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LETTER TO THE EDITOR

Functional assessment of p53 in chronic lymphocytic leukemia

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Several prognostic factors are used in chronic lymphocytic leukemia (CLL) to predict disease progression at diagnosis and to help guide therapeutic choices. Among these factors, the detection of deletions of the short arm of chromosome 17, where the tumor suppressor gene *TP53* is located at the 17p13 locus, predicts resistance to standard treatments and poor prognosis.[1] Deletions of 17p13 are observed in 5–7% of CLL patients at diagnosis and in 25–40% of cases with advanced refractory disease. Other markers of poor prognosis have been described, such as unmutated *IGHV* gene status, high levels of thymidine kinase and soluble CD23, CD38 and ZAP-70 expression, as well as other chromosomal aberrations, such as 11q23 deletion. However, defects in the *TP53* pathway consistently appear as the most significant adverse prognostic factor in CLL.[2]

About 80% of patients with 17p13 deletion display a mutation in the remaining *TP53* allele, resulting in loss of function of the p53 protein, but mutations without a deletion are observed in 4–5% of cases.[3] Mutations of *TP53* have been identified that are consequently associated with an unfavorable outcome, but all mutations do not predict similar consequences on the p53 pathway. In addition, a minority of patients with 17p13 deletion have an indolent clinical course, suggesting that p53 function is preserved. Finally, it has appeared that p53 dysfunction is the result of several intricate factors that have not been clearly defined, and several questions still remain to be addressed regarding the biological consequences of *TP53* deletions and the various types of mutations.

Consequently, it has become evident that new techniques to determine p53 function are required to identify patients with *TP53* abnormalities (deletion and/or mutation), despite normal function, and patients who have *TP53* neither mutated nor deleted, but which is dysfunctional for other reasons, including

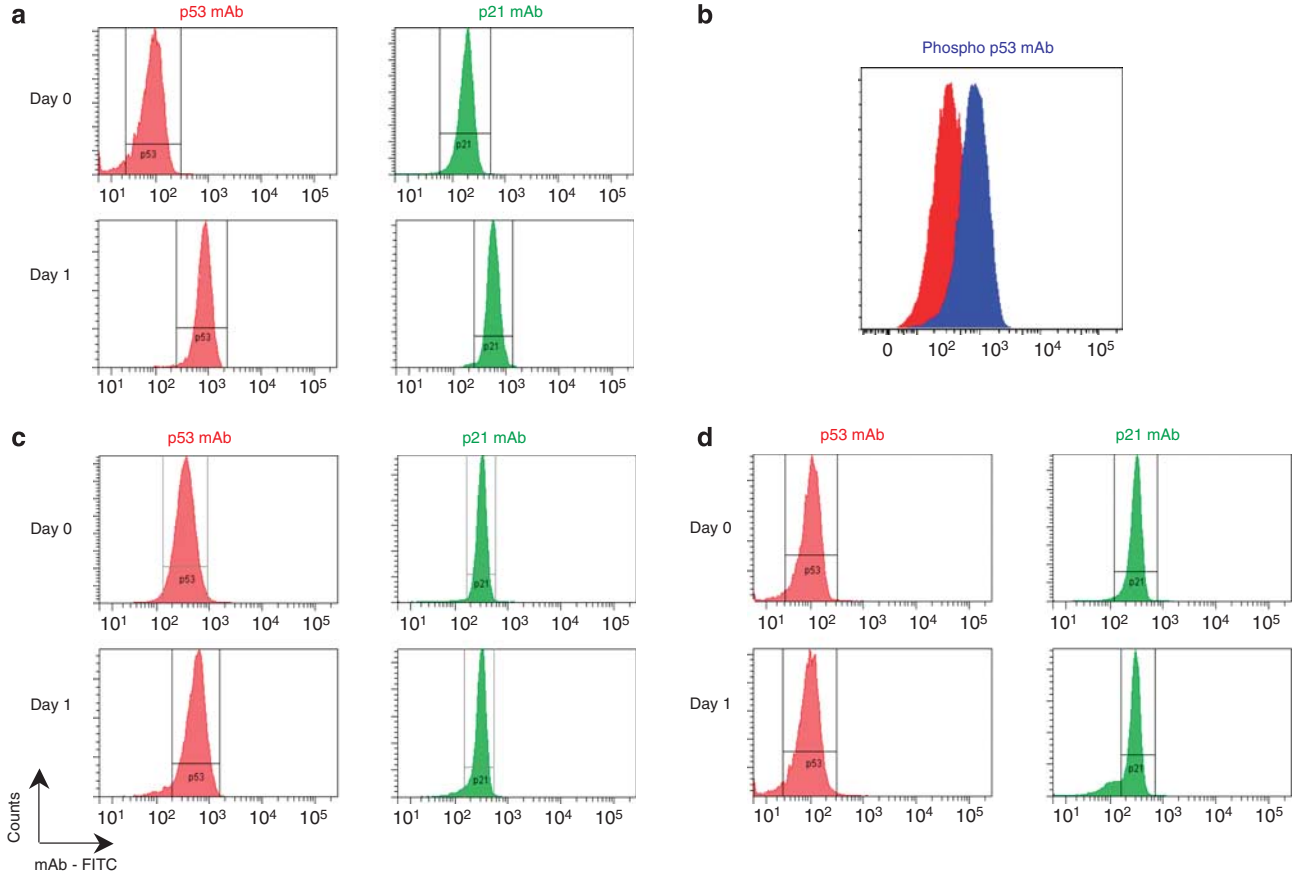

Figure 1 Cytometric functional test for p53/p21 proteins in cells from patients with chronic lymphocytic leukemia (CLL). Profiles of response after 24-h *in vitro* culture of CLL cells in the presence of etoposide and nutlin-3a. (**a**) Type 1 profile: normal p53 function with undetectable p53 protein on day 0, p53 and p21 increased by day 1. (**b**) B cells from a representative type 1 profile were untreated (red) or treated with etoposide and nutlin-3a for 6 h (blue), and p53 Serine 15 phosphorylation was assessed by flow cytometry. (**c**) Type 2 abnormal profile, with a high baseline level of p53 protein and no increase in p53/p21 by day 1. (**d**) Type 3 abnormal profile, with undetectable baseline p53 protein and no increase in p53/p21 by day 1.

Table 1 Cytogenetics, mutational status and functional cytometric profiles of *TP53* in patients with chronic lymphocytic leukemia

Patient no.	*Functional category (1,2,3)*	*FISH*[a]		*TP53 mutation*
		TP53 del[b]	*ATM del*[b]	*Exon/intron*
1	1	–	–	UM
2	1	–	–	UM
3	1	–	–	UM
4	1	–	–	UM
5	1	–	–	UM
6	1	–	–	UM
7	1	–	–	UM
8	1	–	–	UM
9	1	–	–	UM
10	1	–	–	UM
11	1	–	–	UM
12	1	–	–	UM
13	1	-	–	UM
14	1	–	–	UM
15	1	–	+ (72%)	UM
16	1	–	+ (65%)	UM
17	1	–	+ (55%)	UM
18	1	–	+ (92%)	UM
19	1	–	+ (95%)	UM
20	1	–	+ (91%)	UM
21	1	+ (21%)	+ (97%)	UM
22	1	+ (17%)	–	UM
23	1	–	+ (76%)	Exon 6/p.R196P
24	2	+ (86%)	–	Exon 8/p.R273H
25	2	+ (80%)	–	Exon 5/p.H178D
26	2	+ (70%)	–	Exon 5/p.R175H
27	2	+ (94%)	–	Exon 8/p.R273H
28	2	+ (79%)	–	Exon 7/p.R248E
29	2	+ (6%)	–	Exon 7/p.R249S
29 bis	1	–	–	UM
30	2	+ (53%)	–	Exon 5/p.P142R and exon 6/m.P222L
31	2	+ (91%)	–	Exon 8/p.V272M
32	2	–	–	Intron 9/IVS9+2A>G
33	2	–	+ (84%)	UM
34	3	+ (87%)	–	Intron 4/IVS4+1G>A
35	3	+ (88%)	–	Intron 5/IVS5+1G>T
36	3	+ (52%)	–	Intron 6/IVS7−2A>A
37	3	+ (17%)	–	Exon 8/p.Del264L

Abbreviations: FISH, fluorescence *in situ* hybridization; UM, unmutated.
[a]200 Interphasic nuclei were counted.
[b]Monoallelic deletion.

epigenetic gene silencing or post-translational modification. These considerations prompted us to determine in a series of 37 CLL patients the functional status of p53 by using a cytometric method that was adapted from the methodology previously described by Best *et al*.[4] We then compared results with the cytogenetic data and mutational status of *TP53* of this population of patients.

Determination of the functional status of p53 in CLL cells by flow cytometry was based on induction of p53 and p21 protein expression using etoposide and nutlin-3a. Three types of response were observed. The type 1 profile corresponded to normal function (Figure 1a): as expected the baseline expression of p53 on day 0 was undetectable (threshold: mean fluorescence intensity <100), but by day 1, a clear-cut increase in the percentage of p53 expression was observed (increased from 100 to 600%). In parallel, a moderate increase of p21 expression was also observed on day 1 ($>10\%$). To confirm the functionality of p53 protein, we tested its phosphorylation at Serine 15 in response to etoposide and nutlin-3a treatment (Figure 1b). This phosphorylation, which results in p53 stabilization and accumulation in the nucleus, is a typical hallmark showing p53 activation.[5] The type 2 profile (Figure 1c) was defined by a high level of p53 protein on day 0 without any (or with a slight) increase in p53 or p21 by day 1 in the presence of etoposide and nutlin-3a. The type 3 profile (Figure 1d) was characterized by undetectable p53 protein on day 0, and no change in p53 and p21 expression after *in vitro* culture in the presence of etoposide and nutlin-3a.

Cytogenetic data, mutational status and results of the functional tests are listed in Table 1. We observed that a normal p53/p21 expression profile (type 1) corresponded to patients without any *TP53* abnormalities (20 patients, no. 1–20) or with a monoallelic *TP53* abnormality (3 patients, no. 21–23), even though an ATM deletion may be present (cases no. 15–21, 23). In these patients with a normal response, the only case displaying a *TP53* mutation had a monoallelic mutation on exon 6 (no. 23). It is important to note that eight patients with a normal p53/p21 response, who had been tested for p53 phosphorylation, displayed normal results in all cases: this allowed us to confirm the functionality of the protein.

Nine out of 10 patients with a cytometric type 2 expression profile had biallelic abnormalities: 8 patients (no. 24–31) had

Table 2 Results of p53/p21 measured by flow cytometry, according to the types of expression profiles

	n	*Baseline p53*	*% Increase p53*	*% Increase p21*
Type 1	23	50 (15–90)	314 (118–496)	84 (15–395)
Type 2	10	246 (108–775)	52 (0–134)	3 (0–22)
Type 3	4	62 (37–87)	24 (0–71)	2 (0–6)

Baseline p53: average values and extremes correspond to the mean fluorescence intensity.

17p deletion and *TP53* gene mutations on exons 5, 7 or 8, and 1 patient had a biallelic mutation on a splice intron site (intron 9: IVS9 + 2A > G; no. 32).

The last patient of this group with type 2 dysfunctional p53 (no. 33) had no *TP53* abnormality (no deletions or mutations). To note that he had an aggressive disease resistant to treatment, and he died rapidly after a relapse. This pejorative outcome may be explained by a dysfunctional p53 pathway.

In the subgroup of the four patients with a type 3 p53/p21 expression profile, all had biallelic abnormalities: three patients had mutations located in a splice intron site (introns 4, 5 and 6; no. 34–36) and the fourth had a punctual deletion in an exon (no. 37). The results of these functional tests are summarized in Table 2.

Our data show that this functional assay is very sensitive. Indeed, we detected a non-functional p53 in two patients in whom a small clone was detected by fluorescence *in situ* hybridization (no. 29: 6% and no. 37: 17%), and in whom mutations were not detected by the initial mutational analyses. For one of these two patients, *TP53* status could be studied longitudinally by comparing the *TP53* abnormalities (no. 29) with a previous sample taken 5 years before (no. 29 bis). Very interestingly, this previous sample did not have any *TP53* abnormalities (no deletions or mutations), and our cytometric test concluded that it had functional p53 at that time (functional category 1).

Other aberrations than deletions/mutations of *TP53* gene that can alter function of p53 pathway have been described. Deletion of chromosome 11 at the locus of the *ATM* gene (11q22 deletion) may decrease p53 activity. Although some authors postulated that in patients with low ATM protein levels, p53 may not be sufficiently activated for induction of apoptosis in response to DNA damaging agents, we found normal function of p53 in 6/7 cases where ATM deletion was observed without any *TP53* abnormality. This may be explained by the demonstration by Kojima *et al.*[6] that Nutlin-3a activates p53 and induces ATM-independent apoptotic activity. The ubiquitine ligase double-minute protein that controls p53 function may be overexpressed in cases of single-nucleotide polymorphism (SNP309), leading to disabled p53 functionality.[7] MicroRNA-34a has been identified as a major downstream target of p53, and it has been demonstrated that low levels of miRNA-34a are linked to chemotherapy resistance and can impair p53 function.[8]

The functional cytometric test described here is a simple and quick method to detect dysfunction of the p53 protein. The results of the present study allow us to identify cases where a functional test could dramatically change the treatment strategy and the prognosis of the patient. This study allowed us to define the feasibility and reliability of this functional assay, and enabled us to propose its routine usage as a therapeutic strategy for CLL patients at least to complete if not to replace cytogenetic and mutational data. In addition, this cytometric functional test contributes to unraveling the complexity of the p53 pathway.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000; **343**: 1910–1926.
2 Oscier DG, Gardiner AC, Mould SJ, Glide S, Davis ZA, Ibbotson RE *et al.* Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood* 2002; **100**: 1177–1184.
3 Zenz T, Kröber A, Scherer K, Häbe S, Bühler A, Benner A *et al.* Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood* 2008; **112**: 3322–3329.
4 Best OG, Gardiner AC, Majid A, Walewska R, Austen B, Skowronska A *et al.* A novel functional assay using etoposide plus nutlin-3a detects and distinguishes between ATM and TP53 mutations in CLL. *Leukemia* 2008; **22**: 1456–1459.
5 Bode Ann M, Dong Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* 2004; **4**: 793–805.
6 Kojima K, Konopleva M, McQueen T, O'Brien S, Plunkett W, Andreeff M. Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. *Blood* 2006; **108**: 993–1000.
7 Gryshchenko I, Hofbauer S, Stoecher M, Daniel PT, Steurer M, Gaiger A *et al.* MDM2 SNP309 is associated with poor outcome in B-cell chronic lymphocytic leukemia. *J Clin Oncol* 2008; **26**: 2252–2257.
8 Zenz T, Mohr J, Eldering E, Kater AP, Bühler A, Kienle D *et al.* miR-34a as part of the resistance network in chronic lymphocytic leukemia. *Blood* 2009; **113**: 3801–3808.