical analysis

The correlation between *TP53* deletion and IHC expression was assessed with the Fisher's exact test using SPSS software (version 15.0).

Results

Patients

Clinical features (Binet stages), treatment, genetic abnormalities other than *TP53* deletion, mutational status of the

IgVH and CD38/ZAP70 expression are summarised in Table 1.

There were 58 males and 45 females, with a median age at diagnosis of 54 years. At the time of this study close to a half of patients had stable stage A and the remaining had stages B and C or progressive stage A. Nineteen cases (18.5%) had not been previously treated, and 84 cases (81.5%) were previously treated prior to the *TP53* analysis. Among the latter, 72 (85.7%) received fludarabine (36 cases as first line of treatment) either alone (19 cases) or in combination (17 cases). Alemtuzumab with or without methyl-prednisolone was given to 31 (36.9%) patients and in six of them as first line. FISH analysis showed the presence of at least one chromosome abnormality in 68 (66%) cases. Deletion of 13q14 was found in over half of the patients and it was associated with either trisomy 12 or del11q (*ATM*) in a third of them. Two thirds of the cases had unmutated IGVH and cells from greater than a third and two thirds of cases were ZAP-70+ and CD38+, respectively (Table 1).

Table 1 Patient characteristics

No. of cases	103
Age, years	54.5 (25–78)
Male–female ratio	1.3 (58 M/45F)
Stage	
A stable	48 (46.6%)
A progressive	15 (14.6%)
B	17 (16.5%)
C	10 (9.7%)
Unknown	13 (12.6%)
Interphase cytogenetics	
del(11q)	19/99 (19.2%)
+12	20/99 (20.2%)
del(13q)	55/99 (55.5%)
IgVH mutation status[a]	
IgH unmutated	45/72 (62.5%)
IgH mutated	27/72 (37.5%)
ZAP70+	35/93 (37.6%)
CD38+	70/97 (72.2%)
No treatment	19/103 (18.5%)
Treatment	84/103 (81.5%)
Fludarabine	72/84 (85.7%)
1st line	36 cases
	19 FL
	17 in combination (12 FC, 5 FCR)
Alemtuzumab	31/84 (36.9%)
1st line	6 cases

[a] Cutoff of 98%

FL fludarabine alone, *FC* fludarabine plus cyclophosphamide, *FCR* fludarabine plus cyclophosphamide plus rituximab

Fluorescence in situ hybridization and IHC analysis of TP53

Out of 103 CLL cases, 21 (20.4%) had ≥10% of cells with deletion of one *TP53* allele and no deletion (<5%) was found in 82 cases (79.6%). Out of the 21 *TP53* deleted cases, 13 had >20% cells with the gene deletion. No single case showed biallelic deletion of *TP53*. By IHC, 16 cases (15.5%) showed strong nuclear protein staining in the majority of cells and 87 (84.5%) were p53 negative. Concordance between FISH and IHC was found in 83.5% cases: 76 cases (73.8%) were negative for both assays (FISH and IHC) and ten cases (9.7%) were positive for FISH and IHC (Table 2). Deletion of *TP53* gene and p53 expression were strongly correlated ($p=0.00006$). The remaining 17 cases (16.5%) had discrepant results as follows: 11 cases showed *TP53* deletion by FISH and were p53 negative (IHC), and six cases had strong expression of p53 (IHC) in >10% of cells and no evidence of *TP53* deletion (FISH). A total of 27 cases showed one or both abnormalities (Fig. 1).

Correlation between TP53 abnormalities and response to fludarabine-based regimens

Responses [25] to fludarabine containing regimens according to *TP*53 status in each group (concordant and discordant cases) are shown in detail together with other prognostic factors in Tables 3 and 4 and summarised in Table 5. The vast majority of patients without abnormalities of *TP53* (86%) responded to fludarabine whilst only a minority (eight cases; 14%) without *TP53* abnormalities by FISH and IHC were resistant (Table 5).

The presence of *TP53* abnormalities by FISH and/or IHC was significantly ($p=0.0003$) associated with resistance to fludarabine alone as well as fludarabine-containing regimens (fludarabine plus cyclophosphamide, fludarabine plus cyclophosphamide plus rituximab).

All patients positive with both methods that received fludarabine were refractory and all these non-responders were CD38 positive and half of them had somatic mutations in the IgVH; no case had *ATM* deletion and two cases had trisomy 12 (Table 4). In this concordant group, three patients received first line Alemtuzumab in combination with high-dose methyl-prednisolone, and one patient Alemtuzumab alone and all four achieved a complete response. Five of the fludarabine refractory patients subsequently received Alemtuzumab after more than three lines of treatment achieving a good response.

In the IHC-positive FISH-negative discordant group, five out of six patients received fludarabine (two alone and three in combination), three of them (60%) being refractory.

Table 2 Correlation between FISH status and IHC results

	IHC negative	IHC positive	Total	
FISH negative	76 (73.8%)	6 (5.8%)	82 (79.6%)	
FISH positive	11 (10.7%)	10 (9.7%)	21 (20.4%)	$p = 0.00006$
	87 (84.5%)	16 (15.5%)	103 (100%)	

FISH positive: in ≥10% of cells TP53 loss

Only one of the non-responders was treated with Alemtuzumab achieving a complete remission.

In the other FISH-positive IHC-negative discordant group, six out of 11 patients received fludarabine (four alone and two in combination) and a third of them were refractory. Two cases received Alemtuzumab as first line treatment achieving a complete remission.

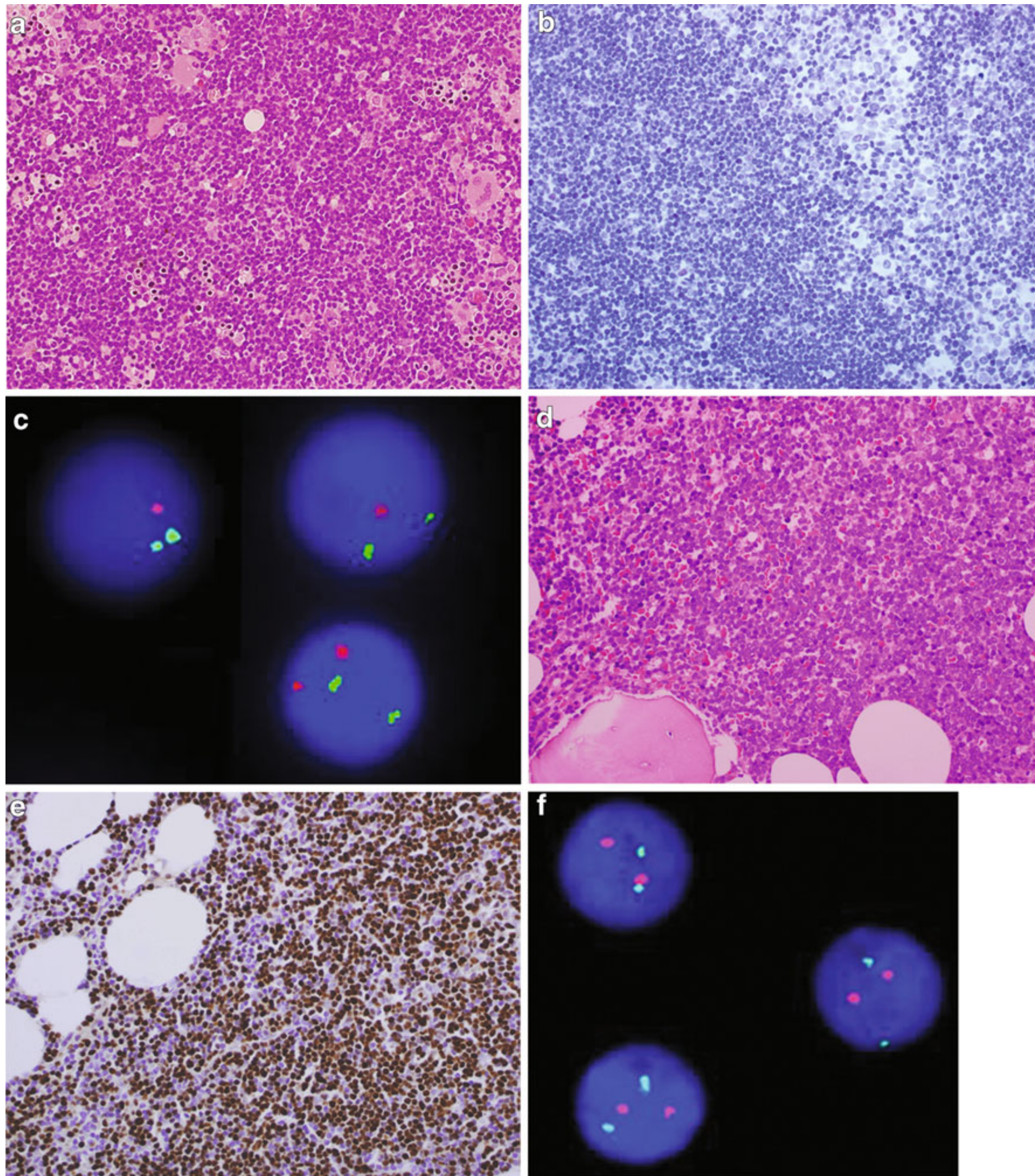

Fig. 1 Bone marrow biopsy of B-CLL with interstitial and nodular infiltration by small lymphoid cells H&E magnification ×40 (**a**). Neoplastic cells are negative for p53 (**b**) with monoallelic TP53 deletion by FISH (**c**). **d** Bone marrow biopsy of B-CLL (H&E magnification ×40) with a nodular and dense infiltrate of lymphoid cells with marked overexpression of p53 (**e**). This case does not have deletion of TP53 by FISH (**f**)

Table 3 Correlation between *TP53* abnormalities and response to therapy

Sample	IHC overexpression	FISH deletion	Treatment	Response to fludarabine
1	+	+	1st FL, 2nd FC	No response
2	+	+	Campred	Complete Remission
3	+	+	FL	No response
4	+	+	Campred	Complete remission
5	+	+	No treatment	
6	+	+	Campred	Complete remission
7	+	+	Campath	Complete remission
8	+	+	1st FL, 2nd FC	No response
9	+	+	FL	No response
10	+	+	FC	No response
11	−	+	Campred	Complete remission
12	−	+	FL	No response
13	−	+	No treatment	
14	−	+	Cyclophosphamide	Autoimmune disease
15	−	+	Chlorambucil	Progression
16	−	+	Campred	Complete remission
17	−	+	FL	Good response
18	−	+	FL	Good response
19	−	+	1st FL, 2nd FC	No response
20	−	+	1st FL, 2nd FC	Good response
21	−	+	1st FL, 2nd FCR	Good response
22	+	−	1st FL, 2nd FC	No response
23	+	−	1st FL, 2nd FCR	No response
24	+	−	Chlorambucil	Autoimmune disease
25	+	−	FL	Good response
26	+	−	1st FL, 2nd FC	Good response
27	+	−	1st FL, 2nd FCR	No response

TP53 deletion + when present in ≥10% of cells

FL fludarabine alone, *FC* fludarabine plus cyclophosphamide, *FCR* fludarabine plus cyclophosphamide plus rituximab

Discussion

The prognostic impact of 17p (*TP53)* deletion in CLL patients has been documented in a number of single centre studies and randomised clinical trials [26–30]. However, the role of p53 protein expression and its correlation with deletion and mutations of the *TP53* is uncertain and not well established. Some reports indicate that p53 overexpression is more sensitive than mutational analysis of the gene for predicting the risk of progression in CLL [31, 32]. In contrast, Döhner et al. [9] have documented that *TP53* deletion is the strongest predictor for survival regardless of the presence of gene mutations analysed by single-stranded conformation polymorphism, and Grever et al. [33] described that *TP53* mutations in the absence of 17p deletion do not have an independent negative impact on progression free survival. Thus, the best and optimal assay to establish the presence of *TP53* abnormalities which is clinically significant is unknown.

We have analysed a series of 103 CLL patients from a single centre and they were selected on the basis that FISH and IHC were investigated at the same time point. There was not a bias for selection but our cohort included younger patients with a higher incidence of trisomy 12, p53 deletion, and unmutated IgVH than are seen in CLL. Our findings have shown that there is a good but not complete concordance between the presence of p53 protein overexpression and deletion of the *TP53* gene. Although there have been several studies analysing the presence of p53 abnormalities in CLL, most of them focused in 17p deletion and *TP53* mutations, and only a few investigated the presence of protein accumulation [34, 35]. The frequency of protein expression (15%) in our study is similar to that reported by Cordone et al. [17] using immunocytochemistry in peripheral blood lymphocytes. It was more often (77%) present in patients with advanced stages or progressive disease supporting previous studies [36, 37]. Flow cytometry (FACS) analysis has been proposed as an objective method for detecting p53 dysfunction [19]. However, unlike IHC, flow cytometry requires fresh or live cells and these are not always available. The advantage of bone marrow biopsies is that it can be done prospectively as well as retrospectively.

As expected, cases with concordant results and treated with fludarabine were resistant to the drug. The possibility

Table 4 Cytogenetic features, IgVH status and CD38 in cases with *TP53* abnormalities

Sample	IHC overexpression	TP53 deletion	+12	del(11q)	del(13q)	CD38	IgVH
1	+	+	−	−	−	+	Mutated
2	+	+	−	−	−	+	Unmutated
3	+	+	+	−	+	+	Mutated
4	+	+	−	−	−	+	Unmutated
5	+	+	−	+	+	+	Unmutated
6	+	+	−	−	−	+	Unmutated
7	+	+	ND	ND	ND	−	ND
8	+	+	ND	−	ND	+	ND
9	+	+	+	−	−	+	Unmutated
10	+	+	−	−	+	+	ND
11	−	+	−	−	−	−	Unmutated
12	−	+	+	−	+	+	Unmutated
13	−	+	−	−	−	−	Unmutated
14	−	+	−	−	+	+	Unmutated
15	−	+	−	−	+	+	Unmutated
16	−	+	−	−	−		Unmutated
17	−	+	−	−	−	+	Mutated
18	−	+	+	−	+	+	Unmutated
19	−	+	−	+	−	−	Unmutated
20	−	+	−	+	+	+	Unmutated
21	−	+	−	−	ND	+	ND
22	+	−	−	−	+	−	Mutated
23	+	−	+	−	−	+	Unmutated
24	+	−	−	−	−	+	ND
25	+	−	−	−	−	+	Unmutated
26	+	−	−	−	+	ND	ND
27	+	−	+	−	−	+	ND

TP53 deletion + when present in ≥10% of cells

ND Not determined

that *TP53* deletion could have been developed following fludarabine treatment by selecting a small resistant clone and/or these patients had been developed myelodysplasia could be entertained. However these scenarios are unlikely to be the responsible of the *TP53* dysfunction as patients were primarily resistant to fludarabine and there was no evidence of dysplastic features in the bone marrow. Drug resistance was not significantly associated with IgVH mutational status, ATM deletion or trisomy 12 in this study.

Of interest is the finding of 17 cases in which the results between del17p and p53 protein expression were discordant, and specially those six cases without deletion and with strong p53 expression. Considering the IHC positive and FISH negative group, two thirds of these cases treated with fludarabine were refractory.

Table 5 Response to fludarabine according to the TP53 status

Fludarabine treated patients/no. of tested	FISH (≥10%)	IHC	% Refractory cases
56/76	Negative	Negative	14% (8/56)
5/10	Positive	Positive	100% (5/5)
5/6	Negative	Positive	60% (3/5)
6/11	Positive	Negative	33% (2/6)

Mutations of the *TP53* gene were carried out by single-strand conformation analysis and confirmed by direct sequencing [38] in 25 of the 27 patients that had *TP53* abnormalities by one or both methods (data not shown). Mutations of the gene were identified in five of the 25 samples tested. Out of these five mutated cases, three had both *TP53* deletion (>20% of cells) and marked over-expression of the protein, one case had p53 protein expression and no gene deletion and the remaining case had *TP53* deletion (88% of cells) and no protein expression. Therefore there was evidence of *TP53* dysfunction by the two or at least one method in all five mutated cases. Despite the lack of detectable mutations in the remaining cases, patients with deletion of the gene or protein expression were fludarabine resistant.

In agreement with recently published data [26, 39–41], most of the non-responders to fludarabine in our series responded to Alemtuzumab alone or in combination with

prednisolone supporting the efficacy of these schedules in patients with 17p deletion.

In conclusion, our findings demonstrate that IHC is a simple and reliable method and a useful prognostic tool complementary to FISH analysis for the evaluation of *TP53* abnormalities in CLL. Both methods can be carried out in routine practice to identify patients with a high chance to be resistant to fludarabine-containing regimens. Although the number of patients positive with both methods is low and studies on larger numbers of cases are needed, our findings suggest that IHC may allow identifying refractory patients without FISH abnormalities of the *TP53* gene. The potential benefit of combining p53 protein expression by IHC and FISH for 17p deletion to detect poor risk CLL should be considered.

Acknowledgment This work has been partially supported by Fondo de Investigaciones Sanitarias. PM is in receipt of research grant from the ISCIII.

Conflict of interest The authors declare that they have no conflict of interest.

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