Papers

p53, c-myc p62 and proliferating cell nuclear antigen (PCNA) expression in non-Hodgkin's lymphomas

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Abstract

Aims—To investigate the immunohistochemical expression of p53 protein in non-Hodgkin's lymphomas (NHL) and its relation to that of c-myc p62 oncoprotein and proliferating cell nuclear antigen (PCNA).

Methods—Paraffin wax embedded tissue from 90 non-Hodgkin's lymphomas (72 B cell and 18 T cell) was stained immunohistochemically for p53 protein, c-myc p62 oncoprotein, and PCNA using the monoclonal antibodies DO7, c-myc 1-9 E10, and PC-10, respectively.

Results—Of the non-Hodgkin's lymphomas studied, 55 (61%) stained positively for p53 protein. The proportion of positive cases increased from low grade through intermediate to high grade non-Hodgkin's lymphoma and was higher in tumours of T cell origin. The percentage of positive cells (labelling index or LI) was significantly lower in low grade non-Hodgkin's lymphoma, but no difference was established between intermediate and high grade non-Hodgkin's lymphoma. In a large proportion of low grade non-Hodgkin's lymphoma the LI was below 1%. c-myc p62 immunoreactivity was identified in all cases. A significant positive correlation was established between p53 LI and c-myc p62 LI ($r = 0.453$) as well as between p53 LI and PCNA LI ($r = 0.338$).

Conclusions—p53 immunoreactivity was present in about half the cases of non-Hodgkin's lymphoma and was related to the grade of malignancy and possibly to the B or T cell origin of the tumour. It was also associated with the proliferation state as expressed by PCNA LI and c-myc p62 expression, indicating that the expression of these three cell cycle-related genes might be interrelated.

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The p53 gene, located on chromosome 17p13, encodes a nuclear phosphoprotein which seems to be a negative regulator of cell growth and division.¹ Wild type p53 can suppress or inhibit the transformation of cells in culture by viral or cellular oncogenes,^{2,3} reduce or eliminate the tumorigenic potential

of a cell line in culture, and arrest the cell cycle of a transformed cell at the G₁ phase.^{4,5} It is therefore regarded as a tumour suppressor gene or anti-oncogene. It has also been associated with apoptosis; its role is to hold a damaged cell in G₁ phase while DNA repair is taking place. Cells which try to oppose the G₁ block may end up by activating the suicide (apoptosis) pathway.⁶

Mutations in the p53 gene are turning out to be the most common genetic alterations in many human cancers.^{7,8} Such mutations, when occurring within the coding region of the gene, can convert normal p53 into a dominant oncogene. There is evidence that mutant p53 protein may exert its oncogenic effect in two ways. First, the mutant protein can form an inactive oligomeric complex with a wild type subunit (dominant loss of function mutation). Alternatively, mutant p53 may gain a new function which overcomes the negative regulation by small quantities of wild type protein (gain of function mutation).⁹

Wild p53 protein has a short intracellular half-life of 15 minutes¹⁰; mutant p53 protein forms complexes with heat shock protein 70 in the cytoplasm and has a longer half-life of several hours.² Therefore, it has been suggested that the immunohistochemical detection of p53 protein corresponds to a mutation and not to up-regulation of the wild type gene.¹¹⁻¹³ There is some evidence from studies in acute myelogenous leukaemia, however, that prolongation of the half-life of the wild p53 protein, which facilitates its immunohistochemical detection, is not necessarily related to an underlying genetic mutation. This implies that changes occur in the post-translational processing of the protein.¹⁴

Abnormal expression of p53 has been reported in cases of acute and chronic lymphoid leukaemia.¹⁵ CD30 positive anaplastic lymphomas¹⁶ and in a variety of non-Hodgkin's lymphomas.¹⁷⁻²²

The human c-myc oncogene is considered to be another cell cycle related gene. It is induced during the transition from G₀ to G₁ phase.²³ It is expressed in a wide range of tissues at a level correlated with cell proliferation.²⁴ c-myc can act as a bivalent regulator, determining either cell proliferation or apoptosis. It may be associated in vivo with a high turnover state in which cell proliferation and apoptosis coexist.²⁵ Experimentally induced

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	<i>p53</i> immunostaining			<i>c-myc p62</i> LI	<i>PCNA</i> LI
	negative	LI < 1%	LI > 1%	mean(SD)	mean(SD)
B cell lymphomas					
Low grade					
B-CLL	8/13	2/13	3/13	17(13)	9(9)
lymphocyte predominant immunocytoma					
(lymphoplasmacytic/					
lymphoplasmacytoid subtype)	2/2	0/2	0/2	5(0)	5(0)
centrocytic lymphoma	0/1	1/1	0/1	5	20
centroblastic-centrocytic					
lymphoma	4/11	4/11	3/11	20(11)	19(11)
monocytoid lymphoma	1/1	0/1	0/1	5	10
plasmacytic lymphoma	0/1	1/1	0/1	5	3
Total	15/29	8/29	6/29	16(12)	15(15)
Intermediate-grade					
centrocytic lymphoma	2/2	0/2	0/2	23(11)	14(8)
centroblastic-centrocytic					
lymphoma	6/12	2/12	4/12	37(27)	31(20)
centroblastic lymphoma	3/17	3/17	11/17	41(21)	60(23)
lymphocyte predominant immunocytoma					
(polymorphic subtype)	4/5	1/5	0/5	41(10)	8(4)
Total	15/36	6/36	15/36	39(20)	41(28)
High grade					
immunoblastic lymphoma	1/3	0/3	2/3	85(7)	85(9)
lymphoblastic lymphoma	2/3	0/3	1/3	62(24)	53(31)
plasmacytic lymphoma	0/1	1/1	0/1	25	10
Total	3/7	1/7	3/7	67(27)	61(33)
T cell lymphomas					
Low grade	1/3	1/3	1/3	20(13)	14(18)
Intermediate grade	1/8	4/8	3/8	14(15)	26(12)
High grade	0/7	1/7	6/7	56(18)	66(16)
Total	2/18	6/18	10/18	31(25)	40(26)

LP = lymphocyte predominant.

lymphocyte stimulation causes an increase in *c-myc* expression followed by an increase in DNA and RNA synthesis.²⁶ *c-myc* encodes two nuclear phosphoproteins, of which p62 constitutes its main protein product.²⁷

The detection of *c-myc* expression in various tissues can be performed by means of molecular biology and immunohistochemical techniques. The latter are of value in studying the *c-myc* p62 distribution in neoplastic and reactive cells, a discrimination which is especially important in non-Hodgkin's lymphoma.²⁸ Overexpression of *c-myc* has been reported in many haematopoietic neoplasms, including non-Hodgkin's lymphoma.^{21 28-32} This may reflect either a structural genetic abnormality or merely an increased rate of cell proliferation—it may be a consequence rather than a cause of tumorigenesis.³³

Proliferating cell nuclear antigen (PCNA) is a 36 kilodalton nuclear protein that acts as an auxiliary factor of DNA polymerase δ and is considered to be involved in the cellular replication machinery.^{34 35} PCNA concentrations are maximally increased at the late G₁ and S phases of the cell cycle and correlate directly with the rates of cellular proliferation and DNA synthesis.^{36 37} PCNA immunolocalisation in paraffin wax sections has been used as an index of cell proliferation in malignant lymphomas.^{32 38 39}

Methods

Ninety cases of non-Hodgkin's lymphoma coming from the files of the pathology departments of Athens School of Medicine and Tzanion Hospital (Piraeus) were examined. Diagnosis was made on the basis of routine histology and immunohistochemical tests. Seventy-two cases were diagnosed as B cell

and 18 as T cell lymphomas. All cases were classified into three grades of malignancy according to the Working Formulation⁴⁰ (table).

All specimens had been fixed in 10% formalin and routinely processed for paraffin wax embedding. Sections were cut at 3 μ m and mounted on Vectabond treated glass slides. The slides were dried at 37°C for at least 24 hours and then stained immunohistochemically for p53 protein using the monoclonal antibody DO7 (Dakopatts, High Wycombe, England), for *c-myc* p62 protein with *c-myc* 1-9 E10 (kindly provided by Professor D Spandidos), and for PCNA with PC-10 (Dakopatts, High Wycombe, England). DO7 recognises an epitope in the N-terminus of the human p53 protein between amino acids 35 to 45. It reacts with both wild and mutant types of p53 protein. For immunostaining with the DO7 antibody the three-step avidin-biotin technique (Vecta-stain Elite PK6102, Vector) was used. The antibody was diluted 1 in 40 in phosphate buffered saline (PBS) (pH 7.4). *c-myc* 1-9 E10 was used at a dilution of 1 in 80, as described before.³¹ PC-10 was diluted 1 in 150 with overnight incubation according to the procedure described by Hall *et al.*⁴¹

Staining was assessed blind (without knowing the histological diagnosis) by one observer. Positive non-neoplastic cells or cells showing only cytoplasmic staining were not taken into account. In each case nuclei from about 1000 tumour cells (from 10 high power fields) were counted and LI was calculated as the percentage of positive nuclei. Although the intensity of staining varied, all identifiable nuclear staining was recorded as positive. In cases with patchy staining, areas containing

Figure 1 (A) DO7 immunostaining in a low grade lymphoma (B chronic lymphocytic leukaemia). Sporadic positive cells are seen. (B) DO7 immunostaining in an intermediate grade T cell lymphoma. Several positive cells are seen.

(A)

(B)

the highest and lowest number of positive cells were selected and the percentages were averaged to give the LI.

The results were evaluated statistically using Wilcoxon's rank sum test, the χ^2 test, and Spearman's rank correlation coefficient.

Results

Of the 90 non-Hodgkin's lymphoma cases studied 55 (61%) stained positively for p53 protein. p53 immunoreactivity was clearly evident as granular nuclear staining and was confined to neoplastic cells alone (figs 1 and 2). In many cases a patchy distribution of positive cells throughout the tumour was observed. A characteristic pattern of staining was noted in cases of low grade lymphocytic lymphoma (B chronic lymphocytic leukaemia—B CLL)

Figure 2 DO7 immunostaining in a high grade B cell lymphoma. Most cells are positive.

where p53 staining was mostly observed in paraimmunoblasts (fig 1A). The proportion of cases with positive cells varies according to the grade of malignancy (50% for low grade, 64% for intermediate grade, and 79% for high grade lymphomas), histological subtype, and whether the tumours were of B or T cell origin (54% for B cell and 89% for T cell lymphomas— $p < 0.01$) (table).

The percentage of positive cells varied from occasional stained cells (less than 1%) to 80% of the neoplastic cells. Cases with less than 1% positive cells were more common in low grade non-Hodgkin's lymphoma (56% of positive low grade lymphomas, 36% of intermediate, and 18% of high grade lymphomas). The p53 LI of intermediate grade non-Hodgkin's lymphoma was significantly higher than that of low grade NHL ($p < 0.05$; Wilcoxon's test) (fig 1), but no significant difference was found between intermediate and high grade NHL (fig 2). A significant difference was also established between B and

Figure 3 c-myc 1-9 E10 immunostaining in a high grade T cell lymphoma showing nuclear and cytoplasmic staining of neoplastic cells.

Figure 4 (A) PC10 immunostaining in a low grade (centroblastic-centrocytic) lymphoma showing scattered positive cells. (B) PC10 immunostaining in an intermediate grade (centroblastic) lymphoma. Many positive cells are seen.

T cell lymphomas, the latter displaying a higher LI ($p < 0.05$; Wilcoxon's test). This difference was not reproduced when the comparison was made within each grade of malignancy separately.

c-myc p62 immunoreactivity was identified in all cases ranging from 1% to 90% and was mainly nuclear, although some cytoplasmic staining was also observed (fig 3). Large cells with cleaved or non-cleaved nuclei, paraimmunoblasts, immunoblasts and lymphoblasts were stained more often. A varying number of non-lymphoid positive cells (histiocytes, reticulum, and endothelial cells) was also found.

Similarly, PCNA immunostaining was observed in all cases studied (fig 4). The staining pattern was of granular, diffuse, or mixed type in the nuclei, while in a small percentage of cases a weaker cytoplasmic staining was also seen. PC10 showed a predilection for blastic cells. This was best exemplified in low grade lymphomas, such as B CLL, where positive staining was mostly confined to paraimmunoblasts. The mean *c-myc* p62 and PCNA LI for each subgroup of non-Hodgkin's lymphoma are shown in the table.

The p53 LI correlated positively with the *c-myc* LI on the one hand and with the PCNA LI on the other, although the rank correlation was not strong in either case (Spearman's rank correlation: r_s 0.453, $p < 0.001$, and r_s 0.0338, $p < 0.001$, respectively).

Discussion

In a previous study of a large series of non-Hodgkin's lymphoma we showed that *c-myc* p62 expression is increased in most cases of non-Hodgkin's lymphoma. It was also shown that *c-myc* p62 and PCNA expression are both related to malignancy grade and that

there is a moderate linear correlation between them ($r = 0.55$).³²

In the present study we observed p53 immunoreactivity in 61% of cases of non-Hodgkin's lymphoma. This percentage is similar to that reported by Villuendas *et al.*¹⁸ and higher than that found by Soini *et al.*,¹⁷ Pezzela *et al.*¹⁹ and Said *et al.*²⁰ Positive cells were identified in 50% of low grade lymphomas and in most intermediate (69%) and high grade non-Hodgkin's lymphoma (79%). These findings also agree with those of Villuendas *et al.*¹⁸ The LI was low ($< 1\%$) in many cases, especially low grade malignancy, confirming previous reports.¹⁷⁻²⁰ The LI was significantly lower in low grade lymphomas compared with intermediate and high grade non-Hodgkin's lymphoma, but no difference was found between the two latter groups. Positive cells in low grade lymphomas did not exceed 15%; in higher grades more than one third of the cases had an LI greater than 15%. This is similar to the finding of Villuendas *et al.*¹⁸; in their series, however, the p53 LIs in low grade lymphomas were lower than 5%. A difference was also established between B cell and T cell non-Hodgkin's lymphoma, but this may have been partly attributable to the higher proportion of intermediate and high grade cases in the T cell group.

It has been suggested that greater p53 immunoreactivity in higher grade lymphomas and its predilection for the paraimmunoblasts in B CLL may reflect the progression of low to high grade in some cases.^{17,18} This view is supported by data provided by sensitive molecular biology techniques²¹ by means of which p53 mutations were detected in B CLL and particularly in its stage of progression known as Richter's transformation. To explain the presence of sporadic positive cells in several cases it has been hypothesised by some authors^{17,18} that a non-mutational mech-

anism could be responsible for the immunohistochemical detection of p53 protein in these instances. According to this theory, a high proliferation rate may result in detectable concentrations of wild p53 protein.^{17,18} This is supported by the experimental finding of detectable amounts of wild p53 protein in phytohaemagglutinin stimulated, rapidly proliferating lymphocytes,⁴² and by the occurrence of sporadic positive cells in thymus and reactive lymphoid tissue.¹⁸

The correlation of p53 expression with the grade of malignancy may indicate that a high p53 LI has an adverse prognostic value. Indeed, Cabanillas *et al*⁴³ have shown that patients with abnormalities in chromosome 17, where the p53 gene is located, tend not to respond to chemotherapy and have a poor survival.

Published data show that p53 protein has a regulatory effect on the PCNA gene: the wild type protein selectively down-regulates PCNA mRNA and protein expression⁴⁴ probably by inhibiting the function of the PCNA promoter⁴⁵ whereas the mutant p53 seems to activate directly PCNA promoter.⁴⁶ That a general activation of cellular growth may be the cause of PCNA promoter activation cannot be relevant.⁴⁶ The weak positive correlation between p53 and PCNA expression in non-Hodgkin's lymphoma established in our series may indicate that in most cases the p53 protein we detected immunohistochemically is of the mutant type. If the reverse were true a negative correlation would be expected. A similar correlation has been shown in primary lung carcinomas (Korkolopoulou *et al*, unpublished data) but not in central nervous system neoplasms.⁴⁷

It has also been shown that p53 is a transcriptional modulator of c-myc expression: normal p53 inhibits the expression of c-myc^{2,3} and may initiate apoptosis by causing G₁/S arrest in cells expressing c-myc.²⁵ p53 mutations have been associated with c-myc gene deregulation in Burkitt's lymphomas and L₃ acute lymphoblastic leukaemia,^{21,48} while a recent study indicates that in B cell lymphoid tumours the critical factor determining the involvement of mutant p53 is the maturation stage of transformed B lymphocytes, independently of c-myc deregulation.⁴⁹ In this study we have shown a positive correlation between p53 and c-myc expression. According to the above data, this finding provides indirect evidence that in most cases of non-Hodgkin's lymphoma the p53 protein we detected immunohistochemically is not of the wild type.

To summarise, p53 immunoreactivity is present in most cases of non-Hodgkin's lymphoma and is related to the grade of malignancy and possibly to the B or T cell origin of the tumour. It is also correlated with the proliferation rate, as expressed by PCNA LI and c-myc p62 expression, indicating that the expression of these three cell cycle related genes is interrelated. The co-expression of c-myc and p53 may be linked to the proliferation rate or may suggest a role for these onco-

genes in the pathogenesis of some cases of non-Hodgkin's lymphoma.

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