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Expression of cyclin D3 and cyclin E and identification of distinct clusters of proliferation and apoptosis in diffuse large B-cell lymphomas

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Summary. In the present study 79 cases of de novo Diffuse Large B-cell Lymphomas (DLBCL) were studied in order: a) to analyse the expression of cyclin D3, cyclin E and cyclin D1 in relation to other proliferative features (expression of Ki67, cyclin A and cyclin B1), the apoptosis status and the expression of p53, Rb, p16 and p27; and b) to determine whether distinct clusters of proliferation and apoptosis could be identified in DLBCL. Overexpression of cyclin D3 and cyclin E was found in 35/79 (43%) and 18/79 (22%) cases, respectively, whereas overexpression of cyclin D1 was not detected in any case. In most cases (39/46) overexpression of cyclin D3 and cyclin E was mutually exclusive possibly reflecting different underlying pathways inducing deregulated expression of these cyclins. In most cases (29/35) overexpression of cyclin D3 was mutually exclusive with Rb/p16 aberrant expression status supporting an oncogenic role for cyclin D3 and suggesting that the pathogenetic effect of cyclin D3 overexpression occurs through perturbation of the Rb1 pathway. Combined alterations of the P53 and the Rb/p16/cyclin D3 expression status were significantly associated with higher mean values of cyclin A (p=0.023) and cyclin B1 (p=0.033) indicating that concurrent impairment of the p53 and Rb1 pathways induces increased tumour cell proliferation in DLBCL. Cluster analysis of the apoptosis and the proliferation status permitted separation of DLBCL into distinct groups with low (44 cases) and high (18 cases) apoptotic activity and into distinct groups with low (32 cases), intermediate (36 cases) and high (11 cases) proliferative activity. The identification of distinct clusters with respect to the proliferation and the apoptosis status indicates that groups with distinct cellular kinetic properties can be defined in the histological group of DLBCL.

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Introduction

Normal tissue homeostasis is maintained by the regulation of the cell proliferation and death and there is now a body of evidence to suggest that the impairment of various regulators of the cell-cycle progression and apoptosis plays a crucial role in oncogenesis (Gil-Gomez et al., 1998; Lopez-Saez et al., 1998; Lundberg and Weinberg, 1999; Hanahan and Weinberg 2000; Zhou and Elledge 2000; Evan and Vousden, 2001; Zhu and Skoultchi 2001).

Cell-cycle progression is regulated by a group of cyclins and cyclin–dependent kinases (CDK) acting at different phases of the cell cycle (Lundberg and Weinberg, 1999; Sherr, 2000). Cyclins are divided in two main families. The G1 family includes cyclins C, D1, D2, D3 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 cyclins A and B. Cyclin A is involved in DNA replication in the S-phase and is usually not detectable at the onset of the mitosis while cyclins B1 and B2 ensure irreversible entry into mitosis. Cyclin–dependent kinase inhibitors (CDKIs) play important roles in the cell-cycle progression, by negatively regulating the kinase activity of the complexes composed of cyclins and cyclin–dependent kinases (CDK) (Lundberg and Weinberg, 1999; Sherr, 2000). There are two known families of CDKIs. The INK4 inhibitors (p16/INK4A, p15/INK4B, p18/INK4C, and p19/INK4D) are specific for CDK4 and 6, while the CIP/KIP inhibitors (p21/CIP1, p27/KIP1 and P57/KIP2) target CDK 2, 4 and 6.

The p53 pathway and the Rb1 pathway are two major growth regulatory pathways (Lundberg and Weinberg, 1999; Sherr, 2000). The p53 pathway regulates apoptosis and cell-cycle arrest in G1 phase,

depending on the cell type and cell state. Moreover, recent findings showed that p53 regulates not only the G1/S but also the G2/M transition (Taylor and Stark 2001). The Rb1 pathway inhibits transcription of genes necessary for the transition from G1 to S phase (Lundberg and Weinberg, 1999; Sherr, 2000). Central to this pathway is the regulation of phosphorylation of the Rb protein (pRb). Hypophosphorylated pRb binds and inactivates transcription factors, notably the E2F1, important for the transition from G1 to S phase. When pRb is phosphorylated the E2F1 transcription factor is released. Phosphorylation of pRb is stimulated by cyclinD-CDK4/6 complexes and inhibited by the p16 and other CDKIs including p27. Recent evidence has linked the two pathways through the 9p21 locus in which two CDKI genes reside, the CDKN2A and CDKN2B genes (Lundberg and Weinberg, 1999; Sherr, 2000). The CDKN2A gene encodes p16 protein which is involved in the RB1 pathway whereas the p14/ARF protein binds to mdm2 and promotes mdm2 degradation thereby abrogating the mdm2-mediated degradation of p53.

Diffuse Large B-cell Lymphomas (DLBCL) represent the most common type of non-Hodgkin's lymphomas in Western countries and are characterised by heterogeneous clinical, immunophenotypic and genetic features (Chan et al.,1997; Nicolaides et al., 1998; Kuppers at al., 1999; Alizadeh et al., 2000). Reflecting their biological heterogeneity, alterations of several cell-cycle regulators have been identified in DLBCL by using immunohistochemical and molecular biology techniques (Sanchez-Beato et al., 1997, 1999, 2001; Villuendas et al., 1997, 1998; Erlanson et al., 1998; Pinyol et al., 1998; Stefanaki et al., 1998; Moller et al., 1999, 2000; Gronbaek et al., 2000; Sanchez-Aguilera et al., 2002). In this respect, we have recently shown (Bai et al., 2001a) that low expression of p27 protein combined with altered p53 and Rb/p16 expression status is associated with increased expression of cyclin A and cyclin B1 in 80 cases of de novo DLBCL. In the present study we have additionally evaluated the apoptosis status and the expression of cyclin D3, cyclin D1 and cyclin E in the same series. The aims of the present study were: a) to analyse the expression of cyclin D3, cyclin E and cyclin D1 in relation with other proliferative features (expression of Ki67, cyclin A and cyclin B1), the apoptotic index and the expression of p53, Rb, p16 and p27; and b) to determine whether distinct clusters of proliferation and apoptosis could be identified in DLBCL.

Materials and methods

Materials

Seventy-nine cases of de novo diffuse large B-cell lymphomas (37 nodal and 42 extranodal) classified according to the WHO classification (Jaffe et al., 2001) were selected from the files of the Department of Pathology of the University of Ioannina on the basis that complete clinicopathological parameters were available.

Immunohistochemistry

Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the labelled streptavidin avidin biotin (LSAB) method. A step of microwave pretreatment was used as described previously (Tzardi et al., 1996). Monoclonal antibodies (MoAbs) directed against cyclin D3 (DCS-22, Novocastra, dilution 1:10), cyclin D1 (DCS-6, Novocastra, dilution 1:10) and cyclin E (13A3, Novocastra, dilution 1:10) were used in the present study. The MoAbs directed against p53 protein (DO-7, Dako SA, Glostrup, Denmark, dilution 1:50), Rb protein (Rb1, Dako SA dilution 1:20), proliferation-associated nuclear antigen Ki67 (Dako SA dilution 1:20), cyclin A (6E6, Novocastra, Newcastle upon Tyne, dilution 1:10), cyclin B1 (7A9, Novocastra, dilution 1:10), p16 (F-12, Santa Cruz Biotechnology, USA, dilution 1:100) and p27 (IB4, Novocastra, dilution 1:20) and the corresponding positive controls and evaluation approach were reported previously (Bai et al., 2001a). The counting of immunopositive cells was performed as described previously (Bai et al., 2001a). Briefly, a continuous score system was adopted by using the x40 objective lense and counting at least 5 fields selected on the basis that they contained immunopositive cells. The number of immunopositive cells was divided by the total number of the counted cells and the expression was defined as the percentage of positive cells in the total number of the counted cells. Overexpression of cyclin D3, cyclin D1 and cyclin E was considered when more than 10% of large tumour cells were positive (Moller et al., 2000). Positive controls for cyclin D3, cyclin D1 and cyclin E were positive cases of reactive lymph nodes, mantle cell lymphoma and breast carcinoma, respectively.

TUNEL method

The TdT (terminal deoxynucleotidyl-transferase)-mediated in-situ labelling technique (TUNEL) (Apotag Peroxidase kit, Oncor) was carried out as described previously (Czader et al., 1996; Bai et al., 2001b). Briefly, after deparaffinization and dehydration, slides were rinsed in phosphate buffer solution (PBS, pH 7.2). Tissue sections were then digested by incubation for 20 minutes with proteinase K (20 μg/ml; Oncor) at room temperature and then were rinsed in distilled water .The peroxidase activity was blocked by incubation for 5 minutes in 2% hydrogen peroxide in PBS. After the application of an equilibration buffer, slides were incubated in working-strength TdT enzymes that contained dUTP-digoxigenin for 1 hour at 37 °C. The reaction was stopped by a prewarmed at 37 °C working-strength stop/wash buffer and then slides were rinsed in PBS. The incorporated nucleotides were identified by

adding peroxidase conjugated anti-digoxigenin antibody. Slides visualized by anti-digoxigenin-peroxidase were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Oncor) and counterstained lightly with Harris' haematoxylin. Positive controls (germinal centers in reactive lymph nodes and thymuses) and negative controls (sections without TdT) were included in every staining (Kanavaros et al., 2001b). The evaluation of the apoptotic cells was performed as previously described (Kanavaros et al., 2001a). Briefly, morphologically intact TUNEL-positive cells and cells with morphological criteria of apoptosis in haematoxylin-eosin stained slides (i.e., condensed, hyperchromatic, ring-like, crescentic or beaded nuclear chromatin and chromatin-containing cytoplasmic remnants) 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 lense and by counting the apoptotic cells in 10 randomly selected fields, corresponding to a total of 2000 to 3000 cells. The apoptotic index (AI) was determined as the number of apoptotic cells expressed as a percentage of the total number of counted cells.

Statistical analysis

Pearson's and Spearman's correlation coefficients, k-means method of cluster analysis, analysis of variance (ANOVA) and multiple analysis of variance (MANOVA) were used for the statistical analysis. The results were considered as statistically significant when $p<0.05$. The program SPSS for Windows Release 10 was used for statistical analysis.

Table 1. Immunohistochemical overexpression of the cyclins D3, E and D1.

PROTEIN OVEREXPRESSION	POSITIVE/TOTAL CASES
Cyclin D3	35/79 (43%)
Cyclin E	18/79 (22%)
Cyclin D1	0/79

Results

Immunohistochemical results

Expression of cyclin D3 (range 1-95%, mean value 17.86%, standard deviation 25.50%) and cyclin E (range 1-45%, mean value 6.59%, standard deviation 11.90%) was found in 56/79 (70%) and 28/79 (35%) cases, respectively. Overexpression of cyclin D3 (Fig. 1) and cyclin E (>10% of large tumour cells positive) was found in 35/79 (43%) and 18/79 (22%) cases, respectively, whereas overexpression of cyclin D1 was not detected in any case (Table 1). Overexpression of cyclin D3 and cyclin E was mutually exclusive in 39/46 (84%) cases whereas concomitant overexpression of both cyclins was found in only 7/46 (16%) cases. The expression of p27, p53, Rb, p16, Ki67, cyclin A and cyclin B1 proteins was reported in previous details (Bai et al., 2001a). Briefly, p27 expression was low/null in large tumour cells in 58/79 cases and intermediate/high in 21/79 cases. Increased expression of p53 protein was observed in 38/79 cases. Decreased expression of Rb and p16 proteins was mutually exclusive and was observed in 5/79 and 14/79 cases, respectively. Ki67 (Fig. 2), cyclin A and cyclin B1 (Fig. 3) expression was detected in all cases. In most cases (29/35) overexpression of cyclin D3 was mutually exclusive with Rb/p16 aberrant expression status. Totally, aberrations in the combined Rb/p16/cyclin D3 expression status were detected in 48/79 (60%) cases. Using Pearson's and Spearman's correlation coefficients

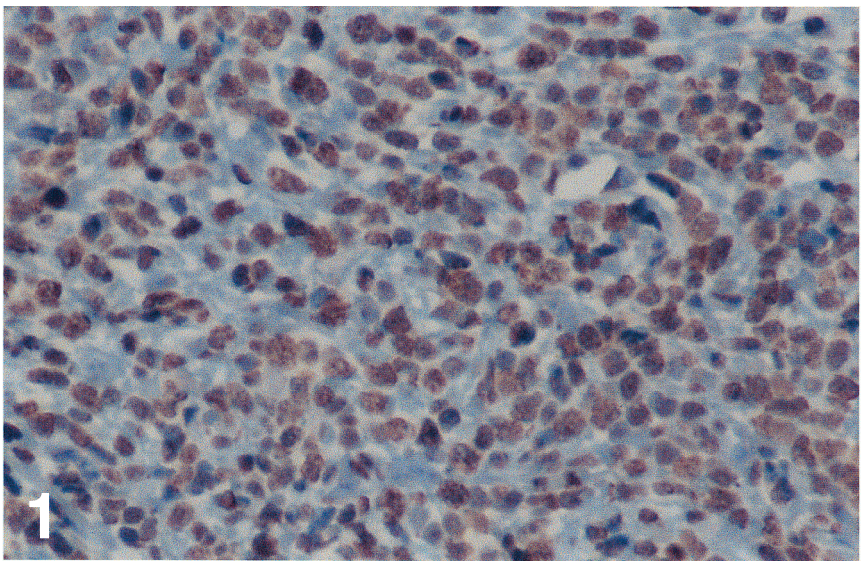

Fig. 1. Cyclin D3 overexpression in tumour cells. x 400

significant positive correlations ($p<0.05$) were found between a) Ki67 and cyclin A, cyclin B1, cyclin D3 and Rb, b) cyclin A and cyclin B1, p53 and Rb, and c) cyclin B1 and p53 and Rb.

Apoptosis status

Cells with and without morphological criteria of apoptosis were found to be positive by the TUNEL staining. Non-labeled cells that satisfied the morphological criteria of apoptosis were also detected. Small, apparently reactive, lymphocytes were very rarely TUNEL positive. Apoptotic cells were sometimes surrounded by a clear halo. On the basis of these results, the apoptotic index (AI) could be evaluated in 62 cases [range of values 0.26-9.7%, mean value 2.79%, standard error for mean 0.25%, 95% confidence interval for mean (lower bound 2.29% and upper bound 3.29%), variance 3.89%, sum of values 173.47%, standard deviation 1.97%, median value 2.21%]. In the remaining 17 cases

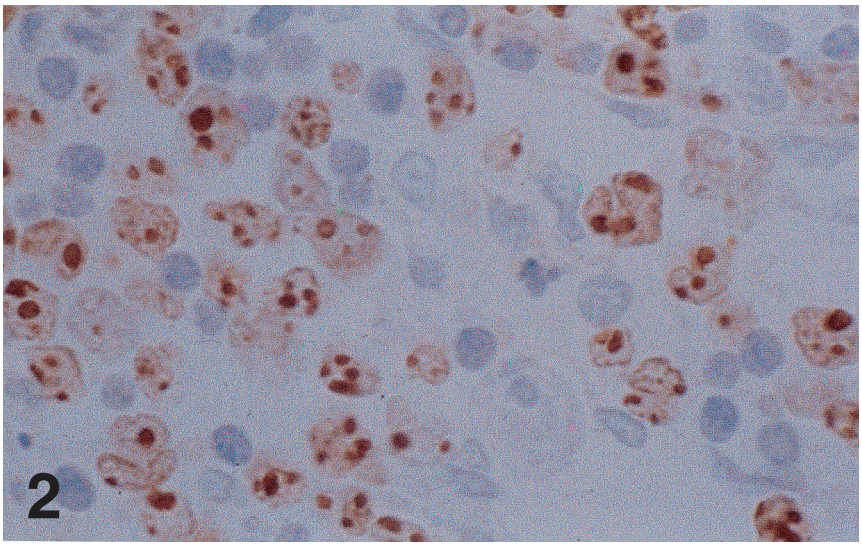

Fig. 2. Ki67 expression in tumour cells. x 1,000

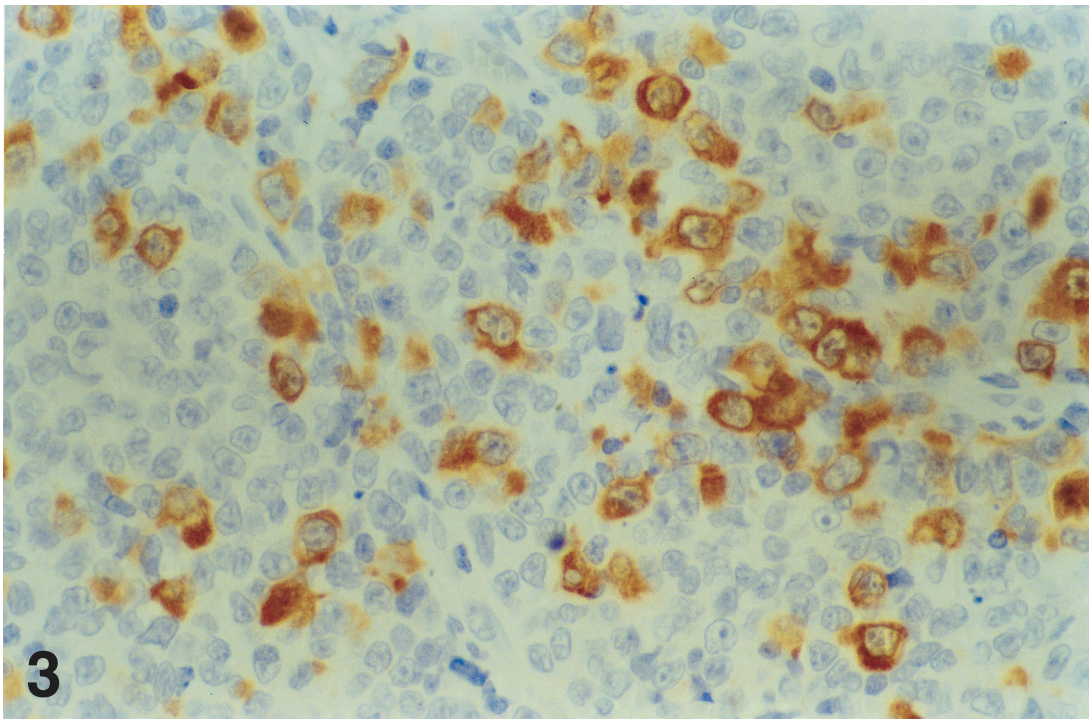

Fig. 3. Cyclin B1 expression in tumour cells. x 400

the TUNEL method did not yield satisfactory results because of either extensive background staining or equivocal staining. Using Pearson's and Spearman's correlation coefficients AI showed significant positive correlation ($p<0.05$) with cyclin A, cyclin B1, Ki67 and Rb expression.

Cluster analysis of the apoptotic index and the proliferation-associated parameters Ki67, cyclin A and cyclin B1

The values counted for AI and for each of Ki67, cyclin A and cyclin B1 expression (these proliferation-associated proteins were chosen because they were detectable in all cases) were allocated to clusters by the k-means method of cluster analysis. This method produced a clear-cut two-cluster solution for apoptosis: cluster 1: low apoptosis (44 cases) and cluster 2: high apoptosis (18 cases) (Table 2). Using analysis of variance the two cluster mean values were significantly different for every cluster ($p<0.001$). The k-means method also produced a clear-cut three-cluster solution for the proliferation-associated parameters Ki67, cyclin A and cyclin B1: cluster 1: low proliferation (32 cases); cluster 2: intermediate proliferation (36 cases); and cluster 3: high proliferation (11 cases) (Table 2). Using analysis of variance the three-cluster mean values were significantly different for every variable ($p<0.001$). Multiple analysis of variance was performed between the cluster as the independent variable and all three variables (Ki67, cyclin A and cyclin B1) simultaneously as the dependent vector. This method produced a Wilks lamda of 0.151 ($p<0.001$).

Table 2. Clusters of the proliferation-associated parameters Ki67, cyclin A and cyclin B1 and clusters of the apoptotic index (AI).

	N	MEAN VALUE	STD. DEVIATION
Ki67	32 (LP)	36.81%	12.24%
	36 (IP)	71.53%	12.58%
	11 (HP)	82.27%	11.48%
Cyclin A	32 (LP)	18.19%	13.64%
	36 (IP)	32.89%	14.34%
	11 (HP)	64.55%	10.11%
Cyclin B1	32 (LP)	10.41%	9.92%
	36 (IP)	20.00%	11.95%
	11 (HP)	41.09%	19.34%
AI	44 (LA)	1.73%	0.78%
	18 (HA)	5.39%	1.53%

N: number of cases; LP: cluster of low proliferation; IP: cluster of intermediate proliferation; HP: cluster of high proliferation; LA: cluster of low apoptosis; HA: cluster of high apoptosis.

Correlations of the apoptotic index and the proliferation profile with the combined p53/Rb/p16/cyclin D3 expression status

Using analysis of variance the cases with combined alterations in both the p53 and Rb/p16/cyclin D3 expression status showed significantly higher mean values of cyclin A ($p=0.023$) and cyclin B1 ($p=0.033$) than the cases without combined alterations in both the p53 and Rb/p16/cyclin D3 expression status (Table 3). Multiple analysis of variance produced a Wilks lamda of 0.918 ($p=0.039$). The alterations in the p53 and Rb/p16/cyclin D3 expression status were also analysed with respect to the clusters of apoptosis and proliferation but no significant correlations were found.

Discussion

In the present study, overexpression of cyclin D3 and cyclin E was found in 35/79 (43%) and 18/79 cases (22%), respectively, whereas overexpression of cyclin D1 was not detected in any case. These results are in agreement with previous immunohistochemical data (Doglioni et al., 1998; Erlanson et al., 1998; Sanchez-Beato et al., 1999; Teramoto et al., 1999; Ferreri et al., 2001; Moller et al., 2001; Filipits et al., 2002). Overexpression of cyclin D3 and cyclin E was mutually exclusive in 39/46 cases whereas concomitant overexpression of both cyclins was found in only 7/46 cases. The mutually exclusive overexpression of these

Table 3. Groups of the proliferation-associated parameters Ki67, cyclin A and cyclin B1 and groups of the apoptotic index (AI) in relation to the P53/Rb/p16/cyclin D3 expression status.

	GROUP	N	MEAN VALUE	STD. DEVIATION	P VALUES
KI67	1	54	55.98%	22.19%	NS
	2	25	65.40%	21.64%	
Cyclin A	1	54	27.85%	20.30%	0.023
	2	25	38.88%	18.04%	
Cyclin B1	1	54	16.55%	14.54%	0.033
	2	25	24.68%	17.21%	
AI	1	42	2.78%	2.16%	NS
	2	20	2.83%	1.54%	

N: number of cases; Group 1: cases without combined alterations of the p53 and Rb/p16/cyclin D3 expression status; Group 2: cases with combined alterations of the p53 and Rb/p16/cyclin D3 expression status; NS: not significant correlation

cyclins may reflect different underlying pathways inducing deregulated expression. This possibility could be suggested since cyclin D3 is involved in the Rb/p16/cdk4,6/cyclin D growth-inhibitory pathway (Sherr, 2000), whereas there is evidence that cyclin E can bypass this pathway downstream of pRb activation (Alevizopoulos et al., 1997). Indeed, Alevizopoulos et al. (1997) demonstrated that cyclin E is sufficient to bypass growth-arrest by p16 and allows cell proliferation in the presence of active pRb family proteins. Considering the present and previous findings together (Ott et al., 1997; Doglioni et al., 1998; Erlanson et al., 1998; Leoncini et al., 1999; Sanchez-Beato et al., 1999; Teramoto et al., 1999; Wolowiec et al., 1999; Ferreri et al., 2001; Moller et al., 2001; Filipits et al., 2002) it appears that cyclin D3 and cyclin E expression patterns are more restricted than those of cyclin A and cyclin B1, being absent in a substantial part of the DLBCL. With respect to cyclin D3, which is the key D-type cyclin involved in human B-cell cycle progression (Bartkova et al., 1998; Wagner et al., 1998), significant correlation was found with Ki67 but not with other proliferation-associated parameters (cyclin A and cyclin B1) in our series of DLBCL. This suggests that cyclin D3 expression does not merely reflect the proliferation rate, as in normal B-cells (Bartkova et al., 1998), but may also have pathogenetic significance in a part of the DLBCL. This could be supported: a) by our present data and previous findings (Moller et al., 2000) showing that overexpression of cyclin D3 in most cases was mutually exclusive with Rb/p16 aberrant expression status supporting an oncogenic role for cyclin D3 and suggesting that the pathogenetic effect of cyclin D3 overexpression occurs through perturbation of the Rb1 pathway; and b) importantly, by recent molecular findings (Sonoki et al., 2001) implicating the CCND3 gene as a dominant oncogene in the pathogenesis and high-grade transformation of several histological subtypes of mature B-cell malignancies with t(6;14)(p21.1;q 32.3). This latter finding, in addition to the observations that in B-cell lymphomas cyclin D3 is higher in high-grade than low-grade tumours and that high expression of cyclin D3 is associated with poor prognosis (Moller et al., 2001; Filipits et al., 2002), supports a role of cyclin D3 overexpression in B-cell lymphoma progression. On the other hand, it appears that the cyclin D3/ cyclin D2/ cyclin D1 immunostaining pattern may be a useful tool for the differential diagnosis of B-cell malignancies. Indeed, cyclin D3 is expressed in variable proportions of most B-cell malignancies including follicular lymphomas, DLBCL and Burkitt's lymphoma, whereas it is selectively not expressed in mantle cell lymphoma, in which the cyclin D1 gene is rearranged and overexpressed (Ott et al., 1997; Doglioni et al., 1998; Sanchez-Beato et al., 1999; Teramoto et al., 1999; Wolowiec et al., 1999; Moller et al., 2001; Filipits et al., 2002). Moreover, cyclin D2 is not expressed in follicular lymphomas and mantle cell lymphoma whereas it is expressed in most chronic lymphocytic leukaemias and in a few DLBCL (Delmer et al., 1995; Teramoto et al., 1999). With respect to cyclin E expression no significant correlation was observed with the expression of Ki67, cyclin A, cyclin B1 in our series of DLBCL, suggesting that this expression may be deregulated and may have pathogenetic significance in a part of DLBCL since it does not merely reflect the proliferation rate. This can be supported by the findings that: a) aggressive B-cell lymphomas had, in principle, a higher index of cyclin E expression than S-phase fractions (Erlanson et al., 1998); and that b) a high level expression of cyclin E can predispose T-cells for hyperplasia and malignant transformation in transgenic mice (Karsunsky et al., 1999). Moreover a role of cyclin E overexpression in B-cell lymphoma progression has been suggested since its expression was higher in high-grade than low-grade tumours and a high expression of cyclin E was associated with poor prognosis (Erlanson et al., 1998; Ferreri et al., 2001).

In the present study, we asked the question whether distinct clusters of apoptosis and proliferation could be delineated in DLBCL, permitting identification of groups of these tumours with clearly distinct cellular kinetic properties. In a previous study (Leoncini et al., 1999) distinct clusters of proliferation could be defined in a series of diffusely growing B and T-cell lymphomas of various histological types, but the DLBCL have not been analysed as a distinct group. Thus, we have analysed our series of DLBCL by using k-means cluster analysis for the values counted for the apoptotic index (AI) and for the proliferation-associated proteins Ki67, cyclin A and cyclin B1. This method produced clear-cut cluster solution for both the AI and the proliferation profile, thereby permiting clear separation of the DLBCL into distinct groups with low and high apoptotic activity and into distinct groups with low, intermediate and high proliferative activity. The identification of distinct clusters with respect to the status of the apoptosis and the proliferation indicates that groups with distinct cellular kinetic properties can be delineated in the histological group of DLBCL. These findings might be useful for the identification of groups of DLBCL with different clinical behaviour since the rates of apoptosis and proliferation increase in parallel during tumour progression and are associated with aggressive tumour behaviour in B-cell lymphomas. Indeed a) the increase in apoptotic and mitotic indices correlates with the increase in cytological grade of follicular center B-cell lymphoma (Logsdon et al., 1999), b) aggressive high grade B-cell lymphomas, when compared with low grade B-cell lymphomas, display higher proliferative activity, higher apoptotic indices and higher bax (proapoptotic protein)/bcl2 (antiapoptotic protein) ratio (Leoncini et al., 1993; Du et al., 1996; Kiberu et al., 1996; Soini et al., 1998; Wheaton et al.,1998; Dononghue et al., 1999; Leoncini et al., 1999; Soini and Paakko, 1999), and c) higher proliferative activity and higher apoptotic rates are adverse prognostic factors in diffuse large cell lymphomas (Miller et al., 1994) and in

diffuse large B-cell lymphomas (Dononghue et al., 1999), respectively.

Concurrent aberrations involving both the p53 and the Rb1 pathway, and in some instances the p27 protein, are frequently observed in DLBCL and appear to be associated with poor prognosis (Moller et al., 1999, 2000; Gronbaek et al., 2000; Bai et al., 2001a; Sanchez-Beato et al., 2001; Sanchez-Aguilera et al., 2002). In the present study the cases with combined alterations in both the p53 and Rb/p16/cyclin D3 expression status showed significantly higher mean values of cyclin A ($p<0.05$) and cyclin B1 ($p<0.05$) than the cases without combined alterations in the p53/Rb/p16/cyclin D3 expression status. These findings suggest that in DLBCL the combined impairment of the cell-cycle control networks involving the p53 pathway and the Rb1 pathway is associated with profound alterations in the tumour cell kinetics. These alterations comprise mainly a significant enhancement of tumour cell proliferation. To explain these findings it could be suggested that the combined aberrations of the p53 and the Rb1 pathways impair the growth inhibitory activity of these pathways and result in increased tumour cell proliferation (Lundberg and Weinberg, 1999).

In conclusion, overexpression of cyclin D3 and, less frequently, of cyclin E were observed in a part of DLBCL. Overexpression of cyclin D3 was mutually exclusive with that of cyclin E in most instances possibly reflecting different underlying pathways inducing deregulated expression. In most cases overexpression of cyclin D3 was mutually exclusive with Rb/p16 aberrant expression status supporting an oncogenic role for cyclin D3 and suggesting that the pathogenetic effect of cyclin D3 overexpression occurs through perturbation of the Rb1 pathway. Combined impairment of the P53 pathway and the Rb/p16/cyclin D3 expression status is associated with profound alterations in the tumour cell kinetics, comprising mainly a significant enhancement of tumour cell proliferation. The identification of distinct clusters of the proliferation profile and the apoptosis status indicates that groups with distinct cellular kinetic properties can be defined in the histological group of DLBCL.

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References

Alevizopoulos K., Vlach J., Hennecke S. and Amati B. (1997). Cyclin E and c-myc promote cell proliferation in the presence of p16/INK4A and of hyperphosphorylated retinoblastoma protein. EMBO J. 16, 5322-5333.

Alizadeh A.A., Eisen M.B., Davis R.E., Ma C., Lossos I.S., Rosenwald A., Boldrick J.C., Sabet H., Tran T., Yu X., Powell J.I., Yang L., Marti G.E., Moore T., Hudson J., Lu L., Lewis D.B., Tibshirani R., Sherlock G., Chan W.C., Greiner T.C., Weisenburger D.D., Armitage J.D., Warnke R., Levy R., Wilson W., Grever M.R., Byrd J.C., Botstein D., Brown P.O. and Staudt L.M. (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403, 503-511.

Bai M., Vlachonikolis J., Agnantis N.J., Tsanou E., Dimou S., Nicolaides C., Stefanaki S., Pavlidis N. and Kanavaros P. (2001a). Low expression of p27 protein combined with altered p53 and Rb/p16 expression status is associated with increased expression of cyclin A and cyclin B1 in Diffuse Large B-cell Lymphomas. Mod. Pathol. 14,1105-1113.

Bai M., Agnantis N.J., Kamina S.A., Demou A., Zagorianakou P.S., Katsaraki A and Kanavaros P. (2001b). The in vivo cell kinetics in breast carcinogenesis. Breast Cancer Res. 3, 276-283.

Bartkova J., Lukas J., Strauss M. and Bartek J. (1998). Cyclin D3: requirment for G1/S transition and high abundance in quiscent tissues suggest a dual role in proliferation and differentiation. Oncogene 17, 1027-1037.

Chan W.C., Armitage J.O., Gascoyne R., Connors J., Close P., Jacobs P., Norton A., Lister T.A., Pedrinis E., Cavalli F., Berger F., Coiffier B., Ho F., Liang R., Schauer A., Hiddemann W., Diebold J., MacLennan K.A., Mueller-Hermelink H.K., Nathwani B.N., Weisenburger D.D., Harris N.L., Anderson J.R. and Roy P. (1997). A clinical evaluation of the international Lymphoma Study Group classification of non-Hodgkin's lymphoma. Blood 89, 3909-3918.

Czader M., Mazur J., Pettersson M., Liliemark J., Stromberg M., Christennson B., Tribukait B., Auer G., Ost A. and Porwit A. (1996). Prognostic significance of proliferative and apoptotic fractions in low grade follicle center cell-derived non-Hodgkin`s lymphomas. Cancer 77, 1180-1188.

Delmer A., Ajchenbaum-Cymbalista F., Tang R., Ramond S., Faussat A.M., Marie J.P. and Zittoun R. (1995). Overexpression of cyclin D2 in chronic B-cell malignancies. Blood 85, 2870-2876.

Doglioni C., Chiarelli C., Macri E., Dei Tos A.P., Meggiolaro E., Dalla Palma P. and Barbareschi M. (1998). Cyclin D3 expression in normal, reactive and neoplastic tissues. J. Pathol. 185,159-166.

Donoghue S., Baden H.S., Lauder I., Sobolewski S. and Pringle J. (1999). Immunohistochemical localization of caspase-3 correlates with clinical outcome in B-cell diffuse large-cell lymphoma. Cancer Res. 59, 5386-5391.

Du M., Singh N., Husseuin A., Isaacson P.G., and Pan L.X. (1996). Positive correlation between apoptotic and proliferative indices in gastrointestinal lymphoma of mucosa-associated lymphoid tissue. J. Pathol. 178, 379-384.

Erlanson M., Portin C., Linderholm B., Lindh J., Roos G. and Landberg G. (1998). Expression of cyclin E and the cyclin-dependent kinase inhibitor p27 in malignant lymphomas-prognostic implications. Blood 92, 770-777.

Evan G.I. and Vousden K.H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342-348.

Ferreri A.J., Ponzoni M., Pruneri G., Freschi M., Rossi R., Dell'Oro S., Baldini L., Buffa R., Carboni N., Villa E. and Viale G. (2001). Immunoreactivity for p27 (KIP1) and cyclin E is an independent predictor of survival in primary gastric non-Hodgkin's lymphoma. Int. J. Cancer 94, 599-604.

Filipits M., Jaeger U., Pohl G., Stranzl T., Simonitsch I., Kaider A, Skrabs C. and Pirker R. (2002). Cyclin D3 is a predictive and prognostic factor in diffuse large B-cell lymphoma. Clin. Cancer Res. 8, 729-733.

Gil-Gomez G., Berns A. and Brady H.J. (1998). A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. EMBO J. 17, 7209-7218.

Gronbaek K., de Nully Brown P., Moller M.B., Nedergaard T., Ralfkiaer E., Moller P., Zeuthen J. and Guldberg P. (2000). Concurrent disruption of p16INK4a and the ARF-p53 pathway predicts poor prognosis in aggressive non-Hodgkin's lymphoma. Leukemia 14, 1727-1735.

Hanahan D. and Weinberg R.A. (2000). The hallmarks of cancer. Cell 100, 57-70.

Jaffe E.S., Harris N.L., Stein H. and Vardiman J.W. (2001). Pathology and genetics of tumours of the haematopoietic and lymphoid tissues. In: World Health Organization classification of tumours. Kleihues P. and Sobin L.H. (ed). International Agency for Research on Cancer (IARC) Press. Lyon. pp 171-174.

Kanavaros P., Bai M., Stefanaki K., Poussias G., Zioga E., Rontogianni D., Gorgoulis V. and Agnantis N.J. (2001a). Immunohistochemical expression of p53, mdm2, p21/waf-1, Rb, p16, cyclin D1, Ki-67, cyclin A and cyclin B1 proteins and apoptotic index in T-cell lymphomas. Histol. Histopathol. 16, 377-386.

Kanavaros P., Stefanaki K., Rontogianni D., Papalazarou D., Arvanitis D., Vamvouka C., Gorgoulis V., Siatitsas I., Agnantis N.J. and Bai M. (2001b). Immunohistochemical expression of p53, p21/waf1, Rb, p16, cyclin D1, p27, Ki67, cyclin A, cyclin B1, bcl2, bax and bak proteins and apoptotic index in normal thymus. Histol. Histopathol. 16,1005-1012.

Karsunsky H., Geisen C., Schmidt T., Haas K., Zevnik B., Gau E. and Moroy T. (1999). Oncogenic potential of cyclin E in T-cell lymphomagenesis in transgenic mice: evidence for cooperation between cyclin E and Ras but not Myc. Oncogene 18, 7816-7824.

Kiberu S.W., Pringle J.H., Sobolewski S., Murphy P. and Lauder I. (1996). Correlation between apoptosis, proliferation and bcl-2 expression in malignant non-Hodgkin's lymphomas. J. Clin. Pathol. (Mol. Pathol.) 49, M268-272.

Kolar Z., Flavell J.R., Ehrman J., Rihakova P., Macak J., Lowe D., Crocker J., Vojtesek B., Young L.S. and Muray P.G. (2000). Apoptosis in malignant cells in Hodgkin's disase is related to expression of the cdk inhibitor p27KIP1. J. Pathol. 190, 604-612.

Kuppers R., Klein U., Hansmann M.L. and Rajewsky K. (1999). Cellular origin of human B-cell lymphomas. New. Engl. J. Med. 20, 1520-1529

Leoncini L., Del Vecchio M.T., Megha T., Barbini P., Galieni P., Pileri S. Sabattini E., Gherlinzoni F., Tosi P. and Kraft R. (1993). Correlation between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. Am. J. Pathol. 142, 755-763.

Leoncini L., Cossu A., Megha T., Bellan C., Lazzi S., Luzi P., Tosi P., Barbini P., Cevenini G., Pileri S., Giordano A., Kraft R., Laissue J.A. and Cottier H. (1999). Expression of p34(cdc2) and cyclins A and B compared to other proliferative features of non-Hodgkin's lymphomas: a multivariate cluster analysis. Int. J. Cancer 83, 203-209.

Logsdon M.D., Meyn R.E., Besa P.C., Pugh W.C., Stephens L.C., Peters L.J., Milas L., Cox J.D., Cabanillas F., Brisbay S., Andersen M. and McDonnell T.J. (1999). Apoptosis and the Bcl-2 family-patterns of expression and prognostic value in stage I and II follicular center lymphoma. Int. J. Radiat. Oncol. Biol. Phys. 44, 19-29.

Lopez-Saez J.F., de la Torre C., Pincheira J.and Gimenez-Martin G. (1998). Cell proliferation and cancer. Histol. Histopathol. 13, 1197-1214.

Lundberg A.S. and Weinberg R.A. (1999). Control of the cell-cycle and apoptosis. Eur. J. Cancer 35, 1886-1894.

Miller T.P., Crogan T.M., Dahlberg S., Spier C.M., Braziel R.M., Banks P.M., Foucar K., Kjeldsberg C.R., Levy N., Nathwani B.N., Schnitzer B., Tubbs R.R., Gaynor E.R. and Fisher R.I. (1994). Prognostic significance of the Ki-67 associated proliferative antigen in aggressive non-Hodgkin`s lymphomas: A prospective Southwest Oncology Group trial. Blood 83, 1460-1466.

Moller M.B., Ino Y., Gerdes A.M., Skjodt K., Louis D.N. and Pedersen N.T. (1999). Aberrations of the p53 pathway components p53, mdm2 and CDKN2A appear independent in diffuse large B cell lymphoma. Leukemia 13, 453-459.

Moller M.B., Kania P.W., Ino Y., Gerdes A.M., Nielsen O., Louis D.N., Skjodt K. and Pedersen N.T. (2000). Frequent disruption of the RB1 pathway in diffuse large B cell lymphoma: prognostic significance of E2F-1 and p161NK4A. Leukemia, 14, 898-904.

Moller M.B., Nielsen O. and Pedersen N.T. (2001). Cyclin D3 expression is non-Hodgkin lymphoma. Correlation with other cell cycle regulators and clinical features. Am. J. Clin. Pathol. 115, 404-412.

Nicolaides C., Dimou S. and Pavlidis N. (1998). Prognostic factors in aggressive non-Hodgkin's lymphoma. Oncologist 3, 189-197

Ott M.M., Bartkova J., Bartek J., Durr A., Fischer L., Ott G., Muller-Hermelink H.K. and Kreipe H. (1997). Cyclin D1 expression in mantle cell lymphoma is accompanied by downregulation of cyclin D3 and is not related to the proliferative activity. Blood 90, 3154-3159.

Pinyol M., Cobo F., Bea S., Jares P., Nayach I., Fernandez P.L., Montserrat E., Cardesa A. and Campo E. (1998). P16 INKA4 gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin`s lymphomas. Blood 91, 2977-2984.

Sanchez-Aguilera A., Sanchez-Beato M., Garcia J.F., Prieto I., Pollan M. and Piris M.A. (2002). p14(ARF) nuclear overexpression is aggressive B-cell lymphomas is a sensor of malfunction of the common tumor suppresor pathways. Blood 99, 1411-1418.

Sanchez-Beato M., Saez A.I., Martinez-Montero .JC., Sol-Mateo M., Sanchez-Verde L., Villuendas R., Troncone G. and Piris M.A. (1997). Cyclin-dependent kinase inhibitor p27/KIP1 in lymphoid tissue. P27/KIP1 expression is inversely proportional to the proliferative index. Am. J. Pathol. 151, 151-160.

Sanchez-Beato M., Camacho F.I., Martinez-Montero J.C., Saez A.I., Villuendas R., Sanchez-Verde L., Garcia J.F. and Piris M.A. (1999). Anomalous high p27/KIP1 expression in a subset of aggressive B-cell lymphomas is associated with cyclin D3 overexpression and P27/KIP1-cyclin D3 colocalization in tumor cells. Blood 94, 765-772.

Sanchez-Beato M., Saez A.I., Navas I.C., Algara P., Sol Mateo M., Villuendas R., Sanchez-Aguilera A., Camacho F.I., Sanchez E. and Piris M.A. (2001). Overall survival in aggressive B-cell lymphomas is dependent on the accumulation of alterations in p53, p16 and p27. Am. J. Pathol. 159, 205-213.

Sherr C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. 60, 3689-3695.

Soini Y. and Paakko P. (1999). Apoptosis and expression of caspases 3, 6 and 8 in malignant non-Hodgkin's lymphomas. APMIS 107, 1043-1050.

Soini Y., Raunio H. and Paakko P. (1998). High-grade malignant non-Hodgkin's lymphomas differ from low-grade lymphomas in the extent

of apoptosis and their expression of bcl-2, mcl-1, bax and p53. Tumour. Biol. 19, 176-185.

Sonoki T., Harder L., Horsman D.E., Karran L., Taniguchi I., Willis T.G., Gesk S., Steinemann D., Zucca E., Schlegelberger B., Sole F., Mungall A.J., Gascoyne R.D., Siebert R. and Dyer M.J. (2001). Cyclin D3 is a target gene of t(6;14) (p21.1;q32.3) of mature B-cell malignancies. Blood 98, 2837-2844.

Stefanaki K., Tzardi M., Kouvidou C., Rontogianni D., Bolioti S., Chaniotis V., Vlychou M., Delides G. and Kanavaros P. (1998). Expression of p53, mdm2, p21, Rb, Ki67, bcl2 and bax proteins in lymphomas of the mucosa-associated lymphoid tissue. Anticancer Res. 18, 2403-2408.

Taylor W.R. and Stark G.R. (2001). Regulation of the G2/M transition by p53. Oncogene 20, 1803-1815.

Teramoto N., Pokrovskaja K., Szekely L., Polack A., Yoshino T., Akagi T. and Klein G. (1999). Expression of cyclin D2 and D3 in lymphoid lesions. Int. J. Cancer 81, 543-550.

Tzardi M., Kouvidou C., Panayiotides J., Stefanaki K., Koutsoubi K., Zois E., Rontogianni D., Eliopoulos G., Delides G. and Kanavaros P. (1996). Expression of p53, mdm2 and p21/waf-1 proteins in non-Hodgkin's lymphomas. J. Clin. Pathol. (Mol. Pathol.) 49, M278-283.

Vrhovac R., Delmer A., Tang R., Marie J.P., Zittoun R. and Ajchenbaum-Cymbalista F. (1998). Prognostic significance of the cell cycle inhibitor p27Kip1 in chronic B-cell lymphocytic leukemia. Blood 94, 1401-1408.

Villuendas R., Pezzella F., Gatter K., Algara P., Sanchez-Beato M., Martinez P., Martinez J.G., Munoz K., Garcia P., Sanchez L., Kocialkowsky S., Campo E., Orradre J.L. and Piris M.A. (1997). $p21^{WAF1/CIP1}$ and mdm2 expression in non-Hodgkin's lymphoma and their relationship to p53 status: a $p53^{+}$, mdm^{2-}, p21-immunophenotype associated with missense p53 mutations. J. Pathol. 181, 51-61.

Villuendas R., Sanchez-Beato M., Martinez J.C., Saez A., Martinez-Delgado B., Garcia J., Sol-Mateo M., Sanchez-Verde L., Beniter J., Martinez P. and Piris M. (1998). Loss of p16/INK4A protein expression in non-Hodgkin's lymphomas is a frequent finding associated with tumor progression. Am. J. Pathol. 153, 887-897.

Wagner E.F., Hleb M., Hanna N. and Sharma S. (1998). A pivotal role of cyclin D3 and cyclin-dependent kinase inhibitor p27 in the regulation of IL-2, IL-4 or IL-10-mediated human B-cell proliferation. J. Immunol. 161, 1123-1131.

Wheaton S., Netser J., Guinee D., Rahn M. and Perkins S. (1998). Bcl-2 and bax protein expression in indolent versus aggressive B-cell non Hodgkin's lymphomas. Hum. Pathol. 29, 820-826.

Wolowiec D., Berger F., Ffrench P., Bryon P.A. and Ffrench M. (1999). CDK1 and cyclin A expression is linked to cell proliferation and associated with prognosis in non-Hodgkin's lymphomas. Leuk. Lymphoma 35, 147-157.

Zhou B.B.S. and Elledge S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature 408, 433-439.

Zhu L. and Skoultchi A.I. (2001). Coordinating cell proliferation and differentiation. Curr. Opin. Gen. Dev. 11, 91-97.

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