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# p53 abnormalities in CLL are associated with excess of prolymphocytes and poor prognosis

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**Summary.** To determine the role of the p53 gene in chronic lymphocytic leukaemia (CLL) and its possible involvement in the pathogenesis of a progressive form of CLL characterized by > 10% prolymphocytes (CLL/PL), we selected 32 cases, 17 with typical morphology and 15 CLL/PL. The extent of inactivation of p53 was examined by assessing loss of heterozygosity (LOH) at 17p13.3, by sequencing the highly conserved region (exons 5–9) of the p53 gene and by analysing p53 protein expression. LOH was detected in 8/28 (29%) cases, p53 mutations in 5/32 (16%) cases and p53 expression in 5/27 (19%) cases. Overall 11 cases (30%) had p53 abnormalities of which eight cases had CLL/PL. There was a significant association between CLL/PL and p53 abnormalities ($P = 0{\cdot}05$); 75% of cases with LOH, 80% of p53 mutations and 80% of cases positive for p53 protein had CLL/PL. Thus, p53 inactivation is the first gene abnormality identified so far to be involved in the development of CLL/PL. All the cases with typical CLL and p53 abnormalities had only one allele affected whereas 4/6 CLL/PL had both alleles inactivated. This difference in the extent of p53 inactivation suggests that accumulation of p53 abnormalities may be associated with progression of CLL to CLL/PL.

CLL cases with p53 abnormalities were characterized by a higher incidence of stage C ($P < 0{\cdot}025$), a higher proliferative rate ($P = 0{\cdot}05$), short survival ($P < 0{\cdot}005$) and resistance to first-line therapy ($P < 0{\cdot}02$) but not to nucleoside analogues. Analysis of the correlation between p53 status and incidence of trisomy 12 by fluorescence *in situ* hybridization (FISH) showed that trisomy 12 was more frequent in cases without p53 abnormalities, suggesting that trisomy 12 and p53 may represent different pathways of transformation in CLL.

**Keywords:** chronic lymphocytic leukaemia, CLL/PL, p53, trisomy 12.

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in Western countries. The disease usually runs a chronic course but a substantial proportion of patients may present with, or develop, a more aggressive disease. One form of transformation is to a high-grade lymphoma (Richter's syndrome) and is seen in 5–10% of CLL cases (Trump *et al*, 1980). A more frequent type of change designated CLL/PL, characterized by > 10% of prolymphocytes, has been associated with an aggressive clinical course and refractoriness to therapy (Melo *et al*, 1987; Scott *et al*, 1987; Vallespi *et al*, 1991). Although the clinical and laboratory features of CLL/PL are in some respects intermediate between those of CLL and PLL, immunophenotypic studies suggest a closer relationship with CLL (Melo *et al*, 1986; Matutes *et al*, 1994). The genetic mechanisms underlying the transition to CLL/PL are poorly understood; the association with trisomy of chromosome 12 (Bird *et al*, 1989; Criel *et al*, 1994; Matutes *et al*, 1996; Que *et al*, 1993) and a higher proliferative rate (Cordone *et al*, 1992; de-Melo *et al*, 1992; Garcia-Marco *et al*, 1996) are so far the only clues to its pathogenesis.

The p53 gene – located on chromosome 17p13.1 – functions as a specific transcription factor of genes controlling the G1 checkpoint of the cell-cycle and also controls the expression of genes involved in the control of programmed cell death (reviewed in Imamura *et al*, 1994, and Newcomb, 1995). Mutant forms of p53 no longer possess the ability to arrest cell growth or to induce apoptosis. In addition to loss of growth suppression, mutant p53 may actively inhibit wild-type p53 in a dominant negative way as a result to complex formation between the two forms (Kern *et al*, 1992; Milner *et al*, 1991). Moreover, some mutant forms may acquire a gain of function, since it has been shown that transformed cells lacking endogenous p53 acquired increase tumourigenicity when certain mutant p53 proteins are

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introduced (Dittmer *et al*, 1993; Halevy *et al*, 1990). Studies in transgenic mice have demonstrated that there is a continuous increase of tumourigenicity after mutation, loss of one allele and inactivation of both alleles (Donehower, 1996). However, the majority of studies on p53 in leukaemia have not analysed mutation and allele loss simultaneously and thus the full potential of p53 inactivation in tumourigenesis may have been underestimated in these conditions.

Mutations of the p53 gene have been found in 10–15% of CLL cases and have been associated with advanced clinical stage, poor prognosis and drug resistance (Fenaux *et al*, 1992; El Rouby *et al*, 1993; Wattel *et al*, 1994; Dohner *et al*, 1995). Moreover, p53 mutations have also been associated with transformation of follicular lymphoma, mantle cell lymphoma and CLL to high-grade lymphoma (Gaidano *et al*, 1991). We have recently shown a high incidence of p53 mutations (58%) in B-PLL (Lens *et al*, 1997). To our knowledge, no data have been published concerning the incidence of p53 abnormalities in the distinct subgroup of CLL/PL.

We have investigated the role of p53 gene in the pathogenesis of CLL/PL by examining loss of heterozygosity (LOH) at 17p, mutations of the p53 gene and aberrant expression of the p53 protein. We have also correlated p53 alterations with clinical features and with two other factors shown to be associated with CLL/PL: trisomy 12 and degree of cell proliferation.

## MATERIAL AND METHODS

*Patients*. 32 patients diagnosed as B-cell CLL were selected for this study. Immunological diagnosis was based on a published score (Matutes *et al*, 1994) from cases available in the laboratory at the time; the majority were not studied at the time of diagnosis. May-Grunwald-Giemsa stained peripheral blood films were reviewed and a minimum of 300 cells evaluated for morphological criteria (Bennett *et al*, 1989). All cases had a varying proportion of cells with features characteristic of CLL with clumped chromatin, no visible nucleoli and a small amount of cytoplasm. 17 cases were classified as typical and 15 cases had > 10% circulating prolymphocytes and were classified as CLL/PL (Melo *et al*, 1986).

Detailed clinical information was available in 30 patients (Table I). According to the Binet staging system (Binet *et al*, 1977), 12 patients were in stage A, six patients in stage B and the remaining 13 were in stage C. 4/30 patients have not required treatment, 24 had received alkylating agents and/or anthracyclines as first-line treatment. 13/24 patients received in addition the nucleoside analogues fludarabine (FDR) or 2′ deoxycoformycin (DCF); one patient received FDR as first therapy. Patients had received treatment prior to the study. Refractory disease was defined as failure to achieve a partial or complete response after stopping therapy.

*LOH analysis*. Conventional Southern blotting was performed as described (Dyer *et al*, 1993). Briefly, high molecular weight DNA was extracted from cryopreserved mononuclear peripheral blood cells by standard methods. 10 $\mu$g of DNA were digested to completion with *Pst1* restriction enzyme (Promega, Madison, Wis.). DNA fragments were electrophoresed in 0·7% agarose and transferred to positively charged nylon membranes (Hybond-N+: Amersham, U.K.) by overnight capillary transfer in alkali. Filters were probed sequentially with p144-D6 (Kondoleon *et al*, 1987), a D17S34 probe released as 5·1 kb *EcoR1* insert and pYNZ22 (Nakamura *et al*, 1988), a D17S30 probe released as 1·7 kb *BambHI* insert. Both probes were obtained from the American Tissue Collection Culture (Rockville, Md.) and were labelled with $^{32}$P dCTP by the method of oligolabelling to a specific activity of $> 3 \times 10^9$ dpm/$\mu$g DNA.

*p53 sequencing*. Sequencing of exons 5–9 of *p53* was performed by polymerase chain reaction (PCR) amplification followed by direct automated sequencing of double-stranded DNA using termination nucleotides tagged with fluorescent dyes.

*PCR amplification*. PCR was performed using 200 ng of DNA, 1·5 mg $MgCl_2$, 200 $\mu$M of each deoxyribonucleoside triphosphate (dNTPs), 1 $\mu$M of each primer, 0·4 $\mu$l of *Taq* polymerase enzyme (Promega, Madison, Wis.) and 5 $\mu$l of 10× Taq buffer (Promega, Madison, Wis.) in a final volume of 50 $\mu$l. CYCLIN profile comprised 30 cycles of denaturation (92°C for 45 s), annealing (61°C for exons 5, 8 and 9; 62°C for exons 6 and 7 for 45 s) and extension (72°C for 45 s). PCR amplification was performed in a Biometra thermal cycler (Gottingen, Germany).

*DNA nucleotide primers*. The primers were obtained from Oswell, University of Edinburgh, and used for PCR and DNA sequencing. These primers derived from intronic sequences immediately flanking the respective exons and were as follows: exon 5 sense strand: 5′ ACTTGTGCCCTGACTTTCAACT 3′; exon 5 antisense strand: 5′ CAATCAGTGAGGAATCAGAGGC 3′; exon 6 sense strand: 5′ TCAGATAGCGATGGTGAGCAG 3′; exon 6 antisense strand: 5′ GCCACTGACAACCACCCTTA 3′; exon 7 sense strand: 5′ GCGACAGAGCGAGATTCCA 3′; exon 7 antisense strand: 5′ GAAATCGGTAAGAGGTGGGC 3′; exon 8 sense strand: 5′ GGAGTAGATGGAGCCTGGTTT 3′; exon 8 antisense strand: 5′ GGTGATGAAAAGTGAATCTGAGGC 3′; exon 9 sense strand: 5′ GGAGACCAAGGGTGCAGTTAT 3′; exon 9 antisense strand: 5′ GTTAGTTAGCTACAACCAGGAGCC 3′.

*Direct DNA sequencing of PCR products*. PCR products were first purified using phenol/chloroform (1:1) extraction and precipitation with isopropanol (100%) and 7·5 M ammonium acetate. The same primers used for generating the PCR products were also used for the sequencing reactions, other reagents were supplied in the ReadyReaction Taq DyeDeoxy termination sequencing kit (Applied Biosystems Inc., Foster City, Calif., U.S.A.). Both strands were sequenced for each exon. The manufacturers protocol (Applied Biosystems Inc.) was followed for the cycle sequencing of PCR products with slight modifications: 1 $\mu$l of the purified PCR product (approximately a total of 40 ng) was used and the primer concentration was 10 pmol/$\mu$l. Unincorporated nucleotides were removed from the extended products using two phenol/chloroform steps followed by an ethanol precipitation step. Samples were loaded on a 0·7% denaturating polyacrylamide gel, and the electrophoresis was carried out on a Model 373A DNA Sequencer (Applied Biosystems Inc.). All PCR

**Table I.** Clinical and laboratory features of CLL patients.

| Case | Diagnosis | Prolymphocytes (%) | Stage | WBC ($\times 10^9$/l) | Phenotype score | Trisomy 12 | Ki67$^+$ (%) | Response to: First-line treatment | Response to: Nucleoside analogue | Survival (months) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CLL/PL | 18 | C | 598 | 4 | No | 4·1 | 0 | NK | 13 |
| 2 | CLL/PL | 35 | C | 174 | 5 | No | 9·8 | 0 | – | 10 |
| 3 | CLL/PL | 19 | C | 117 | 3 | Yes | 0·9 | 0 | – | 16 |
| 4 | CLL | 5 | A | 157 | 4 | No | 2·5 | 0 | 1 | 43* |
| 5 | CLL/PL | 32 | B | 53·2 | 4 | Yes | nd | 1 | – | 18* |
| 6 | CLL/PL | 21 | C | 12 | 4 | No | 3·3 | 2 | 0 | 78 |
| 7 | CLL/PL | 26 | C | 200 | 5 | No | 14·7 | 0 | 0 | 7 |
| 8 | CLL | 8 | C | 38·3 | 4 | No | 14·6 | 0 | – | 64 |
| 9 | CLL | 5 | B | 95·8 | 4 | No | nd | 0 | NK | 21 |
| 10 | CLL/PL | 55 | C | 100 | 4 | nd | 28·3 | 0 | – | 55 |
| 11 | CLL/PL | 50 | C | 120 | 4 | Yes | 9·6 | 0 | 0 | 50 |
| 12 | CLL | 2 | A | 89 | 3 | No | 0·5 | 1 | – | 171* |
| 13 | CLL | 2 | C | 110 | 4 | nd | 1·3 | – | – | 22* |
| 14 | CLL | nd | A | nd | 4 | nd | 0·6 | 1 | – | 21* |
| 15 | CLL | 9 | A | 22 | 4 | Yes | 2·6 | – | – | 81 |
| 16 | CLL | 2 | A | 24 | 4 | No | 1·6 | – | – | 54* |
| 17 | CLL. | 4 | A | 15 | 4 | Yes | nd | 0 | 0 | 50* |
| 18 | CLL | 8 | A | 150 | 5 | No | 0·6 | 2 | 0 | 53* |
| 19 | CLL/PL | 11 | B | 100 | 4 | No | 5·6 | 2 | 1 | 128 |
| 20 | CLL/PL | 35 | C | 55 | 5 | Yes | 12·9 | 0 | 1 | 63 |
| 21 | CLL/PL | 21 | A | 50 | 4 | Yes | 9·7 | 1 | – | 52* |
| 22 | CLL/PL | 11 | A | 60 | 4 | Yes | 0·8 | NK | 0 | 278* |
| 23 | CLL | 10 | B | 209 | 4 | Yes | 2·8 | – | 0 | 21 |
| 24 | CLL | 10 | C | 87 | 4 | nd | 4·5 | NK | NK | 75* |
| 25 | CLL/PL | 35 | C | 290 | 2 | Yes | 4·5 | 2 | 0 | 21* |
| 26 | CLL/PL | 11 | B | 236 | 4 | No | 1·2 | NK | NK | 94 |
| 27 | CLL | 9 | nd | nd | 5 | Yes | 2 | 1 | 1 | nd |
| 28 | CLL | 3 | A | nd | 2 | nd | nd | 1 | – | 75* |
| 29 | CLL | nd | B | 80 | 3 | No | 1·9 | 2 | 0 | 199* |
| 30 | CLL/PL | 25 | B | 157·8 | 4 | Yes | 21·6 | 0 | – | 39 |
| 31 | CLL | 4 | A | 50·2 | 5 | No | nd | 0 | 0 | 16* |
| 32 | CLL | nd | C | 120 | 5 | Yes | nd | – | – | 21 |

Patients 1–11: p53 abnormalities; patients 12–32: p53 abnormalites not found. nd: not determined; NK: not known; –, not treated. 0 = no response (primary resistant); 1 = good response (CR or PR); 2 = response but subsequent resistance (secondary resistant).

*Patient alive.

products were sequenced in both orientations. To overcome the possibility of *Taq* polymerase DNA incorporation errors, PCR products of the mutated cases were sequenced from two separate PCR reactions.

*Immunocytochemistry.* Immunocytochemistry was performed on cytospin preparations of peripheral blood or spleen mononuclear cell suspensions using the monoclonal antibody (McAb) PAb1801 (Oncogene Science Inc., Cambridge, Mass.), which recognizes the amino acids 1–45 at the N terminal epitope of mutant and wild-type *p53* protein, and an indirect immunoperoxidase sandwich technique (Mason *et al*, 1982). Briefly, after 10 min fixation in acetone, cytospins were incubated for 30 min with PAb1801 in phosphate-buffered saline (PBS), followed by a peroxidase-conjugated goat anti-mouse antibody for 30 min. Peroxidase–anti-peroxidase complexes (PAP) were added to increase the staining intensity. The peroxidase reaction was carried out by incubating the slides for 10 min in a developing solution containing diaminobenzidine tetrahydrochloride 0·6 mg/ml (Dako) in PBS with 0·1% of hydrogen peroxide. Cells were counterstained with Haematoxylin Gill (H.D. Supplies, U.K.). Positive control for p53 expression was the CEM T-cell line, which carries a *p53* mutation and expresses abundant p53. Peripheral blood from a healthy donor was used as negative control. 500 cells were evaluated for every case.

*Cell proliferation.* Evaluation of the tumour growth fraction was performed on mononuclear cells spread on cytospins using the McAb Ki-67 at a 1:20 dilution (Dakopatts, High Wycombe) by indirect immunoperoxidase as described above. The proportion of Ki-67-positive cells was evaluated by light microscopy under high power (magnification ×1000) and at least 500 cells were scored from each sample.

*Fluorescence in situ hybridization (FISH)*. Fixed cell suspensions from the conventional cytogenetic preparations were used for FISH with a biotinylated alpha-satellite chromosome 12 PCR53 probe as previously described (Garcia-Marco *et al*, 1996). The slides were pre-treated with RNAse (100 $\mu$g/ml in 2×SSC) (Sigma, Poole), denatured in 70% formamide/2×SSC and serially dehydrated in ethanol. The chromosome 12 probe was ethanol precipitated and denatured at 70°C for 10 min in a hybridization buffer containing 20 ng of probe, 0·5 ng/$\mu$l salmon sperm DNA, 50% formamide, 10% dextran sulphate, 1% Triton X, and 2×SSC. After denaturation, 10 $\mu$l of the hybridization buffer was placed onto each slide, sealed under a coverslip and hybridized overnight at 37°C. Post-hybridization washes and detection of hybrids were performed as published (Que *et al*, 1993). Peripheral blood lymphocytes from normal donors were included as controls of hybridization sensitivity and specificity of the chromosome 12 probe and 500 cells were scored from each case. A diagnosis of trisomy 12 in CLL samples was made when > 3% of cells showed three hybridization signals (percentage of cells with three signals greater than the mean ± 3 SD of control samples).

*Statistical analysis*. Comparisons of clinical and laboratory parameters between patients with and without a p53 gene abnormality were performed using the Wilcoxon rank sum test (quantitative variables) and the Fisher's exact test (binary variables). Survival time, measured from diagnosis and date of sample, was plotted using Kaplan-Meier estimate differences between two survival curves were analysed by the log-rank test.

## RESULTS

### *p53 abnormalities in CLL*

The incidence and extent of p53 inactivation in CLL and CLL/PL was assessed by LOH, p53 sequencing and protein immunostaining. Table II summarizes the cases with p53 abnormalities.

*Loss of heterozygosity.* We tested the presence of loss of heterozygosity (LOH) at 17p13.3 using two highly informative polymorphic probes mapping telomeric of *p53* at 17p13.3 (Kondoleon *et al*, 1987; Nakamura *et al*, 1988). Loss of one allele can be assessed within 95% confidence limits when both probes show a loss of one hybridizing band, even when corresponding normal tissue is not available for comparison (Nigro *et al*, 1989). Results of this type of experiment are shown in Fig 1. 8/28 (29%) of cases showed only one band with both probes (Table II) and therefore these cases were considered as having LOH.

*Sequencing analysis of p53 gene*. Mutations at the highly conserved region of p53 were detected in five of 32 cases. Giving the relatively high rate of base misincorporation in PCR (Nigro *et al*, 1989), all the mutations were confirmed by performing a second PCR reaction and resequencing the products. As shown in Table II, these mutations consisted of: missense mutations in four cases involving exon 7 (one case) and exon 8 (three cases) and a nonsense mutation in exon 9. In all these cases the mutations led to alterations in the amino acid sequence. Three of five alterations were transitional mutations occurring at CpG dinucleotides and two were transversions (one C → G and one G → T) (Table II). Analysis of the chromatogram at the site of mutation showed that in four of five mutated cases no residual signal of the wild-type allele was visualized. In the remaining case (no. 5) the chromatogram showed the persistence of both wild-type and mutated sequences (Fig 2).

*Expression of p53 protein*. Immunostaining was performed in 28/32 CLL cases. The CEM cell line, used as a positive control, showed strong nuclear immunostaining in > 90% of cells. PB from a healthy donor was used as negative control. Five CLL cases (8%) expressed p53 protein with a percentage of positive cells ranging from 45% to 80% (Table II).

**Table II.** Summary of p53 abnormalities in CLL.

| | | | | p53 mutation† | | | |
|---|---|---|---|---|---|---|---|
| Patient | Diagnosis | 17p13 LOH | p53 protein* (%) | Codon | Nucleotide substitution | Amino acid substitution | Type of mutation |
| 1 | CLL/PL | Yes | ++ (80%) | 273 | C**C**T → C**A**T | Arg → His | Ts CpG |
| 2 | CLL/PL | Yes | ++ (65%) | 278 | C**C**T → C**G**T | Pro → Arg | Tv |
| 3 | CLL/PL | Yes | - | 326 | **G**AA → **T**AA | Glu → TERM | Tv |
| 4 | CLL/PL | Yes | nd | 248 | **C**GG → **T**GG | Arg → Trp | Ts CpG |
| 5 | CLL | No | nd | 273 | C**G**T → C**A**T | Arg → His | Ts CpG |
| 6 | CLL/PL | Yes | - | | | | |
| 7 | CLL/PL | Yes | - | | | | |
| 8 | CLL | Yes | - | | | | |
| 9 | CLL | Yes | + (45%) | | | | |
| 10 | CLL/PL | No | ++ (52%) | | | | |
| 11 | CLL/PL | No | ++ (78%) | | | | |

*Intensity of immunocytochemical staining: +, moderate; ++, strong; nd, not determined.
†Not found in cases 6–32.

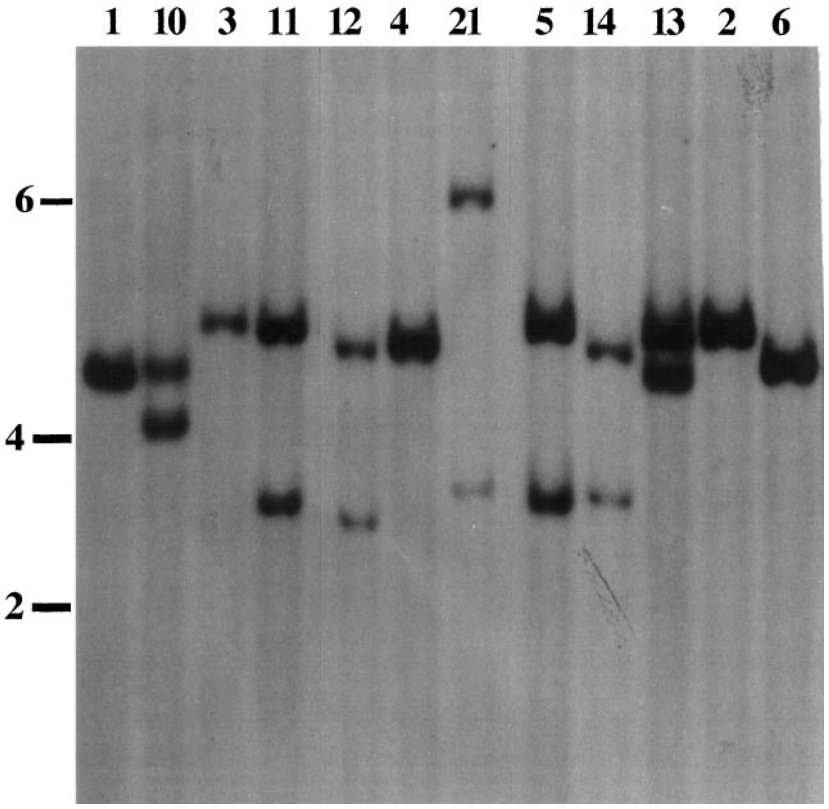


**Fig 1.** Representative loss of heterozygosity analysis at 17p13.3 in CLL. Genomic DNA from the samples studied were digested with *PstI*, subjected to Southern blot and probed with the clone pYNZ22. Numbers at the top of the gel correspond to case numbers in Table I. Cases were scored positive for loss of heterozygosity when only one allelic band with both (p144-D6 and pYNZ22) probes was obtained.

*Relationship between LOH, p53 mutation and p53 protein expression.* Four out of five p53 mutated cases had LOH, suggesting a biallelic inactivation of p53 gene in these cases. The remaining case (no. 5) did not show LOH by Southern blot and sequencing analysis. This was the only case with p53 mutation and typical CLL morphology (Table II). In addition, four cases (nos. 6–9) had 17p allelic deletion (LOH) without mutation of the p53 gene. The five cases of CLL with positive immunostaining comprised two cases with p53 mutation and three cases with normal exons 5–9 by sequencing. The cases with mutation but negative for protein expression were cases 3 and 5. Case 3 harboured a

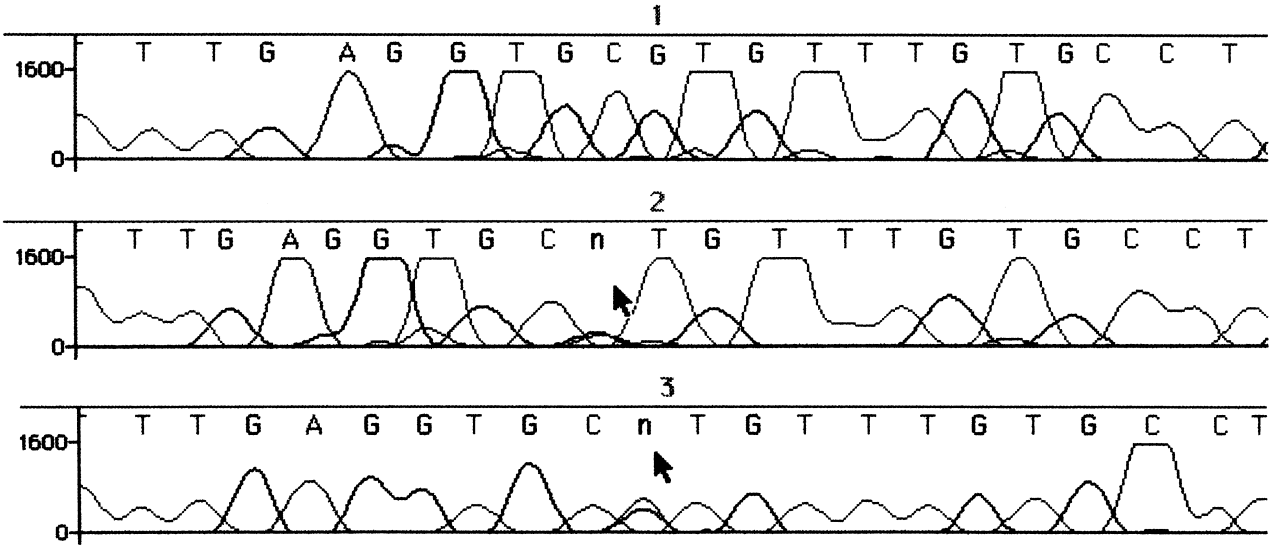


**Fig 2.** Analysis of a p53 mutation by PCR direct sequencing using fluorescent termination nucleotides. A chromatogram of control DNA is shown in (1). (2) and (3) correspond to chromatograms of the PCR product of exon8 of p53 from a mutated case sequenced from both ends. Arrows point to the site of mutation. The germline nucleotide (G) remains present but its intensity is decreased in 50% and is accompanied by a new signal (A) indicating that the mutation occurred in one allele and the other allele is still present.

**Table III.** Clinical and laboratory data of CLL patients with and without p53 abnormalities.

| | p53 abnormalities | No p53 abnormalities | *P* value |
|---|---|---|---|
| No. of patients | 11 | 21 | |
| Age | 58 ± 12 | 64 ± 11 | NS |
| Sex (female/male ratio) | 3/8 | 7/14 | NS |
| Stage | | | |
| A | 2 (18%) | 10 (50%) | <0·025 |
| B | 1 (9%) | 5 (25%) | |
| C | 8 (73%) | 5 (25%) | |
| WBC | 151 ±159 | 105 ± 77 | NS |
| Prolymphocytes (%) | 27 ± 16 | 12 ± 11 | < 0·02 |
| Morphology | | | |
| Typical | 3 | 14 | 0·05 |
| CLL/PL | 8 (73%) | 7 (33%) | |
| FISH | | | |
| Diploid 12 | 7 | 7 | NS |
| Trisomy 12 | 3 (30%) | 10 (59%) | |
| Ki-67$^+$ cells (%) | 12·0 ± 14·5 | 4·4 ± 5·6 | 0·05 |

Mean and standard deviation values are given for quantitative variables.

nonsense mutation whereas case 5 showed a missense mutation without loss of the remaining allele.

*Correlation between p53 and clinical and laboratory data*

The clinical and laboratory features of the patients with and without p53 abnormalities are summarized in Table II. 73% (8/11) of the p53 abnormalities were found in cases of CLL/PL, namely 6/8 (75%) of cases with LOH, 4/5 (80%) with p53 mutation and 4/5 (80%) of cases with positive p53 protein. The correlation between p53 abnormalities with the percentage of prolymphocytes and CLL/PL morphology was statistically significant ($P<0·02$ and $P=0·05$).

**Table IV.** Correlation between response to treatment and p53 abnormalities in CLL (analysis carried out after treatment).

| | Treatment required | Resistance to first-line therapy* | Resistance to nucleoside analogues† |
|---|---|---|---|
| p53 abnormalities | 11/11 (100%) | 9/11 (82%) | 3/4 (75%) |
| No p53 alterations | 14/19 (72%) | 4/13 (31%) | 7/10 (70%) |
| *P* value | NS | <0·02 | NS |

* Chlorambucil ± anthracyclines.
† Fludarabine (FDR), deoxycoformycin (DCF).

When the extent of p53 inactivation was correlated with CLL morphology, all cases with typical CLL morphology and p53 gene abnormalities had only one of the two alleles involved (either by mutation or allele loss) with no abnormality of the remaining allele, suggesting partial inactivation of p53 gene. In contrast, all cases with a biallelic p53 alteration (by mutation and allele loss) were CLL/PL (Table II).

Analysis of the proliferative rate assessed with Ki-67 was performed on 28/32 samples and cases with p53 abnormalities had a significantly higher proportion of Ki-67$^+$ cells than cases with normal p53 (Table III).

FISH analysis for trisomy of chromosome 12 was performed in 27 patients. 10/17 (59%) cases without p53 alterations exhibited trisomy 12 whereas only 3/10 (30%) cases with p53 abnormalities had this abnormality. There was therefore no statistical correlation between p53 abnormalities and chromosome 12 status.

CLL cases with p53 abnormalities were characterized by a higher incidence of stage C (Table III) which was statistically significant. All 11 patients with p53 abnormalities had progressive and/or advanced disease and required treatment (Table IV). All but two cases were resistant to first-line treatment (alkylating agents plus/minus anthracyclines). One of the responding patients subsequently became

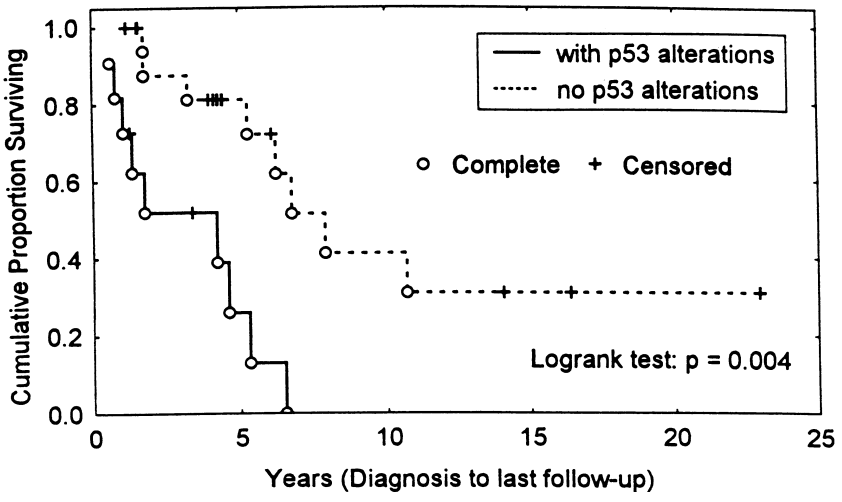


**Fig 3.** Survival curve in CLL according to the presence of p53 abnormalities. A significant difference was observed between both groups ($P<0·005$).

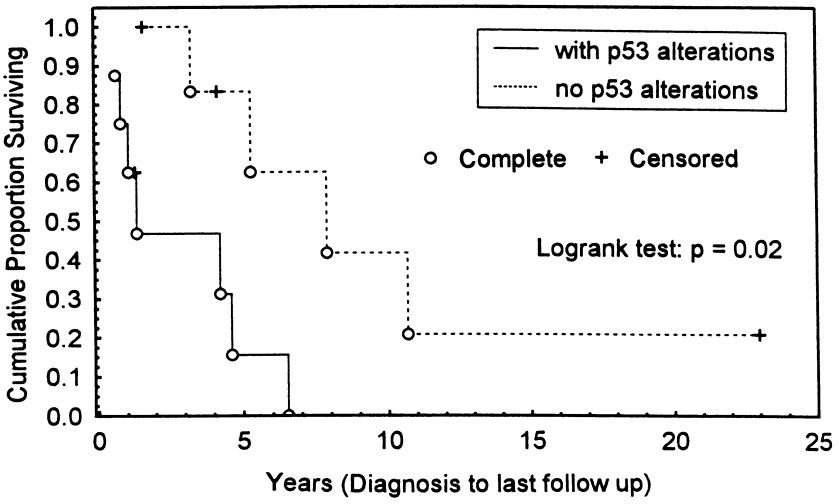


**Fig 4.** Survival curve within the CLL/PL group according to the presence of p53 abnormalities. Cases with p53 alterations fared worse than cases without p53 abnormalities.

resistant. Four cases received therapy with nucleoside analogues but only one of these responded. Of the cases without p53 abnormalities, five had stable disease and did not require therapy. Nine out of 13 cases treated with first-line therapy, responded at some stage of the disease but four of them became resistant later during the evolution of the disease (Table IV). There was a significant correlation between p53 abnormalities and response to first line treatment ($P<0{\cdot}02$), but no such difference was observed in relation to the response to nucleoside analogues.

The actuarial survival from diagnosis was significantly shorter in cases with p53 abnormalities as compared with cases with no p53 alterations (median 5·2 *v* 8 years, $P<0{\cdot}005$) (Fig 3). The difference was also significant ($P=0{\cdot}02$) when survival was considered from the date of analysis (data not shown). When survival analysis was performed within the CLL/PL group, patients with p53 abnormalities fared worse than those without p53 abnormalities ($P=0{\cdot}02$) (Fig 4). To analyse the effect on survival of the p53 gene and chromosome 12 status, cases were divided into four groups: patients with neither trisomy 12 nor p53 alterations, patients with one of the two alterations, and patients with both alterations. The median survival was 10·8 years for the group with no p53 or chromosome 12 alterations, 5·5 years for the group with trisomy 12, 1·8 years for the group with p53 abnormalities alone, and 1·2 for the group with both alterations; the survival probability was significantly different between the four groups ($P<0{\cdot}001$).

## DISCUSSION

p53 is the most common genetic abnormality in cancer and has been extensively studied in B-cell malignancies. In low-grade lymphomas and in CLL, p53 mutations have often been associated with transformation to a high-grade lymphoma (Gaidano *et al*, 1991; Lo Coco *et al*, 1993). We have selected a high proportion of cases with CLL/PL to investigate the possible role of p53 abnormalities in this event. The extent of p53 inactivation was analysed by studying LOH at 17p, mutation of p53 gene and aberrant expression of p53 protein in the same population. LOH analysis showed 29% of cases with only one band. Since there is a 95% of chance of detecting a difference between the restriction fragment lengths with these probes if two alleles are present, it is likely that cases with a single band had one allele of chromosome 17p13 deleted (Nigro *et al*, 1989). Analysis of p53 mutations was limited to the highly conserved region between exons 5 and 9, as prior studies have shown that this region contains 85–95% of mutations in most human cancers including haematological malignancies (Hollstein *et al*, 1991; Newcomb, 1995). Mutations of the p53 gene were found in 16% of cases and all the mutations were base substitutions that produce a change in the amino acid constitution of the protein. By comparison with p53 databases (Cariello *et al*, 1994; Hollstein *et al*, 1994), the pattern of mutation found parallels those previously reported in lymphoid malignancies: three of the mutations were transitions at CpG dinucleotides, localized at hot spot codons of the p53 mutation (Arg248 and Arg273).

The most common mechanism of loss of p53 function consists of the inactivation of one allele by mutation and loss of the second allele by deletion, resulting in LOH for DNA markers adjacent to the tumour suppressor gene. However, the percentage of cases with a p53 mutation that have also lost the remaining allele varies significantly among the different reports published in chronic lymphoid disorders. For instance, Gaidano *et al* (1994) showed that only 60% of CLL cases with p53 mutations had allele loss, assessed by Southern blot using polymorphic probes adjacent to the p53 region, whereas Wattel *et al* (1994) reported that 75% of mutated CLL had loss of the wild-type sequence at the site of the mutation. Baldini *et al* (1994) showed that 85% of cases with splenic lymphoma presenting p53 mutations had loss of the remaining allele whereas Wada *et al* (1993) studied 359 cases of haematological malignancies and found in all but one of the cases with mutations a migrating wild-type band together with the altered band, suggesting that only 22% of these cases with p53 mutation had loss of the remaining p53 allele. In this study there was no evidence of an additional

signal corresponding to the normal p53 sequence in four out of five CLL cases with p53 mutations, which indicates a close correlation between p53 mutation and deletion of the remaining allele. Therefore no normal p53 could be synthesized by the malignant clone in these cases.

When p53 mutation and LOH results were compared, allele loss was also demonstrated in four out of five CLL cases with mutation by Southern blot analysis, confirming the DNA sequencing results. In addition, four CLL cases had LOH but not mutation, which suggests that either allelic deletion may represent the first of two mutation events, with an unaffected second allele, or that mutation may reside outside exons 5–9, although such mutations are rare. An alternative explanation is that other gene(s) may be the target of these allelic deletions. Evidence of a second gene at 17p has been found in other neoplasias (Casey *et al*, 1993; Cogen *et al*, 1992; Cornelis *et al*, 1994; Makos Wales *et al*, 1995). Further studies combining the analysis of p53 mutations and loss of heterozygosity along the 17p region in sequential tumour samples may help to clarify this issue.

Positive immunostaining of p53 reflects the presence of a mutated protein, as mutation usually results in protein stabilization and increase of its half life. However, the relationship between p53 mutation and protein accumulation is more complex than initially thought, and several investigators have reported examples of a breakdown in the correlation between immunocytochemistry positive findings and mutations, in both 'directions' (Hall *et al*, 1991; Wynford-Thomas, 1992). We found no strict correlation between mutation and detectable p53 expression: two cases with p53 mutation were negative for p53 protein, of which one was a nonsense mutation. As previously reported, nonsense mutations generally lead to absent or reduced levels of an unstable p53 protein which cannot be detected by immunocytochemistry (Harris & Hollstein, 1993; Soussi *et al*, 1994). In contrast, three cases which expressed p53 protein had no detectable p53 mutations. This may be due to mutations localized outside the highly conserved region or to mechanisms other than mutation that result in overexpression and/or stabilization of p53 protein. Overall, these results indicate that immunocytochemistry may not always correlate with p53 mutation; similar results have been reported in other lymphoid malignancies and by us in B-PLL (Villuendas *et al*, 1992; Kocialkowsky *et al*, 1995; Piris *et al*, 1995; Lens *et al*, 1997).

We have shown here that 75% of cases with p53 abnormalities were CLL/PL. This observation identifies p53 gene as the first gene involved in CLL/PL and gives further support to the concept that CLL/PL represents a more aggressive subgroup within CLL. However, since sequential samples were not available for cases harbouring p53 alteration, it was not possible to determine whether p53 changes have been acquired at some stage during the progression of CLL to CLL/PL or whether cases harbouring p53 abnormalities are more likely to evolve to CLL/PL.

The different tumourigenic effects that have been found in experimental models after one allele mutation, one allele loss and inactivation of both alleles (Donehower, 1996), may also play a role in tumour development in humans. Recently, Du *et al* (1995) reported that in low-grade MALT tumours most p53 defective cases showed either one allele mutation or one allele loss, suggesting only partial loss of p53 function, whereas in high-grade tumours the majority of affected cases exhibited both p53 mutations and allele loss, implying complete loss of p53 function. No previous reports on CLL have analysed the full potential of p53 inactivation by studying both p53 alleles (mutation and LOH) in the same group. In this study, differences in the extent of loss of wild-type p53 gene were found to be correlated with morphology; all cases with typical morphology and p53 gene abnormalities had one of the p53 alleles unaffected, whereas four of six CLL/PL cases had both alleles affected. These results favour the view that accumulation of p53 abnormalities may be associated with progression of CLL to CLL/PL.

The presence of p53 abnormalities appear to have implications for the clinical management of CLL. In agreement with previous reports (Fenaux *et al*, 1992; El Rouby *et al*, 1993; Wattel *et al*, 1994; Dohner *et al*, 1995), we have shown that CLL patients with p53 alterations differed from those without abnormalities in several ways: they were more likely to have advanced stage, to require treatment, to have a poor response to first-line therapy and a short survival. In contrast with data recently reported by Dohner *et al* (1995) who found a correlation between p53 deletions and resistance to fludarabine, we did not find a correlation between response to this agent and p53 abnormalities. However, as only 14 of our patients were treated with purine analogues and as most of them were resistant (Table IV), we can draw no definitive conclusions about response to this type of treatment and p53 abnormalities. Also, it is important to point out that since multivariate analysis could not be performed due to the small number of cases, the exact role of p53 as a prognostic factor in CLL and CLL/PL remains an open question.

Given the role of p53 in controlling cell proliferation, it is likely that loss of p53 function may contribute to tumour progression directly by increasing the proliferative rate of CLL cells. This is supported by our findings of a correlation between Ki-67 expression and p53 abnormalities. However, as CLL/PL has been shown to associate with a high proliferative activity (Cordone *et al*, 1992; de-Melo *et al*, 1992), it is not certain whether the association between p53 abnormalities and Ki-67 was an independent factor or was related to the morphology subset.

Previous reports have shown that trisomy 12 is associated with poor prognosis and with atypical morphology in CLL (Que *et al*, 1993; Matutes *et al*, 1996). Here, we have found no statistical association between p53 abnormalities and trisomy 12, suggesting that trisomy 12 was independent of p53 status. Moreover, trisomy 12 appeared to be more frequent in cases without p53 abnormalities than in cases with p53 abnormalities, suggesting that trisomy 12 and p53 may represent independent pathways of transformation. Our survival analysis shows that cases without any of these two abnormalities were the group with longest survival whereas cases with p53 abnormalities fared worse than those with trisomy 12. The few cases with both p53 and trisomy 12 abnormalities fared worst. However, since the number of

patients studied was small, larger studies are required to establish this point unequivocally.

In summary, we have confirmed that p53 is correlated with advanced stage in CLL, low response to chemotherapy and short survival. This is the first report to demonstrate an association between p53 abnormalities and CLL/PL. Although both trisomy 12 and p53 abnormalities appear to be involved in the pathogenesis of CLL/PL, they seem to exert their role independently.

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