

p53 EXPRESSION IN HAEMATOLOGICAL MALIGNANCIES: RESPONSE TO ALKYLATING AGENT IN THE COMET ASSAY

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Abstract. Aims. The P53 gene is a tumour suppressor, often inactivated by deletion and/or point mutation in malignancies. The incidence of p53 mutation in haematological malignancies varies with malignancy type and cell lineage affected. It is associated with disease progression, poor prognosis and resistance to chemotherapy. The wild-type p53 protein has a short half-life and cannot be detected by immunocytochemistry, whereas the mutated form has an extended half-life and can be. **Materials and methods.** Using lymphocyte cytospin preparations from patients with a range of haematological malignancies, p53 mutation was assessed with immunocytochemistry. **Results.** 83 patients showed intracellular p53 in 16 cases, including 4/18 (22%) Non-Hodgkin's lymphoma (NHL), 3/15 (20%) chronic lymphocytic leukaemia (CLL), 2/13 (15%) myeloma, 2/19 (11%) chronic myeloid leukaemia (CML), 1/3 (33%) hairy cell leukaemia (HCL), 4/15 (27%) myelodysplastic syndromes (MDS) including two transformed acute myeloid leukaemia (AML). Using the Comet assay, lymphocytes from the p53 positive NHL, CLL, CML and AML cases showed reduced damage to the alkylating agent, ethyl methanesulfonate (EMS), when compared to p53 negative cases. **Conclusions.** This suggested that mutant p53 positive cases are resistant to damage by cytotoxic agents.

Key Words: Haematological malignancies, p53 mutant protein, lymphocyte cytospin preparations, immunocytochemistry, Comet assay, ethyl methanesulphonate

Rezumat. Obiective. Gena p53 este un supresor al tumorilor, frecvent inactivată în afecțiunile maligne prin deleție și/sau mutații punctiforme. Incidența mutațiilor p53 în cazul bolilor maligne hematologice variază în funcție de tipul de malignitate și de linia de celule afectată. Se asociază cu evoluția bolii, cu prognosticul nefavorabil și cu rezistența la chimioterapie. Tipul sălbatic de proteina p53 are o perioadă scurtă de înjumătățire și nu poate fi detectată prin metode imunocitochimice, în timp ce forma care a suferit mutații a cărei perioadă de înjumătățire este prelungită, poate fi astfel pusă în evidență. **Material și metode.** Mutațiile p53 au fost puse în evidență prin metode imunocitochimice, folosind limfocite citospin preparate provenite de la pacienți cu variate afecțiuni maligne hematologice. **Rezultate.** Dintr-un grup de 83 pacienți, la 16 cazuri a fost pus în evidența p53 intracelular, incluzând 4/18 (22%) limfoame non-Hodgkin-iene (LNH), 3/15 (20%) leucemie limfatică cronică (LLC), 2/13 (15%) mieloame, 2/19 (11%) leucemie mieloidă cronică (LMC), 1/3 (33%) leucemia cu celule păroase (HCL), 4/15 (27%) sindroame mielodisplazice (SMD) incluzând două conversii la leucemie mieloidă acută (LAM). Folosindu-se testul Comet s-au pus în evidența alterări reduse ale agenților de alchilare, a etil-metan-sulfonatului (EMS) în cazul limfocitelor p53 pozitive provenite de la cazurile LNH, LLC, LMC și LAM, prin comparație cu cazurile p53 negative. **Concluzii.** Aceste date sugerează rezistența cazurilor pozitive cu mutații p53 față de acțiunea agenților citotoxici.

Cuvinte cheie: boli maligne hematologice, proteine p53 mutante, limfocite citospin preparate, imunocitochimie, testul Comet, etil-metan-sulfonat

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INTRODUCTION

p53 is a tumour suppressor gene located on the short arm of chromosome 17, mutations in p53 have been found among many kinds of malignancies (1,2).

Normal p53 protein is a multifunctional protein that participates in cell cycle regulation, apoptosis, cell immortality, and cancer cell response to chemotherapeutic agents (3, 4, 5).

Generally p53 mutations have been found to be more frequent in myeloid leukaemias such as chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML) than acute lymphocytic leukaemia (ALL) (depending on severity of type) and chronic lymphocytic leukaemia (CLL) (6). Mutations of the p53 gene have been found in 15-30% of blast crisis CML patients and rarely in the preceding chronic phase and accelerated phase (7, 8). The p53 gene is also infrequently altered in myelodysplastic syndromes (MDS), with a frequency of about 5-10%, most of which are missing mutations (9, 10, 11, 12). The mutations in p53 in MDS occur in subtypes with a prognostically poor French-American-British (FAB) classification e.g. refractory anaemia with excess blast (RAEB), RAEB in transformation, chronic myelomonocytic leukaemia (CMML). Mutations have not been reported in patients with either refractory anaemia (RA) or RA with ring sideroblasts.

The incidence of p53 mutations in ALL is approximately 3%, although this varies with type and severity (10). Up to 50% of ALL L3 and 30% of T-ALL relapsed samples have p53 mutations

(10, 13, 14). In CLL p53 mutations have been found in 15% of patient samples with an increased rate of 40% in the minority of CLL patients with an enhanced propensity to transform to the aggressive lymphoproliferative disorder called Richter's syndrome (15). Aggressive, high grade B-cell Non-Hodgkin lymphoma (NHL) has about a 30% incidence of p53 mutations, whereas indolent B-cell NHL rarely have alterations of p53 (16, 17, 18, 19). Little is known of expression of p53 in plasma cells of patients with multiple myeloma, the mutated p53 is found in about 5-10% of cases (21, 22, 23).

In several types of solid tumours, p53 mutations or p53 over expression detected by immunocytochemistry have been associated with poor prognostic factors including a large tumour mass and/or a highly proliferative tumour, and short survival (24, 25, 26, 27, 28). In fact p53 mutations are mainly seen in advanced or relapsing haematological malignancies suggest that they are also associated with poor response to chemotherapy.

In recent years there has been a rapid increase in the number of reports and papers being published using the Comet assay. The unique design of the Comet assay provides direct determination of the extent of DNA damage in individual cells it is also possible to determine whether all cells within a population demonstrate the same degree of damage. Heterogenous response by cells during treatment can aid in the prediction of tumour response to specific treatment protocols since it is possible to identify small

populations of cells which may be resistant.

The aims of this study are firstly, to investigate the incidence of p53 mutations in a range of haematological malignancies using immunohistochemistry. The second part of the study will look at the response of lymphocytes isolated from p53 positive and p53 negative patients in the Comet assay after treatment with a chemotherapeutic agent.

MATERIALS AND METHODS

Ethical approval

Ethical approval for this study was obtained from the local ethics committee at Airedale NHS Trust, Airedale General Hospital, Steeton, England. Written informed consent was obtained from all participants prior to the collection of any specimens.

Chemicals

The primary DO-7 and the secondary Envision System Kit (DAKO K1393) was purchased from Dako Corp. (Carpinteria, CA). The chemicals for the comet assay were purchased from the following suppliers: RPMI (Rothwell Park Memorial Institute) 1640 medium from BRL Life Technologies, Inc. (Gaithersburg, MD); agarose, low melting agarose, dimethyl sulfoxide (DMSO), ethidium bromide and trypan blue from Sigma Chemical Company (St Louis, USA); sodium chloride, sodium hydroxide and EDTA from BDH Lab Supplies (Poole, England); Lymphoprep cell separation gel from Nycomed Pharma AS (Oslo, Norway) and phosphate buffered saline from Mast Diagnostics (Merseyside, UK).

Sample collection and lymphocyte separation

Samples were collected by venepuncture using Becton Dickinson Vacutainer tubes containing 0.054 ml of 15% K₃EDTA as the anticoagulant. The mononuclear cell fraction was isolated by centrifugation over a lymphoprep cell separation medium of density 1.077g/ml. Two cytospin slides of the isolated lymphocytes were prepared for each patient.

Immunocytochemical detection of p53 protein

Cytospin slides were fixed for 10 min in cold acetone and washed in buffered saline prior to labelling. The immunocytochemical reaction was performed using the avidin-biotin-peroxidase (ABC) technique and was performed as instructed in the DAKO K1393 kit insert. Briefly, to block endogenous peroxidase activity the slides were incubated in H₂O₂-methanol mixture for 20 min after which the slides were washed twice with Tris buffer pH 7.6. The slides were incubated sequentially with 100µL blocking 20% swine normal serum for 10 min, one drop of primary monoclonal antibody for 60 min and finally with horseradish-peroxidase-conjugated avidin-biotin complex for 60 min and then washed twice with Tris buffer pH 7.6.

The reaction was developed using 0.05% diaminobenzidine and 0.01% H₂O₂, and slides were counterstained at 1/20 diluted Giemsa for 30 sec. The negative control was peripheral blood from a normal healthy donor and the p53 positive control was the Raji,

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Burkitt's lymphoma cell line, which overexpresses P53 mutant protein. The proportion of cells positively stained was evaluated by light microscopy using x1000 magnification and at least 500 cells per slide were scored. Positivity was accepted when more than 10% of cells showed nuclear p53 protein staining.

Comet assay

Lymphocytes were isolated from 4 patients who showed positivity to p53 protein immunocytochemistry and another 4 patients with the same disorder but who were negative for p53. One patient was diagnosed as having MDS and at the time of testing had transformed to AML. Of the other three, one was a CML patient, one a CLL patient and one NHL. The preparation of the slides, electrophoresis, staining and slide scoring were as described using the modified method of Singh *et al* (29). Ethyl methanesulfonate (EMS) was used as a model alkylating agent and used in concentrations of 0, 15, 30 and 45 mM.

Statistical analysis

Twenty-five cells/slide from two slides were analysed randomly and all experiments carried out twice (total cells were one hundred for each dose). Normal distributions were checked through the Kolmogorov-Smirnoff test. The data did not have a normal distribution; therefore a non-parametric test (Mann-Whitney) was used. Statistical analysis was carried out using the statistical software SPSS 13.0 for Windows.

RESULTS

Immunocytochemical detection of p53 protein

To detect the presence of mutant p53 protein, immunocytochemistry was performed on 83 patients who were diagnosed with a range of different haematological malignancies. Table 1 shows the number of patients tested for each disorder and the numbers in which mutant p53 protein was detected.

Table 1. Patient profile and number of cases tested positive using immunocytochemistry

Disease	Number of patients	Sex	Age median (Range)	Number positive (%)
Non-Hodgkins Lymphoma (NHL)	18	5 Male 13 Female	69 (49-80)	4 (22)
Chronic lymphocytic leukaemia (CLL)	15	9 Male 6 Female	55 (40-78)	3 (20)
Myeloma	13	7 Male 8 Female	63 (41-80)	2 (15)
Myeloproliferative disorder	19	8 Male 11 Female	67 (49-83)	2 (11)
Hairy cell leukaemia	3	1 Male 2 Female	61 (58-70)	1 (33)
Myelodysplastic syndrome (MDS)	15	6 Male 9 Female	56 (42-71)	4 (27)

The p53 control Raji cells showed intense brown staining, which was confined to the nucleus (fig 1A). The negative control did not exhibit any nuclear staining (fig 1B).

At the time of testing, 2/4 positive MDS patients had transformed into AML and the other two were classed as being in RAEB(t) stage of the disorder. In both of the positive AML

cases more than 75% of the cells were positive for p53 protein (fig 1C). 2/11 of the MDS patient who was classed as being negative for p53 showed nuclear staining in 5-6% of the cells. The 3 positive CLL had total white cell counts of greater than $50.0 \times 10^9/L$ with more than 70% lymphocytes and the percentage of p53 positive cells ranged from 15-33% (fig 1D).

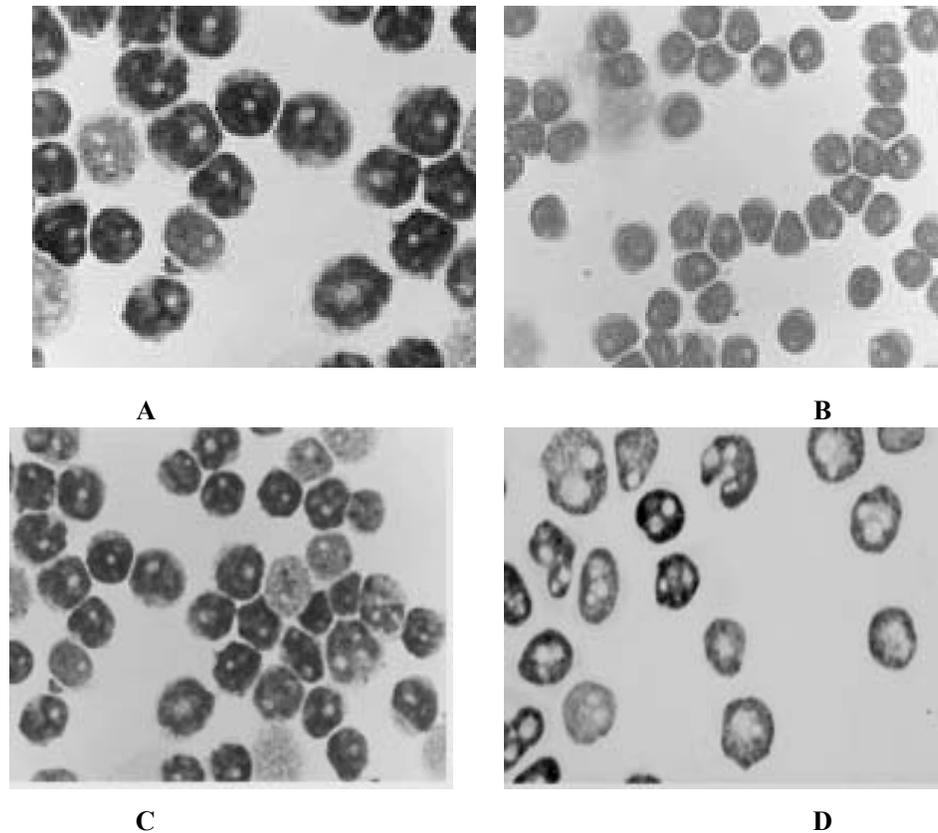


Fig 1. Immunocytochemical staining for p53 protein: positive population show intense brown nuclear staining. (A) cytopsin of Raji cells used as p53 positive control. (B) cytopsin of normal peripheral lymphocytes used as p53 negative control (C) cytopsin of transformed AML in which more than 75% of cells were p53 positive. (D) cytopsin of CLL case showing both p53 positive and p53 negative cells.

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4 of 18 (22%) cases of NHL showed p53 positivity. All 4 positive cases were tumours of B-cell phenotype and histologically were classed as diffuse large B-cell non-Hodgkin's lymphoma. In the myeloma group 15% (2/13) were positive for p53 staining, one of the positive cases had circulating plasma cells in the peripheral blood sample. In the myeloproliferative group 2 of 19

(11%) cases was positive for p53, both of the positive cases were CML with the Philadelphia chromosomes. One of the positive CML was in the accelerated phase with 12% blast cells in the peripheral blood sample.

Effect of EMS in Comet assay

Table 2 show the median tail moments obtained using varying concentrations of EMS.

Table 2. Median tail moments obtained in the Comet assay using varying concentrations of EMS in disorders of NHL-Non-hodgkin's lymphoma, CLL-chronic lymphocytic leukaemia, CML-chronic myeloid leukaemia and AML-acute myeloid leukaemia. In NHL, CML and AML the lymphocytes from p53 positive cases showed significantly reduced sensitivity to EMS ($P<0.05$) when compared to the control lymphocytes and to lymphocytes isolated from p53 negative cases, but in CLL differences between p53+ and p53- cases were not statistically significant (n.s.).

	Concentration of EMS (mM)			
	0	15	30	45
NHL				
p53 negative control	2.22	7.53	13.81	28.92
p53- NHL	1.88	5.98	10.61	24.33
p53+ NHL	2.01	4.66	9.88	12.67*
CLL				
p53 negative control	2.81	9.01	13.61	28.73
p53- CLL	2.98	6.77	13.98	14.22
p53+ CLL	2.03	6.98	11.46	13.21n.s.
CML				
p53 negative control	2.43	8.71	14.21	27.63
p53- CML	1.93	7.71	10.66	12.53
p53+ CML	2.56	5.54	6.99	8.27*
AML#				
p53 negative control	2.35	8.42	12.86	27.98
p53- MDS	3.01	9.32	11.75	15.63
p53+ AML	2.66	8.87	9.32	10.46*

#AML case was a transformed MDS.

In all 4 disorders the lymphocytes from p53 positive cases showed significantly reduced sensitivity to EMS ($P < 0.05$) when compared to the control lymphocytes and to lymphocytes isolated from p53 negative cases (Figures 3(A)-3(D)). The only exception to this was for the CLL case, although the p53

positive lymphocytes showed reduced sensitivity the difference between p53 positive CLL and p53 negative CLL was not statistically significant for this particular case ($P > 0.05$). Figure 2 shows some images of the Comet seen in the AML case.

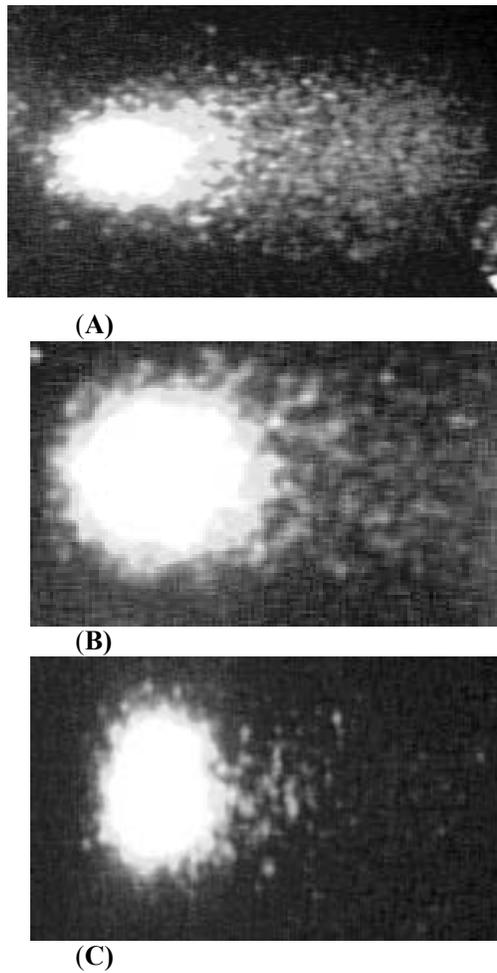


Fig 2. Comet images after treatment with 45 mM of EMS. (A) p53 negative control, (B) p53 negative AML, and (C) p53 positive AML

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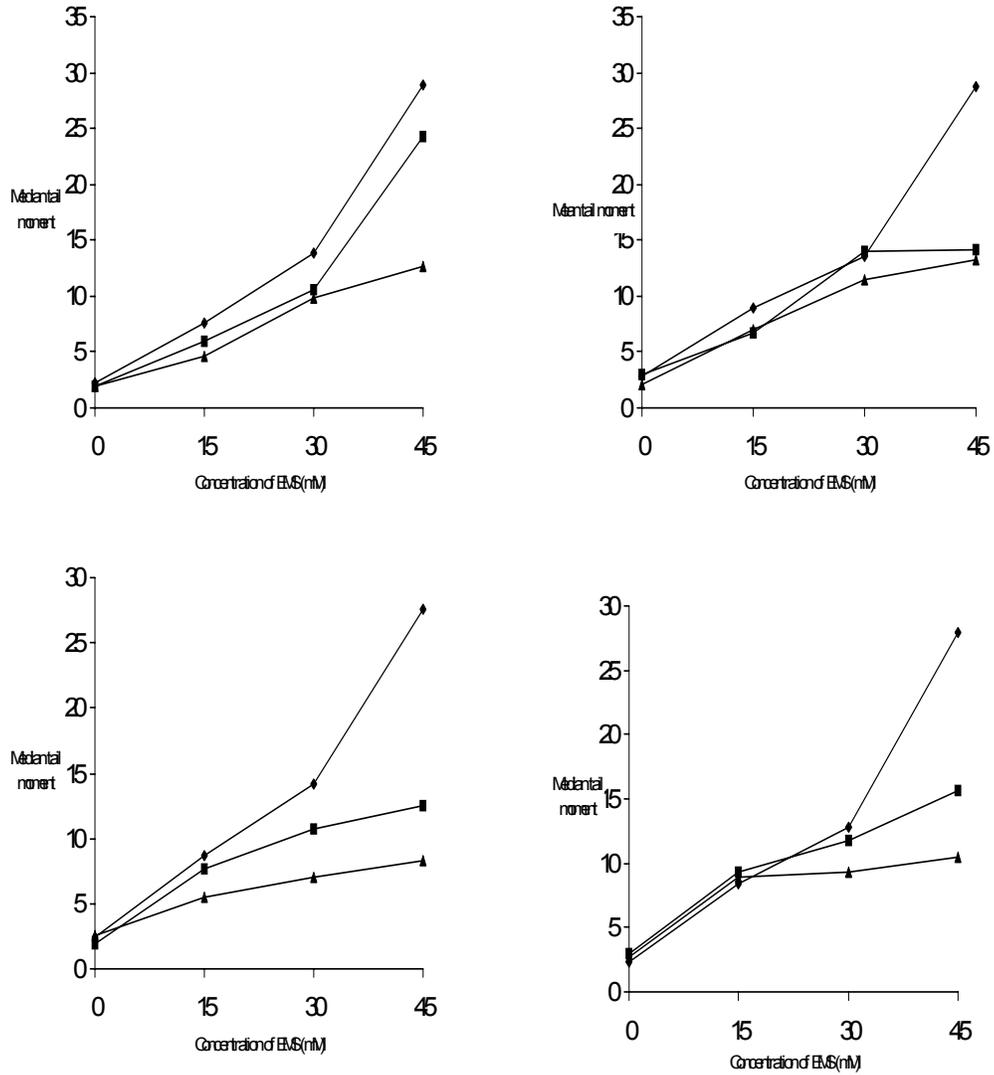


Fig 3. Median tails after treatment with EMS. (A) NHL-◆ p53 negative control, ■ p53 negative NHL, ▲p53 positive NHL. (B) CLL-◆ p53 negative control, ■ p53 negative CLL, ▲p53 positive CLL. (C) CML-◆ p53 negative control, ■ p53 negative CML, ▲p53 positive CML. (D) AML -◆ p53 negative control, ■ p53 negative AML, ▲p53 positive AML.

DISCUSSION

In this study 83 patients with a range of haematological malignancies were investigated for p53 protein using immunocytochemistry. Previous studies have shown that immunocytochemical detection of p53 protein correlates well with molecular techniques for detecting p53 such as single-stranded confirmation polymorphism (SSCP) analysis of DNA (6, 30, 31). One exception to this observation is chain-termination mutations which generally lead to absent or reduced levels of unstable p53 protein, which cannot be detected by immunocytochemistry, however, they are rarely found in tumours (6, 30, 32).

The incidence of p53 detected in this study (table 1) is broadly in agreement with previously reported findings (6, 30, 32, 33). In MDS previous studies have demonstrated that p53 mutations occur in the subtypes of MDS with a prognostically poor FAB classification (e.g. RAEB, RAEB(t)) (31, 33, 34, 35). Similar observations were made in this study of the four p53 positive patients; at the time of testing, 2 had transformed to AML and the remaining 2 were classed as being in the RAEB(t) stage of the disorder.

In CLL p53 mutation is seen in approximately 15% of cases. In this study 3/15 patients (20%) with CLL were found to be p53 positive. Previous studies have shown that the highest frequency of p53 expression is observed in patients at an advanced clinical stage (6, 33, 36). It has been suggested that p53 protein expression observed in progressive CLL may be due

to post transcriptional modifications that induce functional and confirmational alterations (37). However, a study by Cordone *et al* has shown that p53 expression was mainly due to gene mutations (36). A study by El Rouby *et al*. found that in CLL, the response rate to chemotherapy (chlorambucil, fludarabine, or combination chemotherapy) was very low in patients with a p53 mutation as compared with patients without a p53 mutation (38). The prognostic value of p53 mutations for response to chemotherapy persists even after adjustment for other risk factors such as age, sex and disease stage (33). Similar findings were more recently reported by Cordone *et al* who demonstrated that a significantly shorter treatment free interval from diagnosis and a poor response to therapy was observed in p53 positive compared with p53 negative patients (36).

In a study by Wilson *et al*. who investigated the prevalence of p53 mutation in low- and intermediate-grade NHL, abnormality was found in 16 of 75 (21%) of cases (39). In the present study a very similar incidence of p53 mutations was found, 4 of 18 (22%) of cases were p53 positive. Several studies have associated p53 mutations with advanced-stage disease and histological progression of low grade lymphomas, suggesting that p53 mutations are a late event in disease evolution (40, 41). If this was to be the case then patients with mutant p53 might be expected to have a shorter survival as compared to patients without the mutation, possibly because

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of lead time bias. Indeed, two studies of mantle cell lymphoma and one study of relapsed tumours have documented that p53 mutation and/or expression was associated with decreased survival, attributed to the development of new clones that are resistant to therapy (39, 42, 43).

The incidence of p53 mutation in myeloma has been reported to be about 5-20% of cases and is regarded to be a late event in the disease and is associated with an aggressive course (21, 22, 23, 44, 45). In the present study 2 of 13 (15%) cases of myeloma were found to have a p53 mutation. In a recent study by Drach *et al.* using fluorescence in situ hybridisation (FISH) with a DNA probe specific for the p53 locus they found that p53 deletions were present in 32.8% and 54.5% of patients with newly diagnosed and relapsed myeloma, respectively (46). It was also shown that patients with a p53 deletion had significantly shorter survival time compared with those without a deletion, both from the time of diagnosis and from the time of initiation of induction treatment consisting of conventional dose chemotherapy.

In CML 20-30% of patients in the blast crises phase of the disease have alterations in p53, whereas chronic phase CML cells only rarely have detectable p53 alterations (7, 47, 48). Circumstantial evidence suggests that a p53 mutation in the CML clone can result in disease transformation to myeloid blast crises (47). In the present study of the two p53 positive CML one was in the accelerated phase

of the disease at the time of testing, the other was clinically in the chronic phase.

Accumulating evidence suggests that abnormalities in the control of the cell cycle and programmed cell death (apoptosis), a final common pathway through which cytotoxic agents may exert their lethal effects, can lead to drug and/or radiation resistance (49, 50). p53 arrests cells exposed to DNA-damaging agents in G₁ to allow DNA repair or, if essential repairs are not possible, promotes apoptosis (51). Experimentally, loss of p53 function produces cellular resistance to alkylating and topoisomerase-II drug classes, suggesting that loss of p53 function may cause drug resistance (49, 51). In the present study, using EMS as a model alkylating agent it has been demonstrated that lymphocytes isolated from p53 positive AML, CLL, CML and NHL patients show reduced damage in comparison to corresponding p53 negative lymphocytes in the Comet assay (fig. 3). This would suggest that p53 positive lymphocytes show increased resistance to DNA damage by alkylating agents, and in particular EMS when compared to p53 negative lymphocytes.

The exact mechanism by which p53 induces resistance to chemotherapy is not clear, but a number of hypothesis has been put forward. It has been shown that normal p53 suppressed the multidrug resistance (*mdr*₁) gene promoter, whereas mutated p53 could stimulate it (52). Expression of the *mdr*₁ gene has been correlated to

resistance in many tumour types, including AML and MDS (30, 53). Mutated p53 could therefore interfere with response to chemotherapy, by activating *mdr*₁ expression. However, conflicting reports have been published, which show no correlation between p53 mutation and *mdr*₁ expression (11, 54). It has been suggested that some chemotherapeutic agents, including anthracyclin derivatives and anthracyclin-cytosine arabinoside (Ara C) could induce leukaemic cell death, at least in part, by triggering apoptosis (50, 55, 56, 57). Therefore, the ability of these agents to promote apoptosis and hence cell death in mutant p53 would not be possible.

All available evidence broadly suggests that p53 mutations are frequently seen in advanced, progressive or relapsing haematological malignancies and overall are relatively rare in the early stages of the disease. In contrast, Wada *et al.* have shown that in several leukaemic cell lines p53 mutations are already present at diagnosis in a very small proportion of cells and in a larger percentage of cells in relapse (58). This would suggest that a relatively large number of haematological malignancies carry a small population of mutated p53 at diagnosis, which is undetectable by conventional methods (especially SSCP). This population could be resistant to chemotherapy, which would facilitate relapse with a predominantly p53 mutated cell population. Furthermore, using the Comet assay it has been observed that lymphocytes isolated from p53 positive cases show reduced damage to the alkylating

agent EMS, when compared to p53 negative cases, highlighting the role that p53 may play in drug resistance in haematological malignancies.

CONCLUSIONS

The present study has demonstrated that immunocytochemistry is a sensitive method for detection of mutant p53 protein, and the incidence of mutant p53 protein is broadly in agreement with previous reports.

Our results suggested that mutant p53 positive cases are resistant to damage by cytotoxic agents.

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