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p53 allele deletion and protein accumulation occurs in the absence of p53 gene mutation in T-prolymphocytic leukaemia and Sezary syndrome

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Summary. In a series of 24 patients with chronic T-lymphoid disorders [13 T-prolymphocytic leukaemia (T-PLL) and 11 Sezary syndrome] we have studied (i) chromosome 17p abnormalities and p53 allele deletion by fluorescence *in situ* hybridization; (ii) mutation in the exons of the p53 gene by direct DNA sequencing; and (iii) p53 protein expression by immunocytochemistry and, in some cases, also by flow cytometry with DO-1, a monoclonal antibody to the p53 protein. The study revealed p53 deletion and accumulation of p53 protein in the absence of mutation in the exons that included the hot-spots and differs from that described in B-prolymphocytic leukaemia. Seven T-PLL and five Sezary syndrome patients had p53 overexpression, and five T-PLL and nine Sezary syndrome patients showed p53 deletion. Although the majority of cases with p53 accumulation had p53 deletion, the proportion of cells with the deletion did not correlate with the proportion of cells positive for p53 expression. Two cases of T-PLL showed strong p53 expression in the absence of p53 deletion, and one case of Sezary syndrome with p53 deletion in 97% of cells did not express p53. These findings suggest that a non-mutational mechanism exists for the accumulation of p53 protein in these T-cell disorders. The oncogenic effect of the accumulating wild-type protein has been reported in other malignancies. Whether haploidy resulting from p53 deletion contributes to this mechanism has yet to be determined. Alternatively, the frequent loss of the p53 gene could be associated with the deletion of an adjacent gene, which could be involved in the pathogenesis of these diseases.

Keywords: T-prolymphocytic leukaemia, Sezary syndrome, p53 gene, p53 protein.

T-prolymphocytic leukaemia (T-PLL) and Sezary syndrome are two distinct chronic T-cell lymphoproliferative disorders with a post-thymic immunophenotype. T-PLL is an aggressive disease characterized by splenomegaly and high white blood cell (WBC) count with the presence of circulating prolymphocytes (Matutes & Catovsky, 1991; Matutes *et al*, 1991). Sezary syndrome presents with skin lesions, predominantly erythroderma and moderate lymphocytosis, together with the presence of circulating cells in the blood with cerebriform nuclei (Matutes & Catovsky, 1991; Kuzel *et al*, 1991).

Mutations and/or deletions of the tumour-suppressor gene p53 mapped to chromosome 17p13 are the most frequent genetic alterations in cancer and are observed in a wide variety of haematological malignancies. The wild-type p53 contains 11 exons and codes for a 53-kDa phosphoprotein with DNA-binding ability. The loss of one p53 allele with the mutational inactivation of the other is one of the commonest mechanisms by which p53 is made to produce a dysfunctional protein that accumulates. Exons 5–9 are hot-spots for mutations; > 80% of p53 mutations reported in cancer involve these exons (Bonsing *et al*, 1997; Levine, 1997; Preudhomme & Fenaux, 1997). Cytogenetic abnormalities of chromosome 17p have been described in T-PLL and Sezary syndrome. (Mitelman, 1995; Brito-Babapulle *et al*, 1997).

The present study investigates the possible role of p53 gene deletion in 24 patients with mature T-cell leukaemia (T-PLL and SS) by fluorescence *in situ* hybridization (FISH), DNA analysis for gene mutation and p53 protein expression with a view to determining whether p53 allele loss correlates with p53 protein expression and gene mutation.

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PATIENTS AND METHODS

T-PLL patients comprised seven men and six women with a

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median age of 59 years (range 37–76 years). Characteristic disease features were splenomegaly in eight cases, lymphadenopathy in four cases and skin lesions in four cases. All had a high lymphocyte count (median 91×10^9/l; range $20–700 \times 10^9$/l) with prolymphocytes. Three cases had the cerebriform variant of T-PLL (Brito-Babapulle *et al*, 1997). Sezary syndrome patients comprised eight men and three women with a median age of 65 years (range 40–86 years). All had skin lesions, and six had lymphadenopathy. The median WBC count was 23×10^9/l with circulating Sezary cells. The proportion of malignant cells in all cases of T-PLL and Sezary syndrome investigated ranged from 55% to 96%. Five of the 13 T-PLL (case nos 1, 2, 4, 6 and 10) and three of the 11 Sezary syndrome (case nos 5, 7 and 11) were tested for HTLV-1 and found to be negative.

Immunological markers. The majority of cells from all the T-PLL were CD2+, CD3+, CD5+, CD7+. Three cases had a CD4+ CD8− phenotype, four had a CD4− CD8+ phenotype, and cells from six patients co-expressed CD4 and CD8. Cells from all cases of Sezary syndrome were CD3+ CD5+, and four were CD7+. Eight had a CD4+ CD8− phenotype, two cases were CD4 and CD8 negative, and one expressed both CD4 and CD8.

Immunocytochemistry. This was performed on cells cytocentrifuged onto slides and fixed for 10 min in acetone using an immunoperoxidase antiperoxidase (PAP) technique. The monoclonal antibody DO-1 anti-p53 (Novocastra, Newcastle, UK) (Bonsing *et al*, 1997) was diluted 1:20 in phosphate buffered saline (PBS). The second layer was a peroxidase-conjugated goat anti-mouse Ig, and the third layer was soluble PAP complexes. The reactivity was visualized by incubating the slides in a peroxidase developing solution (diaminobenzidene) and counterstaining with haematoxylin. The CEM cell line, which carries a p53 mutation and is known to accumulate p53 protein, was used as a positive control, and peripheral blood lymphocytes from normal individuals were used as a negative control. The DO-1 antibody has produced consistent results in our laboratory for detecting p53 protein by immunocytochemistry and flow cytometry in lymphoproliferative disorders.

Flow cytometry. Mononuclear cells($1–2 \times 10^6$) were washed twice and incubated for 30 min with 2% cold paraformaldehyde, washed twice with buffer containing 1% bovine serum albumin (BSA), 0·1% Na_3N and 0·05% Tween 20, stored at −20°C in 4 ml of 80% ethanol for 2 h and washed twice with buffer. Neat monoclonal antibody DO-1 (5 μl; Novocastra) and 50 μl of 2% AB serum were added to the cells, incubated for 15 min and washed with PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG $F(ab)_2$ fragments (50 μl; ICN Biomedicals, Aurora, USA) diluted 1:25 and 50 μl of 2% AB serum were added and incubated for 15 min. After two washes with PBS, the cells were resuspended in isoton and processed on a FACScan flow cytometer (Becton Dickinson, San Jose, USA) using CELLQUEST software. The CEM cell line was used as the positive control, and lymphoid cells from normal individuals as a negative control.

p53 gene mutation. Direct DNA sequencing was performed on five cases of Sezary syndrome in exons 1–9 (nos 2 and 7–10) and eight cases of T-PLL in exons 1–11 (nos 1, 3, 4, 6, 9–12). Target sequences were amplified by polymerase chain reaction (PCR), which was performed using 100 ng of DNA, 1·5 mM $MgCl_2$, 10 mM each dNTPs, 1 μM each primer, 0·5 l of *Taq* polymerase enzyme (Advanced Biotechnologies, Epsom, UK) and 5 μl of 10 × buffer in a final volume of 50 μl. The cycling profile comprised 10 cycles of denaturation (92°C for 45 s), annealing (61°C for exons 1–5

Table I. Chromosome 17 abnormalities, p53 gene deletion and p53 protein expression in T-PLL.

Case no.	Chromosome 17 abnormalities	Percentage p53 allele loss (FISH)	Percentage cells expressing p53 protein (ICC)
1	–	–	–
2	–	6%	+ +
3	–	–	–
4	–	9%	+
5	–	–	–
6	17, add(17)(q25)	–	+ +
7	17, t(12; 17)(p11; p13)	–	+ +*(77%)
8	NA	–	–
9	17, del(17)(q21)	20%	+ +*(66%)
10	–	–	–
11	17, der(17)t(17;?)(p;?)	88%	+*(65%)
12	17, der(11)t(11; 17)(p15; p12) t(7; 17)(p22; q21)	66%	+ +*(87%)
13	17, t(17;?)(p11;?)	–	ND

+, 10–50% of cells positive for p53 expression; + +, > 50% of cells positive for p53 protein.

* Four cases were also tested by flow cytometry and found to be positive for p53 expression. Cases 1, 3, 4, 6 and 9–12 were studied by direct DNA sequencing of exons 1–11 and had no evidence of mutations in the p53 gene. All except case 11 were screened elsewhere by SSCP (Vorechovsky *et al*, 1997) and were negative for mutations in exons 2–11.

ND, no data; ICC, immunocytochemistry.

and 8–9 and 62°C for exons 6 and 7 for 45 s) and extension (72°C for 45 s) in a thermal cycler). PCR amplification was performed in a Hybaid omnigene.

PCR products for direct DNA sequencing from five patients with Sezary syndrome and eight with T-PLL were purified using phenol/chloroform (1:1) extraction and precipitated with isopropanol (100%) and 7·5 mol/l ammonium acetate. The same primers used for generating the PCR products were also used for the sequencing reactions. Other reagents were supplied in the ready reaction *Taq* Dye Deoxy termination sequencing kit (Applied Biosystems, Warrington, UK). Both strands were sequenced for each exon. PCR product (40 ng) was used with a primer concentration of 10 pmol/l.

Direct DNA sequencing can identify the p53 mutation in a population of cells containing 20% or more malignant cells.

Cytogenetics and FISH. Chromosome studies were performed on blood mononuclear cells cultured for 3–7 d in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) with phytohaemagglutinin (PHA) as mitogen. Cells were harvested and fixed after treatment with hypotonic potassium chloride and G-banded according to standard procedures. Fixed cells were used in FISH experiments with probes LSI p53 gene (spectrum orange) and chromosome 17 centromere CEP17 (spectrum green) from Vysis (Richmond, UK) as described by Coignet *et al* (1996). After hybridization, slides were washed and counterstained with mounting medium containing DAPI (4′-6-amindino-2-phenylindole dihydrochloride; Vector Shield with DAPI Vector). Interphase nuclei were captured using a CCD camera attached to a Zeiss Axioplan fluorescence microscope connected to a computer using smart capture software. positive cut-off point was established by scoring 500 nuclei per slide from cultured peripheral blood lymphocytes from 10 normal individuals. The cut-off level for detection of p53 deletion was the mean + 3 standard deviations of the percentages of nuclei with p53 deletion and was found to be 5%. Between 100 and 200 cells were scored from patient slides and, when p53 was deleted in > 5% of cells, the case was considered positive for p53 deletion. A nucleus with two green signals for centromere 17 and two red signals for p53 was classified as diploid; a nucleus with two green and one red signal as monoallelic p53 deletion; a nucleus with two green signals and no red signals as biallelic p53 deletion; and a nucleus with one red and one green signal as monosomy for chromosome 17.

RESULTS

Cytogenetics

Six out of 13 cases with T-PLL had chromosome 17 abnormalities, of which only four had involvement of 17p (nos 7, 11, 12, 13) (Table I). Karyotypic data was available in five Sezary syndrome cases, of which four had chromosome 17 abnormalities, with the involvement of 17p in three (cases 3, 5 and 7).

Fish

A cut-off point of 5% of cells with p53 deletion was established from lymphocytes of healthy individuals as a control (median ± 3SD). Five T-PLL cases had p53 allele loss by FISH in more than 5% of cells (cases 2, 4, 9, 11 and 12), of which three had 20% or more cells with p53 deletion. Case 9 had no p53 deletion in the metaphases, but 20% deletion was observed in the interphases. (Table I) Nine out of 11 cases of Sezary syndrome had monoallelic p53 deletion in more than 5% (range 12–97%) of cells (Table II; Figs 1 and 2).

P53 gene mutation

Exons 2–11 in 12 cases of T-PLL (nos 1–11 and 13) have been screened elsewhere by single-strand conformation polymorphism (Vorechovsky *et al*, 1997), and eight cases of T-PLL (nos 1, 3, 4, 6 and 9–12) were studied at this institution by direct DNA sequencing of exons 1–11 with no

Table II. Chromosome 17 abnormalities, p53 gene deletion and protein expression in Sezary syndrome.

Case no.	Chromosome 17 abnormalities	Percentage p53 allele loss (FISH)	Percentage cells expressing p53 protein (ICC)
1	–	–	–
2	ND	97%	–
3	17, i(17q)(p10)	90%	ND
4	–	–	ND
5	add(17)(p10) der(17)ins(8; 17)(?; q21)	40%	ND
6	ND	60%	+
7	i(17q),der(3)t(3; 17)(p25; p11)	75%	+ +
8	ND	76%	+ +
9	ND	12%	+ +
10	ND	12%	+ +
11	ND	15%	ND

+, 10–50% of cells expressing p53; + +, > 50% cells expressing p53. Gene mutation was tested in five cases(nos 2 and 7–10) by direct DNA sequencing of exons 1–9 and found to be negative.

ND, no data; ICC, immunocytochemistry.

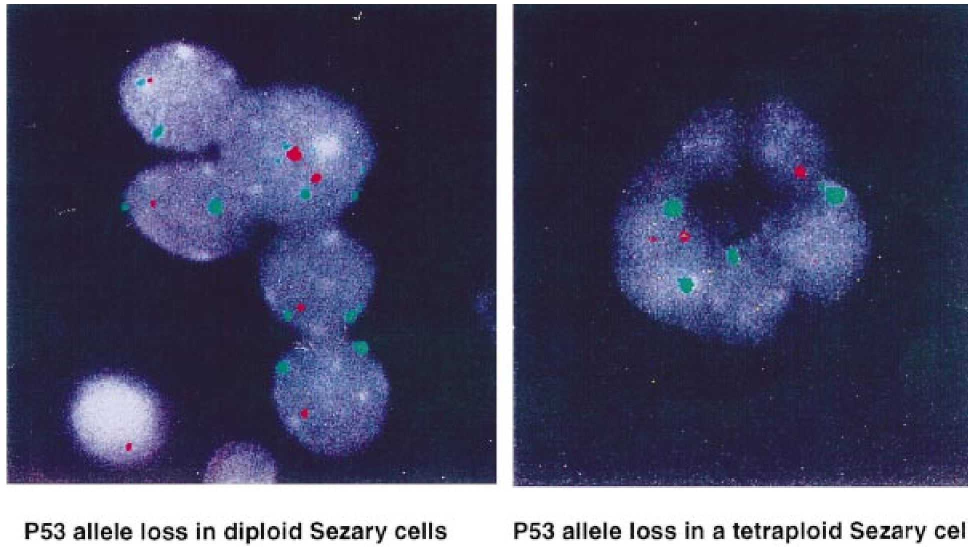

Fig 1. Interphase FISH showing monoallelic p53 deletion in (A) diploid and (B) tetraploid Sezary cell. Red signal, p53. Green signal, chromosome 17 centromere.

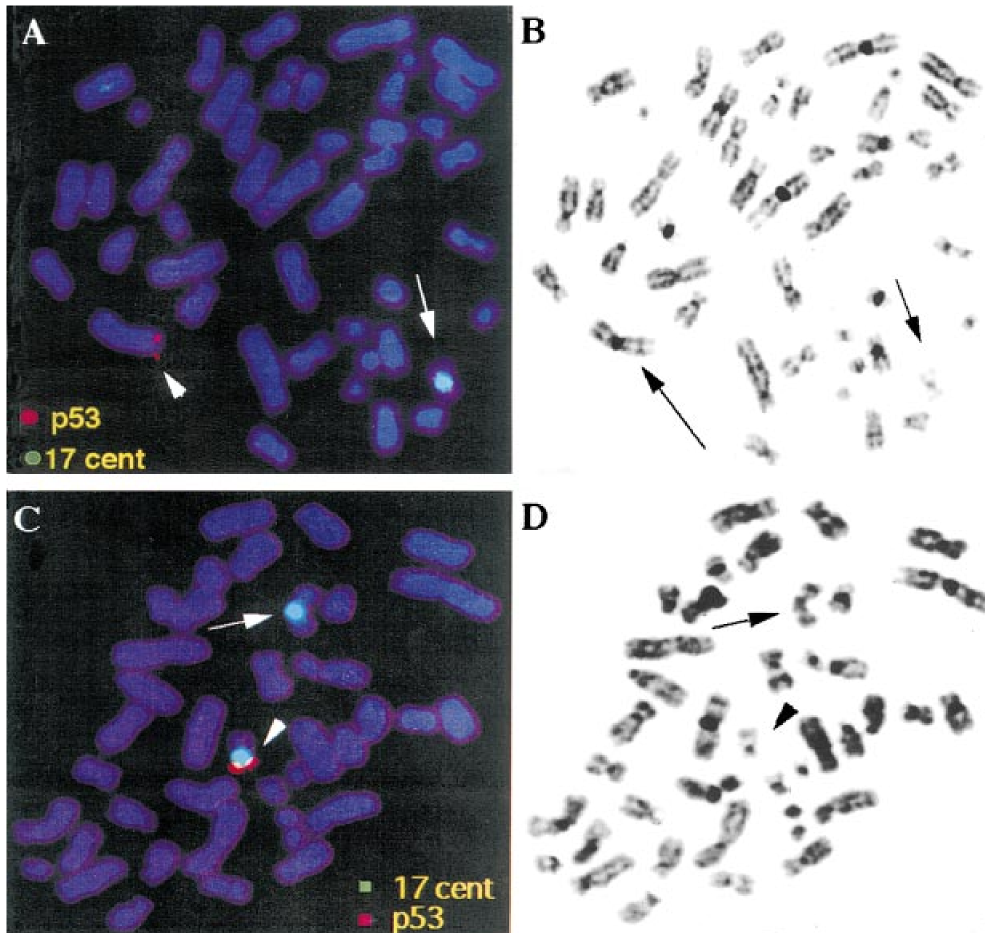

Fig 2. Metaphase FISH. (A) Metaphase from a case of Sezary syndrome (no. 7) showing i(17q) with green signal (appears blue) for centromere and a lack of signal for the p53 gene resulting from loss of the p arm. A normal chromosome 17 is absent, but a translocated p53 allele is present as a result of a der(3)t(3;17)(p25;p11). (B) Banding image of the metaphase. (C) Metaphase from a case of Sezary syndrome (no. 3) showing a normal chromosome 17 with red and green signals and an i(17q) with a green centromere signal and a missing p53 gene. (D) Banding image of the metaphase.

evidence of mutation. Five cases of Sezary syndrome (cases 2, 5 and 8–10) investigated by direct DNA sequencing of exons 1–9 had no mutations in these exons (Fig 3).

Immunocytochemistry

CEM cells used as a positive control showed strong positive nuclear p53 staining, whereas normal blood lymphocytes were negative, with less than 10% of cells showing a weak non-specific cytoplasmic staining (Fig 4A and B). The cut-off point for positive p53 expression was when staining was observed in 10% or more than 10% of the cells. According to this, a variable percentage of cells ranging from 10% to 92% in seven out of 12 T-PLL and from 10% to 97% in five out of seven Sezary syndrome cases expressed p53 protein. The staining was mostly confined to the nucleus, although both nuclear and cytoplasmic staining was observed in some cells (Fig 4C).

Correlations

In T-PLL (Table I), four cases had chromosome 17p abnormalities by conventional cytogenetics (cases 7 and 11–13), of which two had monoallelic loss of p53 by FISH (cases 11 and 12). T-PLL case 9 had metaphases with del 17q and a normal p arm, as observed by G-banding and whole-chromosome painting, and retained both p53 alleles, but 20% of interphase cells showed p53 deletion. In Sezary syndrome (Table II), nine out of 11 cases had p53 deletion. Karyotypic data were available in five cases, of which three had chromosome 17p abnormalities and p53 deletion.

In T-PLL, p53 was expressed in 10–92% of cells in seven cases, of which five had p53 deletion, and the two cases without p53 deletion expressed p53 protein in more than 50% of the cells. In Sezary syndrome, all five cases with p53 expression had p53 deletion, but one case with 97% deletion did not express p53 protein. The proportion of cells with p53 deletion did not correlate with the proportion of cells expressing p53. In both T-PLL and Sezary syndrome, p53 expression was observed in the absence of mutation.

DISCUSSION

Wild-type p53 plays an important suppressor role in tumour formation. p53 acts as a cell cycle check point subsequent to DNA damage and induces G1 arrest or apoptosis. Disruption of the p53 pathway is known to correlate strongly with tumorigenesis. Mutational inactivation of one allele with the loss of the normal allele, often by chromosome 17p rearrangement, is the commonest mechanism by which p53 is made dysfunctional (Bonsing *et al*, 1997; Levine, 1997; Preudhomme & Fenaux, 1997). In normal cells, p53 protein levels are tightly regulated as a result of a short half-life (15–30 min) and are not detectable by immunocytochemistry, as observed in the negative controls in the present study. Missense mutation in the p53 exons can result in a protein with a longer half-life, which accumulates

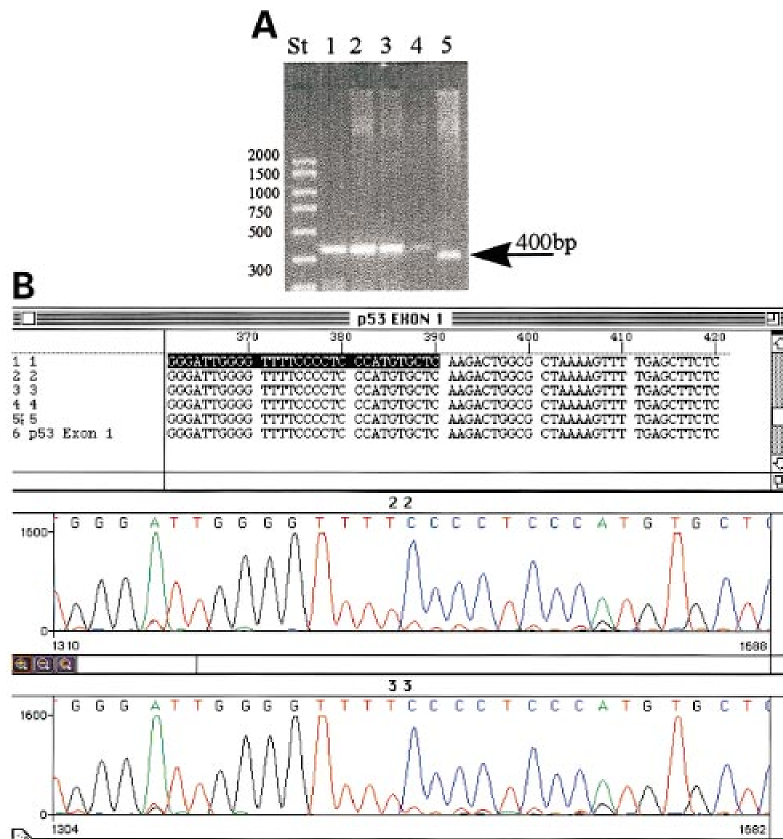

Fig 3. Comparison of a representative p53 gene exon in five patients with Sezary syndrome. (A) PCR of exon 1 in the five cases (lanes 1–5; St, size standard). (B) Direct sequencing of PCR products using fluorescent termination nucleotides in five cases of Sezary syndrome and chromatogram of PCR products in two of them, demonstrating the absence of mutation.

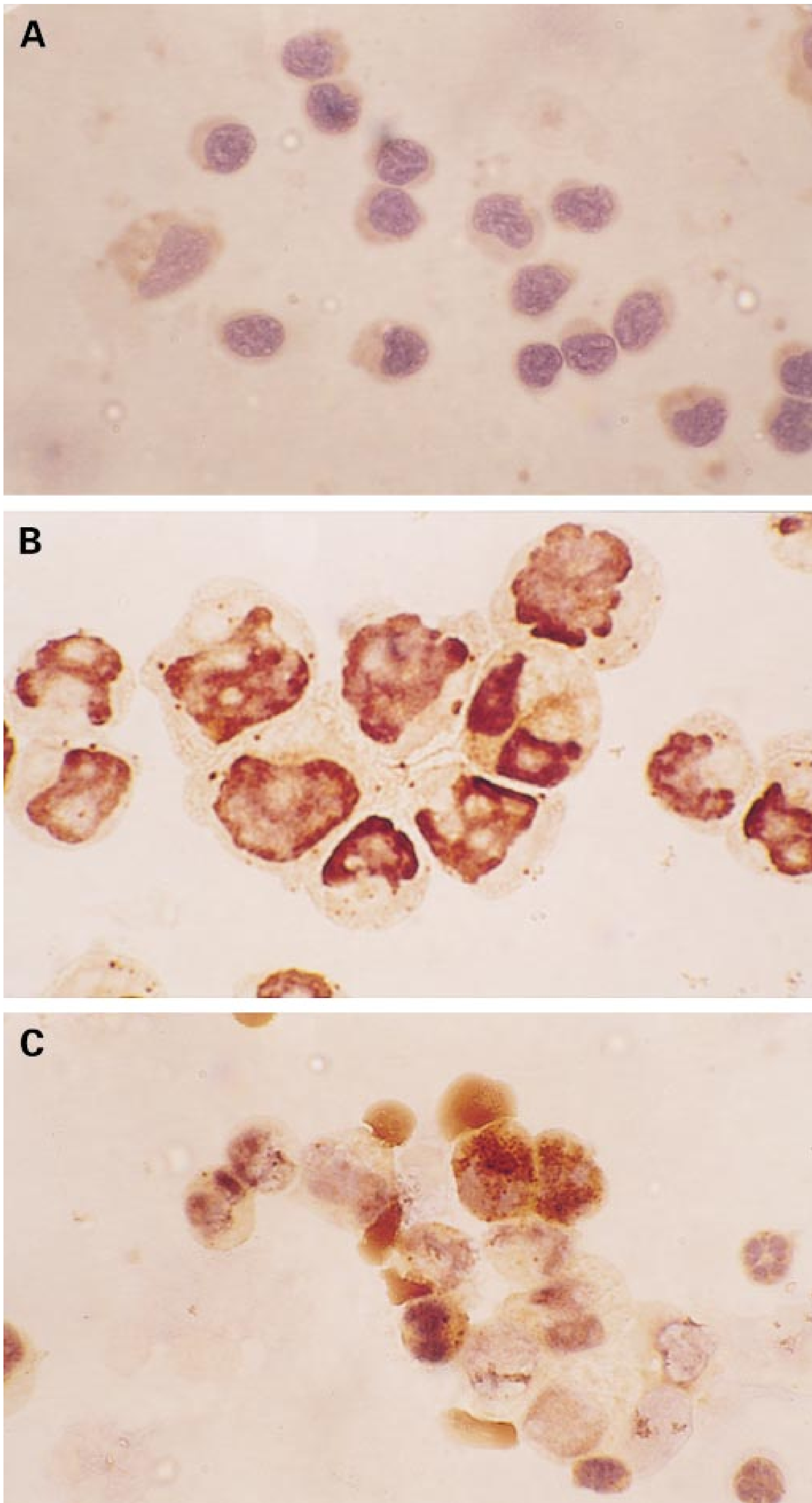

Fig 4. Expression of p53 protein by immunocytochemistry (original magnification × 100). (A) Normal lymphocyte negative for p53. (B) Cells from the CEM cell line carrying p53 gene mutation with strong nuclear staining and weaker cytoplasmic staining. (C) Two large Sezary cells from case no. 8 showing nuclear and cytoplasmic staining for p53 protein.

predominantly in the nucleus. The CEM cell line used as a positive control in the present study is an example of this. Studies carried out on p53 expression in B-prolymphocytic leukaemia (B-PLL) and chronic lymphocytic leukaemia with excess prolymphocytes (CLL/PL) confirmed the above mechanism of p53 inactivation and protein accumulation (Lens *et al*, 1997a,b).

T-PLL and Sezary syndrome are different, because p53 protein accumulation was observed in these cases of chronic T-cell leukaemias with no evidence of gene mutation in the

exons that included the hot-spots, suggesting that a non-mutational mechanism could exist for stabilizing the wild-type protein, which could lead to the abrogation of p53 suppressor function. Binding to viral proteins, overexpression of the MDM2 gene and cytoplasmic sequestration are some of the mechanisms that have been described previously for stabilizing wild-type p53 protein (Bonsing *et al*, 1997). T-lymphocytes transformed by human lymphotrophic virus (HTLV)-1 are often initially interleukin (IL)-2 dependent before progressing to an IL-2-independent state. Increased levels of wild-type p53 with a longer half-life have been demonstrated by immunoprecipitation in IL-2-independent cell lines with no evidence for gene mutation (Reid *et al*, 1993; Gartenhaus & Wang, 1995). However, in our experience, T-PLL and Sezary syndrome are not associated with HTLV-1 infections (Bazarbachi *et al*, 1997; Pawson *et al*, 1997). In some cases of non-Hodgkin lymphoma, p53 overexpression has been observed without detectable p53 gene mutation, and the transactivating function of the protein is retained, resulting in increased p21 expression (Mansukhani *et al*, 1997). The mechanism by which p53 protein is stabilized in T-PLL and Sezary syndrome has yet to be determined. It is important to note that, in the present study, the non-coding regions of the p53 gene were not tested for mutations. A recent report suggests that the presence of a germline mutation in intron 6, with no evidence for mutations in the splice sites and any of the exons, is responsible for stabilization and accumulation of wild-type p53 in diverse childhood malignancies (Avigad *et al*, 1997). Mutations to the promoter region of p53 associated with the abolition or reduction of p53 expression have been described (Kirch *et al*, 1999), but there are no known mutations to the promoter reported that result in the deregulation and expression of wild-type p53.

The other feature of interest was the high incidence of p53 allele deletion, particularly in Sezary syndrome (9/11 cases) with no evidence of mutation in exons 1–9, which covers the hot-spots. A previous study has reported p53 allele loss in 6/18 cases of Sezary syndrome (Marks *et al*, 1996). We are not aware of studies on p53 deletion in T-PLL. Chromosome 17p is frequently altered in human cancers, and allelic losses often coincide with mutations in the p53 gene localized to 17p13.1. However, in some tumour types, allelic loss at 17p is not always associated with p53 mutation (Marks *et al*, 1996; Schultz *et al*, 1996; Chattopadhyay *et al*, 1997). A new candidate gene HIC-1 (hypermethylated in cancer), which codes for a transcription factor, has been identified on 17p13.3 and is expressed ubiquitously in normal tissue but underexpressed in different tumour cells, in which it is hypermethylated. The gene has a p53 binding site in the 5′ flanking region and is activated by wild-type p53. Thus, accumulating non-functional wild-type p53 can prevent its expression (Wales *et al*, 1995).

In conclusion, p53 was found to be expressed in these T-cell leukaemias in the absence of p53 gene mutation in the exons that included the known hot-spots. Although p53 deletion was observed in the majority of cases expressing the protein, the lack of correlation between the proportion of cells with p53 deletion and expression and the presence of some cases with strong p53 expression in the absence of deletion suggests that the relationship between these two parameters, if any, is more complex. It is likely that the high incidence of p53 allele loss in Sezary syndrome in the absence of p53 mutation could mean that a gene adjacent to p53 on 17p, such as HIC-1, is the target gene. The absence of p53 protein accumulation in case 2 of Sezary syndrome with monoallelic p53 loss may represent the presence of one normal allele producing a wild-type p53 protein with the usual short half-life. Future FISH studies on HIC-1 and studies on the expression of p21 and HIC-1 in cases with p53 accumulation to determine whether the transactivation function of the wild-type p53 is retained should provide clues to the mechanism of p53 accumulation in T-PLL and Sezary syndrome.

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