Immunohistochemical expression of the p53, mdm2, p21/Waf-1, Rb, p16, Ki67, Cyclin D1, Cyclin A and Cyclin B1 proteins and apoptotic index in T-cell lymphomas

**P. Kanavaros[1#], M. Bai[2#], K. Stefanaki[3], G. Poussias[2], D. Rontogianni[4], E. Zioga[2],
V. Gorgoulis[5] and N.J. Agnantis[2]**

Departments of Histology [1]University of Thessallia, [5]University of Athens, Departments of Pathology,
[2]University of Ioannina, [3]Agia Sophia Hospital of Athens, and [4]Evangelismos Hospital of Athens, Hellas

Summary. Fifty-seven cases of T-cell lymphomas (TCL) including 5 lymphoblastic (T-LBL) and 52 peripheral TCL (PTCL) were analyzed by immunohistochemistry for the expression of p53, mdm2, p21, Rb, cyclin D1, cyclin A, cyclin B1, and Ki67/MIB1 proteins and 39/52 PTCL were also analyzed for the expression of p16 protein and for the presence of apoptotic cells by the TUNEL method. The aim was to search for abnormal immunoprofiles of p53 and Rb growth control pathways and to determine the proliferative activity and the apoptotic index of TCL. Abnormal overexpression of p53, p21 and mdm2, in comparison to normal lymph nodes, was found in 12/57, 10/57 and 2/57 cases of TCL, respectively. Abnormal loss of Rb and p16 expression was found in 1/57 and 2/39 cases, respectively, whereas abnormal overexpression of cyclin D1 was not detected in any of the 57 cases. Our data revealed entity-related p53/p21/mdm2 phenotypes. Indeed, most nodal and cutaneous CD30+ anaplastic large cell lymphomas (ALCL) showed concomitant overexpression of p53 and p21 proteins (7/8 cases), and mdm2 was overexpressed in 2 p53-positive nodal ALCL. In contrast, over-expression of p53 was found in 3/17 cases of nodal peripheral TCL unspecified (PTCL-UC) and 2/7 non-ALCL cutaneous pleomorphic TCL. Overexpression of p21 protein was detected in 2/3 p53-positive PTCL-UC and in 1/2 p53-positive non-ALCL cutaneous pleomorphic TCL. Finally, all the remaining 25 cases of TCL did not show p53 and p21 overexpression. Overall, the p53+/p21+ phenotype in 10/57 TCL suggests wild-type p53 capable of inducing p21 expression. The highest apoptotic index (AI) was found in ALCL and a positive correlation between apoptotic index and Ki67 index (p<0.001) was detected. Ki67, cyclin A and cyclin B1 expression was found in all 57 TCL and on the basis of the combined use of these 3 variables, 3 groups of proliferative activity could be determined: a) high in ALCL and T-LBL, b) low in mycosis fungoides (MF) and γδ hepatosplenic TCL, and c) intermediate in the remaining TCL entities. The proliferative activity in the 12 p53 overexpressing cases was higher in comparison to the 45 p53-negative cases. Ki67 expresion in more than 25% of tumour cells showed significant correlation with p53 overexpression (p<0.001). Rb expression tended to be parallel to Ki67, cyclin A and cyclin B1 expression in all but one case of nodal PTCL-UC which displayed loss of RB expression. Interestingly, this case was p53-negative, whereas the p53-positive cases were Rb-positive. These findings suggest that different pathogenetic routes may function in some TCL, involving either the p53 or, less frequently, the Rb pathways.

Key words: Cell-cycle proteins, Apoptosis, immuno-histochemistry, T-cell lymphomas

Introduction

There is increasing evidence that deregulation of the p53/mdm2/p21 and p16/Rb growth control pathways and impairment of other components of the cell cycle such as cyclins, cyclin-dependent kinases (CDK) and cyclin-dependent kinase inhibitors (CDKI) may be involved in oncogenesis (Cordon-Cardo, 1995; Sellers and Kaelin, 1997; Gillet and Barnes, 1998; Liggett and Sidransky, 1998).

p53 tumour suppressor gene is important for the control of cell death and proliferation, inducing cell cycle arrest and/or apoptosis in response to various cellular stress (Cox, 1997; Polyak et al., 1997). P-53 dependent G1 arrest is mediated, at least in part, through p53-mediated induction of p21/WAF1/Cip1, an inhibitor of the cyclin-cyclin-dependent kinase (CDK) complexes

(Cox, 1997). The activity and the stability of p53 protein is regulated via interactions with proteins such as mdm2 which allows targeting of p53 to the ubiquitin-mediated proteolytic network (Prives and Hall, 1999). Mdm2 binding also blocks the ability of p53 to interact with the transcriptional apparatus and disruption of the p53-mdm2 interaction is likely to be associated with p53 stabilization after cellular stress or oncogene imbalance (Haupt et al., 1997; Kubbutat et al., 1997; Save et al., 1998; Prives and Hall, 1999).

p53 gene mutations have been described in lymphoid malignancies, more frequently in Burkitt's lymphoma and in transformation of low to high grade B-cell lymphoma (BCL) (Cesarman et al., 1993; Sander et al., 1993; Du et al., 1995; Chilosi et al., 1996; Villuendas et al., 1997). Many studies reported a discordance between low rates of p53 gene mutation and frequent immunoexpression of the protein, which may reflect expression of a stabilized wild-type(wt) p53 protein (Matsushima et al., 1994; Chilosi et al., 1996; Maestro et al., 1997; Villuendas et al., 1997). In this respect, a combined immunohistochemical evaluation of p53 and p21 proteins could be helpful to obtain indirect information about the status of the p53 gene since only wt p53 can induce p21 protein (Chilosi et al., 1996). Although many p53 studies were performed in BCL, only a few studies have focused on p53 abnormalities in T-cell lymphomas (TCL). TCL constitute a morphologically and immunologically heterogeneous group of usually aggressive tumours, which represent 15 to 20% of non-Hodgkin's lymphomas (NHL) (Harris et al., 1994; Gisselbrecht et al., 1998). In these tumours, overexpression of p53 protein is reported in 20-30% of cases, whereas mutations of p53 gene seem to be very rare (Cesarman et al., 1993; Inghirami et al., 1994; Kanavaros et al., 1994; Matsushima et al., 1994; Lauritzen et al., 1995; Pescarmona et al., 1999).

A critical step in cell cycle progression is phosphorylation of the Rb protein (pRb) resulting in release of the E2F transcription factor which plays an important role in the regulation of S-phase entry (Weinberg, 1995). pRb phosphorylation is stimulated by cyclin-D/CDK4 complexes and inhibited by p16. P16 functions as an inhibitor of CDK4. Therefore, p16, cyclin D1 and pRb are suggested to function in a single regulatory pathway of the cell cycle (Liggett and Sidransky, 1998). Unregulated phosphorylation of pRb by CDK4 due, either to cyclin-D1 overexpression or loss of functional p16 could lead to uncontrolled cellular proliferation. Moreover, there is recent evidence for a complex network linking directly the function of Rb and p53 genes via mdm2. Rb binding to mdm2 could overcome the antiapoptotic function of mdm2 on p53-induced apoptosis and could also inhibit the mdm2-targeted p53 degradation, but could not prevent mdm2 from inhibiting the p53-mediated transactivation (Hsieh et al., 1999). With respect to lymphoid malignancies, alterations of p16 gene structure and/or expression have been reported more frequently in T-acute lymphoblastic leukaemias (T-ALL), in adult T-cell leukaemias/lymphomas (ATLL) and in transformation of low to high grade B-cell lymphomas (Siebert et al., 1996; Herman et al.,1997; Uchida et al., 1997; Drexler, 1998; Gerajts et al.,1998; Villuendas et al., 1998). Abnormalities of the Rb gene expression have been described in some high grade B-cell lymphomas (Martinez et al., 1993; Weide et al., 1994; Zhu et al., 1995; Grierson et al., 1996; Jares et al., 1996). On the other hand, cyclins are important molecules for the progression of the cell cycle and are divided into two main families (Gillet and Barnes, 1998). The G1 family includes cyclins C, D1-3 and E, which are important for the passage of cells through the G1 phase and their entry into the S-phase. The other family includes the mitotic cyclins A and B. Cyclin A plays a role in DNA replication in the S-phase. Cyclins B1 and B2, in conjunction with p34cdc2, ensure irreversible entry into mitosis.

Prompted by the above data and because of the paucity of combined immunohistochemical information regarding cell-cycle regulation in TCL we have investigated proteins involved in the p53 and Rb growth control pathways in relation with the proliferation status as determined by the expression of Ki67, cyclin A and cyclin B1 proteins in 57 cases of TCL. The aims were a) to search for immunohistochemical abnormalities of the p53 or Rb networks, b) to determine the immunohisto-chemical proliferation status, and c) to correlate the findings with the histological subtypes of TCL.

Materials and methods

Fifty-two cases of peripheral T-cell lymphomas (PTCL) and five cases of lymphoblastic T-cell lymphomas (T-LBL) were selected at initial diagnosis (Table 1). All TCL cases with available tissue blocks were retrieved from the files of the Departments of Pathology of the University Hospital of Ioannina, the Evangelismos Hospital of Athens and the Venizelion Hospital of Heraklion. The TCL cases were selected on the basis that tumour cells expressed at least one of the CD3, UCHL-1/CD45RO T-cell antigens and were negative for the B-cell marker L26/CD20. Lymphomas were classified according to the REAL classification (Harris et al., 1994) (Table 1). Five reactive lymph nodes were included as control.

Immunohistochemical staining

Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the alkaline-phosphatase/anti-alkaline phospatase (APAAP) procedure with microwave pretreatment. Monoclonal antibodies directed against p53 protein (DO-7; Dako SA. Glostrup, Denmark; dilution 1:50), mdm2 protein (IF-2, Calbiochem NY; dilution 1:50), p21/waf1 protein (EA-10; Calbiochem NY; dilution 1:50), Rb protein (Rb1 Dako SA; dilution 1:20), cyclin D1 (DCS-6 Novocastra; dilution 1:20), proliferation-associated nuclear antigen

Ki67 (MIB-1: Immunotech, Marseille, France; dilution 1:20), cyclin A (6E6, Novocastra; dilution 1:10), cyclin B1 (7A9, Novocastra; dilution 1:10) and p16 (F-12, Santa Cruz; dilution 1:100) were applied. P16 was tested only in 39 cases of PTCL because of lack of material. Positive control slides were included in all cases. They consisted of reactive lymph nodes for Rb, cyclin A, cyclin B1 and Ki67, Hodgkin lymphoma known to be positive for p53 and mdm2 (Kanavaros et al., 2000) normal colon for p21 and breast carcinoma for cyclin D1. A semi-quantitative evaluation of p53, mdm2, p21, and cyclin D1 expression was performed, by using the x40 objective and counting at least 5 fields selected on the basis that they contained considerable numbers of immunopositive cells. P53, mdm2, p21 and cyclin D1 abnormal overexpression was considered when more than 10% of tumour cells were positive since these proteins are either expressed at low levels or are undetectable in the 5 reactive lymph nodes. Thus each case was conventionaly scored by evaluating the percentage of positive tumour cells: 10 to 25%; 25 to 50%; and more than 50%. For angioimmunoblastic lymphomas (AIL), since the neoplastic or reactive nature of the positive cells could not be ascertained, the quantification was assessed as the overall percentage of the positive cells. For Ki67, p16, Rb, cyclin A and cyclin B1, a continuous score system was adopted since the 5 reactive lymph nodes always expressed Rb, p16, Ki67, cyclin A and cyclin B1. Then, the groups of positivity were determined as follows: less than 10%; 10 to 25%; 25 to 50%; and more than 50%. Rb or p16 loss of expression was considered when there was no nuclear staining in the entire specimen (diffuse) or in areas (focal) of the tumour section. Staining of stromal and endothelial cells was required as internal positive control to interpret Rb or p16 loss of expression. A mosaic pattern with positive cells intermingled with negative cells was not considered as loss of expression since the level of p16 protein varies along the cell cycle (Villuendas et al., 1998).

TUNEL

The TdT (terminal deoxynucleotidyl-transferase)-mediated in-situ labelling technique (TUNEL) (Apotag kit, Oncor, Gaithersburg) was carried out as previously described (Czader et al., 1996). Positive controls (reactive lymph nodes) and negative controls (sections without TdT) were included in every staining. Morphologically intact TUNEL-positive cells and apoptotic cells were considered as positive and are referred to as apoptotic cells. Necrotic areas were excluded. The number of apoptotic cells was recorded by using the x40 objective and by counting the apoptotic cells in 10 randomly selected fields, corresponding to a total of 2000 to 5000 cells. The apoptotic index (AI) was expressed as the mean number of labelled cells per 100 intact cells.

Statistical analysis

Statistical analysis was performed using Chi-square test with Yates' correction. Fisher exact test was used when there were less than 5 cases in one group in a table for statistical analysis. Pearson and Spearman correlation analysis were used for the assessment of correlation between continuous variables. The results were considered as statistically significant when $p < 0.05$.

Results

p53, mdm2 and p21 expression

Our results showed that concomitant p53 and p21 protein overexpression was strongly associated with nodal and cutaneous anaplastic large cell lymphoma (ALCL) while it was less frequent or absent in the other clinicopathological entities of TCL (Table 1). Mdm2 overexpression was detected in only 2/57 cases of TCL which were nodal ALCL. In detail, nodal and cutaneous ALCL, showed concomitant overexpression of p53 in 7/8 cases and the 2 mdm2-positive cases were also p53+/p21+ (Table 1) (Fig. 1). In nodal peripheral TCL unspecified (PTCL-UC) overexpression of p53 protein was found in 3/17 cases. P21 overexpression was detected in 2/17 cases which were also p53-positive. In non-ALCL cutaneous pleomorhic TCL, p53 overexpression was found in 2/7 cases and one of these 2 cases also showed p21 overexpression. The lymphomas belonging to the other categories of TCL i.e. 9 AIL, one hepatosplenic γδ, one intestinal enteropathy-type TCL (ETCL), 5 lymphoblastic T-cell lymphomas, 3 MF and 6 other extranodal TCL (Table 1) did not show any

Table 1. Overexpression of p53, mdm2 and p21 proteins in relationship with the clinicopathological entities of T-cell lymphomas (TCL).

	P53	Mdm2	P21
Precursor TCL			
T-LBL	0/5	0/15	0/5
Nodal TCL			
AIL-TCL	0/9	0/9	0/9
PTCL-UC	3/17	0/17	2/17
ALCL nodal	5/5	2/5	5/5
Extranodal TCL			
MF	0/3	0/3	0/3
ALCL cutaneous	2/3	0/3	2/3
Non-ALCL cutaneous pleomorphic TCL	2/7	0/7	1/7
ETCL	0/1	0/1	0/1
HPS γδ TCL	0/1	0/1	0/1
Other extranodal TCL*	0/6	0/6	0/6
TOTAL	12/57	2/57	10/57

T-LBL: T-lymphoblastic lymphoma; AIL-TCL: Angioimmunoblastic T-cell lymphoma; ALCL: Anaplastic large cell lymphoma; PTCL-UC: Peripheral TCL-unclassified; ETCL: Enteropathy-type intestinal T-cell lymphoma; HPS γδ TCL: Hepatosplenic γδ T-cell lymphoma; *: three cases with initial diagnosis in muscle tissue, 2 cases with initial diagnosis in adipose tissue and 1 case with initial diagnosis in tongue tissue.

significant overexpression of p53 and p21 proteins.

Ki67, cyclin A, cyclin B1, cyclin D1, Rb and p16 expression

Ki67, cyclin A and cyclin B1 expression was found in all 57 TCL (Table 2) and on the basis of the combined use of these 3 variables, 3 groups of proliferative activity could be determined (with very rare cases being outside the following limits): a) high in ALCL and T-LBL (mean rate >25% for each variable), b) low in MF and γδ hepatosplenic TCL (mean rate <5 % for each variable), and c) intermediate in the remaining TCL entities (mean rate 5-25% for each variable). In detail, Ki67 expression

Table 2. Expression of p53, mdm2, p21, Rb, p16, cyclin D1, cyclin A, cyclin B1 and Ki67 proteins in T-cell lymphomas (TCL).

	<10%	10-25%	25-50%	>50%
P53	8*	9	3	-
P21	11*	10	-	-
Mdm2	7*	2	-	-
Rb	1	32	21	3
P16#	-	19	17	3
Cyclin D1	2*	-	-	-
Cyclin A	15	31	11	-
Cyclin B1	27	14	6	-
Ki67	17	29	9	2

*: these cases were not considered to overexpres the corresponding proteins; #: only 39 cases of PTCL were studied for p16 expression. Two cases with 10-25% positive cells displayed focal loss of p16 expression.

varied from 2 to 51%. The highest expression was found in ALCL (mean rate 38.3%). In contrast, MF showed the lowest Ki-67 expression with mean rate 1.9%. Cyclin A (Fig. 2) and cyclin B1 expression (Fig. 3) increased in parallel to Ki-67 expression with positivity varying from 2 to 36% (cyclin A) and 1 to 31% (cyclin B1). The highest expression was found in ALCL (mean rates 31% for cyclin A and 24.8% for cyclin B1). In contrast, MF showed the lowest cyclin A and cyclin B1 expression with mean rates of 1.4% and 1.1%, respectively. Rb diffuse or mosaic expression (Table 2) was found in all but one TCL and tended to parallel Ki67, cyclin A and cyclin B1 expression. The highest Rb expression was found in ALCL (mean rate 47.4%). Loss of Rb expression with concomitant intermediate proliferative activity was found in one nodal PTCL-UC. In AIL, Rb expression was found in the majority of larger cells whereas most small cells were Rb-negative. In MF and the hepatosplenic Tγδ case about 10% of tumour cells were Rb-positive but this was not interpeted as loss of expression because of the low proliferative activity of these tumours. Cyclin D1 overexpression was not detected. Of the 39 tested cases of PTCL, 1 case of nodal PTCL-UC and 1 case of non-ALCL cutaneous pleomorphic TCL, displayed focal loss of p16 expression in areas composed mainly of large cells which showed increased proliferative activity. The focal absence of p16 expression in these 2 cases was interpreted as loss of expression because in reactive lymph nodes higher levels of p16 expression were found in those compartments where cell proliferation was increased. P16 diffuse or mosaic expression was found
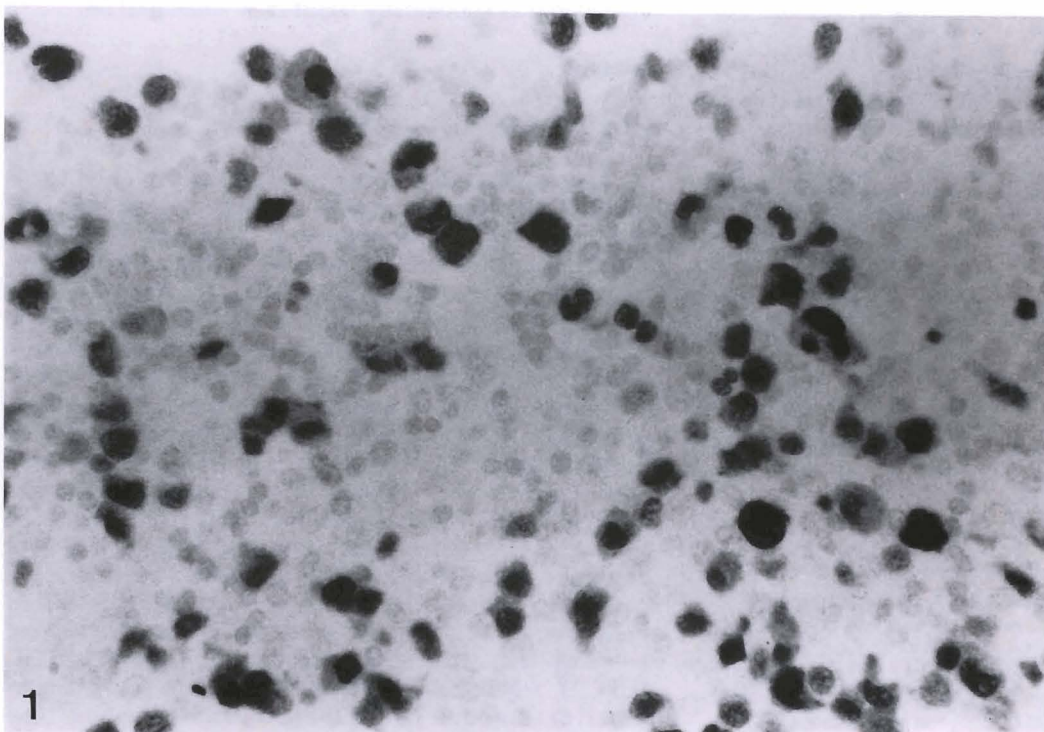

Fig. 1. Expression of p53 in nodal ALCL. x 400

in all the remaining 37 PTCL studied, comprising 4 nodal and 2 cutaneous ALCL, 15 nodal PTCL-UC, 9 AIL, 1 ETL, 3 non-anaplastic large cell cutaneous pleomorphic TCL, and 3 other extranodal PTCL. The highest p16 expression was found in ALCL (mean rate 49.2%).

Apoptotic index

The group of 39 cases which was studied for p16, was also investigated by the TUNEL method and interpretable results were obtained in 35 of them (Table 3). The 4 nodal and 2 cutaneous ALCL showed the highest apoptotic index, varying from 1.1 to 2.3. Of the remaining 29 cases, only 3 cases of nodal PTCL-UC and

Table 3. Apoptotic index (AI) and Ki67 index in relationship to the clinicopathological entities in 39 cases of peripheral T-cell lymphomas (PTCL).

	Number of cases	AI Range	Ki67 index Range
Nodal TCL			
AIL-TCL	9	0.1-0.9	7.6-12.3
PTCL-UC	16	0.4-1.7	11.3-39.5
ALCL	4	1.1-2.3	25.2-51
Extranodal TCL			
ALCL cutaneous	2	1.2-1.3	25.7-45.6
Non-ALCL cutaneous pleomorphic TCL	4	0.5-1.4	19.1-35.8
ETCL	1	0.4	24.4
Other extranodal TCL*	3	0.6-0.9	16.8-39.7
Total	39	35#	39

*: four cases were not interpretable

1 case of non-ACL cutaneous pleomorphic TCL showed AI>1, whereas in the remaining 26 cases the AI varied from 0.1 to 0.9.

Comparison of abnormal P53 and Rb phenotypes with proliferation profile

We have observed the following abnormal phenotypes: a) p53 overexpression (12 cases); b) Rb loss of expression (1 case); and c) P16 loss of expression (2 cases). Phenotypes involving combination of the above abnormal patterns were not observed. All the cases with the above abnormal phenotypes exhibited an intermediate to high proliferation profile. The mean rates of Ki67, cyclin A and cyclin B1 expression in the 12 p53-positive cases were higher in comparison to the 45 p53-negative cases. The Ki67 expression in more than 25% of tumour cells showed statistically significant correlation with p53 overexpression ($p<0.001$) and Rb expression in more than 25% ($p<0.001$).

Comparison between apoptotic index and Ki67 index

A positive correlation was found between apoptotic index (AI) and Ki67 index (Pearson coefficient $r=0.739$, $p<0.001$ and Spearman coefficient $r=0.655$, $p<0.001$) (Table 3). There was a statistically significant correlation between AI>1 and p53 overexpression ($p<0.001$).

Discussion

Our results show that p53 overexpression in TCL is strongly associated with ALCL but it is sporadic or
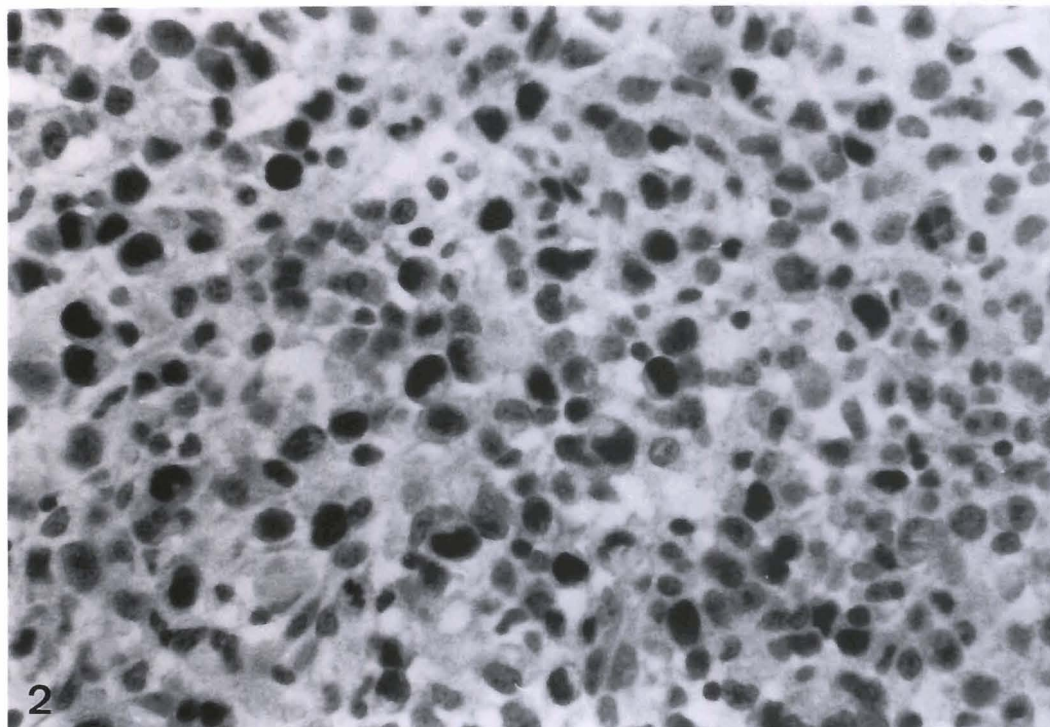

Fig. 2. Expression of Cyclin A in nodal ALCL. x 400

absent in the other clinicopathological entities. In total, most ALCL (nodal and cutaneous) but only a proportion of nodal PTCL-UC, and non-ALCL cutaneous pleomorhic TCL show p53 protein overexpression. In contrast, p53 protein overexpression was undetectable in other histological subtypes such as T lymphoblastic lymphomas (T-LBL), AIL, MF, ETCL, other non-cutaneous extranodal PTCL and hepatosplenic T-$\gamma\delta$ lymphoma. The finding of p53 overexpression in most ALCL, but only in some nodal PTCL-UC and non-ALCL cutaneous pleomorhic TCL concurs with previous data and appears to be very rarely related to p53 gene mutations (Cesarman et al., 1993; Matsushima et al., 1994; Lauritzen et al., 1995; Pescarmona et al., 1999). Thus, these tumours may display overexpression of the wild-type (wt) p53 protein pathway. To further test this possibility and on the basis of previous studies (Chilosi et al., 1996; Villuendas et al., 1997), we have analyzed the expression of the wt p53-induced p21 protein, which blocks cell cycle progression at the G1 to S interface (Cox, 1997). In our study, p21 overexpression was found in all cases of ALCL displaying p53 overexpression. This suggests that the p53 protein is of wild type (wt) and could induce p21 expression, which may function as control of the tumour cell proliferation (Cox, 1997). The stabilization of transcriptionally active wt p53 protein could reflect impairment of the mdm2-dependent degradation of p53 and inhibition of the mdm2-dependent negative regulation of the transactivation activity of p53 (Hsieh et al., 1999). Impairment of the interaction between mdm2 and p53 can occur through a)

covalent modifications of the proteins (i.e. phosphorylation), and b) the induction and action of the protein ARF which is the product of the alternative reading frame of the p16 locus (reviewed in Prives and Hall, 1999). However, despite the very frequent overexpression of wt-p53 and p21 proteins in ALCL, the high proliferation rates of ALCL, as evidenced by the high Ki67 (MIB-1), cyclin A and cyclin B1 expression, suggest that the presumptive p53-induced p21-mediated growth arrest is impaired and somehow overridden in these tumours. The inability of p21 to induce growth arrest could be related to p21 gene structural abnormalities, but these are rare in human tumours and rather absent in lymphomas (Cox, 1997; Maestro et al., 1997). Alternatively, the amount of p21 protein may be insufficient to inhibit the activity of the cdk4/cyclin D complex, since in vitro studies have shown that a low level of p21 protein promotes the assembly of active kinase complexes, whereas at higher concentration it inhibits kinase activity (Labaer et al., 1997). Abnormalities in the cell cycle downstream the step of regulation of p21 could also be involved i.e. alteration of p21 targets as described in melanomas (Wolfel et al., 1995). Interestingly, 2 cases of ALCL with concomitant p53/p21 overexpression, also showed mdm2 overexpression, in keeping with previous studies comprising a few ALCL (Martinez et al., 1995; Tzardi et al., 1996). Despite its overexpression, mdm2 does not seem to inhibit the p53-mediated transactivation, in view of the p53+/p21+ phenotype. However, in these 2 cases, the increased amounts of mdm2 might be sufficient to
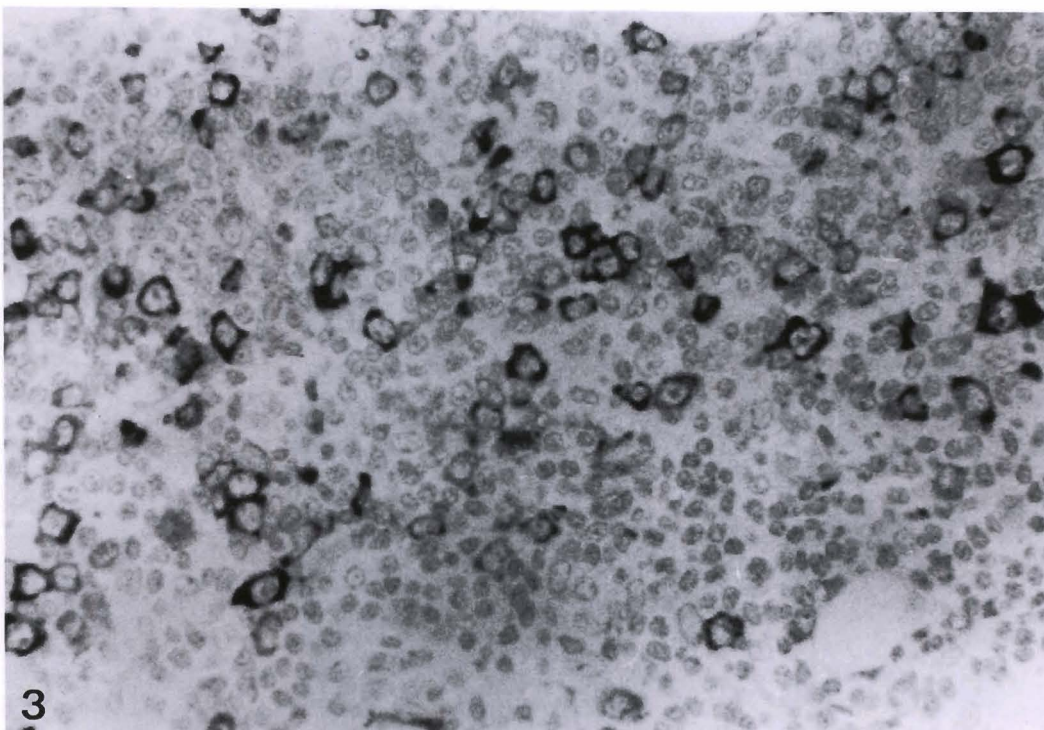

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Fig. 3. Expression of Cyclin B1 in nodal ALCL. x 400

induce functional inactivation of pRb despite its normal immunohistochemical expression, since mdm2 is able to interact physically and functionally with Rb protein (Xiao et al., 1995). This could be an additional hit for uncontrolled cell proliferation.

Besides their high proliferative activity, ALCL in our study were also characterized by increased numbers of apoptotic cells in comparison to other PTCL, as evidenced by the TUNEL method. This suggests a tendency for positive correlation between apoptosis and proliferation, which was confirmed in the 35 studied PTCL of our series. This tendency probably reflects a general phenomenon in tumours and implies that apoptosis and proliferation are inseparable events during tumour growth (Du et al., 1996; Kiberu et al., 1996). Thus, it is possible that in ALCL, the presumptive inability of the overexpressed wt p53 protein to induce cell cycle arrest may, alternatively, lead to activation of the p53-induced apoptotic program (Cox, 1997). This may result, at least in part, from p53-induced upregulation of the proapoptotic bax protein and the downregulation of the antiapoptotic bcl2 protein. The findings of Schlaifer et al. (1996) in ALCL might support this assumption. It is possible that the ratio of p53/p21 protein levels plays a role in the cellular decision to arrest or to die but there is some evidence that apoptosis is dominant over cell-cycle arrest irrespective of p21 levels (reviewed in Cox, 1997).

While all p53-positive ALCL were also p21-positive, 1 nodal PTCL-UC and 1 non-ALCL cutaneous pleomophic TCL were p53-positive/p21-negative, suggesting functional inactivation of overexpressed p53 unable to induce p21 expression in these 2 tumours (Cox, 1997). One hypothesis is p53 gene mutations but they are very rare in PTCL. Another hypothesis is inhibition of wt p53 protein-mediated transactivation by basal levels of mdm2 protein undetectable by immunohistochemistry. Indeed, the p53-mdm2 binding prevents p53-mediated transactivation but if the pRb, which is normally expressed in the above 2 cases binds to mdm2, then the mdm2-induced degradation of p53 can be inhibited (Hsieh et al., 1999).

In our study, Rb expression tended to parallel Ki67, cyclin A and cyclin B1 expression in all but one case of nodal PTCL-UC. This tendency suggests that Rb expression follows the proliferative activity, representing a growth inhibitory response to control cell proliferation, but it is necessary to determine the pRb phoshorylation status in TCL. Analogous findings of parallel pRb/Ki67 expression have been previously reported in B-cell lymphomas (BCL) (Martinez et al., 1993; Jares et al., 1996). We found loss of Rb expression concomitant with intermediate proliferative activity in only one nodal PTCL-UC. Our findings showing loss of Rb expression in about 6% (1/17 cases) of nodal PTCL-UC are somewhat different from those of Pescarmona et al. (1999) who analyzed only nodal PTCL-UC and reported such a loss in about 20% (10/45 cases) of them. Case selection could be an explanation for this difference. We

have then analyzed the expression of cyclin D1 and p16 proteins which are involved in the cyclin D1/p16/Rb growth control pathway (Liggett and Sidransky, 1998). While no overexpression of cyclin D1 was detected in the 57 cases of TCL, 2/39 tested cases of PTCL dislayed focal loss of p16 expression. This is in keeping with the findings of Villuendas et al. (1998), who described a focal loss of p16 expression in 7/14 PTCL. Pinyol et al. (1998) reported loss of p16 expression in 2 studied ALCL with concomitant hypermethylation in one of them. Regarding molecular mechanisms, Martinez-Delgado et al. (1997) reported hypermethylation of p16 gene in 3/20 TCL, all cases being of high-grade histology. Baur et al. (1999), reported hypermethylation of p16 gene in 1/7 PTCL and 1/2ALCL with concomitant absence of gene expression, assessed by RT-PCR, in this latter case. However, Otsuki et al. (1995) reported no deletion or mutation of p16 gene in 35 PTCL. In the review of Drexler (1998), 47/332 mature TCL showed p16 deletions, but 44 of them were ATLL while none of the 27 PTCL showed p16 deletions. Taken together, the above findings indicate that a) loss of p16 expression is not frequent in PTCL, and b) hypermethylation of p16 gene may be the principal mode of p16 inactivation in PTCL whereas neither deletions nor mutations appear to be relevant mechanisms of p16 silencing in these tumours. Further combined immunohistochemical and molecular investigations are required to determine the p16 status in PTCL.

Interestingly, the 2 cases of our study, with focal loss of p16 expression had neither loss of Rb expression nor p53 overexpression. Moreover, the one case with loss of Rb expression was p53-negative and the 12 p53-positive cases were Rb-positive. These findings suggest that different pathogenetic routes may function in some TCL, involving either the p53 or, less frequently, the Rb pathways.

The comparison of TCL with B-cell lymphomas (BCL) reveals the following points with respect to the abnormalities of p53 and Rb pathways. 1) The p53/p21 pathway is affected in a proportion of "high-grade" BCL, more frequently in cases of tumour progression from "low-grade" BCL, where p53 overexpression is frequently associated to p53 gene mutations. In TCL, abnormalities of the p53 pathway are rare except for ALCL and they are not associated to p53 gene mutations (Matsushima et al., 1994; Villuendas et al., 1997; Pescarmona et al., 1999). 2) The Rb/p16 pathway is affected in a substantial proportion of "high-grade" BCL, more frequently in cases of tumour progression from "low-grade" BCL, where loss of p16 expression is frequently associated to hypermethylation of the p16 gene. In TCL, abnormalities of the Rb/P16 pathway are relatively uncommon but when loss of p16 expression occurs, is also frequently associated to hypermethylation of the p16 gene as in BCL (Weide et al., 1994; Martinez-Delgado et al., 1997; Villuendas et al., 1998; Pescarmona et al., 1999). Regarding lymphoid

leukaemias, abnormalities of the p53 pathway (i.e. p53 gene mutations) were detected in a proportion of B and pre B-cell acute lymphoid leukaemias (ALL) but not in most T-ALL (ProcDcimer and Rotter, 1994). In contrast, the p16 gene was frequently deleted in T-ALL (>50%) and in precursor B-ALL (>30%) but rarely in chronic lymphoid leukaemias (CLL); inactivation of the Rb-1 gene is rare in ALL and CLL but loss of Rb protein seems to be more frequent (Hangaishi et al., 1996; Sellers and Kaelin, 1997; Drexler, 1998).

In our study Ki67, cyclin A and cyclin B1 expression was found in all 57 TCL and on the basis of the combined use of these 3 variables, 3 groups of proliferative activity could be determined: a) high in ALCL and T-LBL; b) low in MF and γδ hepatosplenic TCL; and c) intermediate in the remaining TCL entities. Analogous findings in a series of lymphomas including cases of TCL were recently reported by Leoncini et al. (1999). Interestingly, we observed that the 12 cases with p53 overexpression exhibited higher proliferation activity than the 45 cases without p53 overexpression. In addition, the Ki67 expression in more than 25% of tumour cells showed statistically significant correlation with p53 overexpression (p<0.001). This concurs with a previous study (Pescarmona et al., 1999), and provides further support for the hypothesis that p53 growth control pathway is impaired in this subset of TCL.

In summary, the unique findings to T-cell lymphomas were that ALCL represent a lymphoma strongly associated with overexpression of p53 and p21 along with high proliferative activity, elevated apoptosis and high pRb and p16 expression. This suggests: a) impairment of the wild-type p53/p21 protein pathway, unable to induce cell-cycle arrest, but possibly able to increase apoptosis, and b) normal expression of the Rb/p16 pathway in relation to the proliferative activity of tumour cells. P53 and p21 overexpressions were sporadic or absent in the other entities of TCL. Mdm2 overexpression does not appear to play a prominent role in TCL. Overall, immunohistochemical abnormalities of the Rb/P16/Cyclin D1 pathway were not frequent in our series of TCL. Rb protein was normally expressed in relation to the proliferative activity of tumours in all but one case of TCL and p16 protein expression was focally lost in 2/39 PTCL studied. This suggests involvement of the abnormal Rb or p16 expression in the pathogenesis of a few TCL.

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