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$p27^{KIP1}$ is abnormally expressed in Diffuse Large B-Cell Lymphomas and is associated with an adverse clinical outcome

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Summary Cell cycle progression is regulated by the combined action of cyclins, cyclin-dependent kinases (CDKs), and CDK-inhibitors (CDKi), which are negative cell cycle regulators. $p27^{KIP1}$ is a CDKi key in cell cycle regulation, whose degradation is required for G1/S transition. In spite of the absence of $p27^{KIP1}$ expression in proliferating lymphocytes, some aggressive B-cell lymphomas have been reported to show an anomalous $p27^{KIP1}$ staining. We analysed $p27^{KIP1}$ expression in a series of Diffuse Large B-cell Lymphoma (DLBCL), correlating it with the proliferative index and clinical outcome, to characterize the implications of this anomalous staining in lymphomagenesis in greater depth. For the above mentioned purposes, an immunohistochemical technique in paraffin-embedded tissues was employed, using commercially available antibodies, in a series of 133 patients with known clinical outcomes. Statistical analysis was performed in order to ascertain which clinical and molecular variables may influence outcome, in terms of disease-free survival (DFS) and overall survival (OS). The relationships between $p27^{KIP1}$ and MIB-1 (Ki-67) were also tested. An abnormally high expression of $p27^{KIP1}$ was found in lymphomas of this type. The overall correlation between $p27^{KIP1}$ and MIB-1 showed there to be no significant relationship between these two parameters, this differing from observations in reactive lymphoid and other tissues. Analysis of the clinical relevance of these findings showed that a high level of $p27^{KIP1}$ expression in this type of tumour is an adverse prognostic marker, in both univariate and multivariate analysis. These results show that there is abnormal $p27^{KIP1}$ expression in DLBCL, with adverse clinical significance, suggesting that this anomalous $p27^{KIP1}$ protein may be rendered non-functional through interaction with other cell cycle regulator proteins.

Keywords: $p27^{KIP1}$; MIB1; CDK inhibitors; diffuse large B-cell lymphoma; survival analysis

Cell cycle progression is promoted by the action of the cyclin-dependent kinases (CDKs) and their activating sub-units, the cyclins. The G1/S transition in mammalian cells is believed to be regulated by complexes of Cyclin E/CDK2 and Cyclin D/CDK4 or CDK6; the kinase activity of these CDKs requires an association between a catalytic sub-unit, the CDK, and a regulatory sub-unit, the cyclin. This kinase activity can be inhibited by various CDK inhibitors (CDKis). There are two main groups of CDKis, the CIP1/KIP1 ($p21^{WAF1/CIP1}$ (El-Deiry et al, 1993; Harper et al, 1993), $p27^{KIP1}$ (Polyak et al, 1994; Toyoshima and Hunter, 1994) and $p57^{KIP2}$ (Lee et al, 1995; Matsuoka et al, 1995) and the INK4 ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ (Serrano et al, 1993; Guan et al, 1994; Hannon and Beach, 1994; Chan et al, 1995)) families. Members of the CIP1/KIP1 family share sequence homology, and are able to inhibit a broader range of cyclin–CDKs. Nevertheless, members of the INK4 family mainly inhibit the kinase activity of CDK4-CDK6. All of these CDKis cause G1 arrest when overexpressed in transfected cells.

$p27^{KIP1}$ protein inhibits CDK activity by mediating cell cycle arrest (Polyak et al, 1994; Toyoshima and Hunter, 1994). In vitro, $p27^{KIP1}$ has been found to be involved in G1 arrest in response to extracellular signals such as transforming growth factor (TGF)-β in epithelial cells (Polyak et al, 1994), cell–cell contact in fibroblasts (Fang et al, 1995), cAMP in murine macrophages (Kato et al, 1994) and rapamycin in T-lymphocytes (Nourse et al, 1994), all of which are agents known to inhibit cell cycle progression. $p27^{KIP1}$ has been implicated as the CDKi involved in mitogen-dependent CDK regulation, as a key protein in passage through the restriction point (Couts et al, 1996), thus making the $p27^{KIP1}$ degradation a necessary step in the irreversible decision to enter the S phase (Cheng et al, 1998). $p27^{KIP1}$ protein is present in quiescent cells, and levels descend dramatically when cells are stimulated by growth factors (Kato et al, 1994; Nourse et al, 1994). Besides this key role in cell cycle control, additional functions for $p27^{KIP1}$ in cell differentiation (Hengst and Reed, 1996), apoptosis induction (Bani et al, 1995; Wang et al, 1997) and chemotherapy resistance (Croix et al, 1996) have been proposed. Knockout mice for $p27^{KIP1}$ show an increase in the size of organs and the cell number, confirming its inhibitory role in cell cycle control, in the same regulatory pathway as the gene Retinoblastoma (Rb) (Fero et al, 1996; Kiyokawa et al, 1996; Nakayama et al, 1996).

According to this model, quiescent non-tumoural lymphocytes express high levels of $p27^{KIP1}$ protein, which is down-regulated following cell activation. Thus, proliferating lymphoid cells, in either peripheral blood or peripheral lymphoid organs, express low or undetectable levels of $p27^{KIP1}$ protein (Croix et al, 1996; Sánchez-Beato et al, 1997). In lymphomas, when all histological types are taken into consideration, this relationship between $p27^{KIP1}$ expression and proliferation is generally preserved, with an inverse expression for $p27^{KIP1}$ and MIB-1 proteins. Tumours with a low

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proliferative index were mostly positive, while tumours characterized by a higher growth fraction had low $p27^{KIP1}$ protein levels (Sánchez-Beato et al, 1997), although in an earlier study performed by this group some exceptions to this rule were found in the group of aggressive B-cell lymphomas, including Burkitt's lymphoma and diffuse large B-cell lymphoma (DLBCL). Only very rare genetic alterations have been found in the $p27^{KIP1}$ gene (Shiohara et al, 1994; Kawamata et al, 1995; Morosetti et al, 1995; Pietenpol et al, 1995; Watanabe et al, 1995), suggesting that if inactivation of this CDKi takes place in human tumours, it would be through a mechanism other than genetic mutation, unlike the INK4 family.

Initial studies performed on breast, lung and colorectal cancer and melanoma, coincide in indicating that a high level of $p27^{KIP1}$ expression is associated in these tumours with a lower degree of malignancy (Fredersdorff et al, 1997) or a better clinical outcome (Bani et al, 1997; Catzavelos et al, 1997; Esposito et al, 1997; Loda et al, 1997; Porter et al, 1997), in spite of the fact that in these tumours there is no clear relationship between $p27^{KIP1}$ expression and growth fraction. Further studies offer differing data showing that a high level of $p27^{KIP1}$ expression may be associated with a more aggressive course in breast cancer and B-cell lymphocytic leukaemia (B-CLL) (Sgambato et al, 1997; Vrhorac et al, 1998). The presence of an elevated level of $p27^{KIP1}$ protein expression in subsets of tumours with high proliferative indexes suggests that the abnormally expressed protein lacks the functional capacity to inhibit cell cycle progression (Fredersdorf et al, 1997; Sgambato et al, 1997; Quintanilla-Martinez et al, 1998; Yatabe et al, 1998).

DLBCLs form a heterogeneous group in terms of clinical presentation, histology, immunophenotype, genetic alterations, response to treatment and prognosis (Harris et al, 1994). Recent findings suggest that the overall outcome for patients with DLBCL is decided by a combination of different clinical and biological variables, the expression of some of these cell cycle regulator proteins being specifically associated with different clinical features of tumours. Overall analysis showed that an extended overall survival (OS) time is associated with low growth fraction, low expression of p53 and MDM2 proteins and high Rb expression. In contrast with this finding, higher disease-free survival (DFS) time is mainly associated with low bcl2 expression (Slymen et al, 1990; Yamanaka et al, 1992; Miller et al, 1994; Piris et al, 1994; Martin et al, 1995; Hermine et al, 1996; Hill et al, 1996; Kramer et al, 1996; Gascoyne et al, 1997; Grogan et al, 1988; Sánchez et al, 1998)

Earlier studies in non-Hodgkins lymphomas (NHLs) pointed to the presence of a small group of DLBCL characterized by an unusually high level of $p27^{KIP1}$ expression (Sánchez-Beato et al, 1997; Quintanilla-Martinez et al, 1998), in which the opposing relationship between $p27^{KIP1}$ and MIB1 expression seemed to be lost. The main purpose of this work was to analyse the expression of this protein in greater depth, and to determine the clinical relevance of $p27^{KIP1}$ expression in this lymphoma type. To this end the clinical characteristics of a series of 133 cases of DLBCL were analysed, while simultaneously studying the relative clinical relevance of MIB1 expression, which is a growth fraction marker.

MATERIALS AND METHODS

Tissue samples, patients and clinical variables

Tumour specimens from 133 DLBCLs were obtained from the routine files of the Virgen de la Salud Hospital, and diagnosed according to the criteria used in the REAL classification (Harris et al, 1994). Consecutively diagnosed patients from 1980 to 1995 were included in the study if their cases fulfilled the following conditions:

- The initial diagnosis of DLBCL had to be made on a representative biopsy obtained before treatment.
- Paraffin-embedded, formalin-fixed tissue blocks from the diagnostic biopsy had to be available for immunohistochemical studies.
- All patients had to be treated with the aim of achieving a cure.

Untreated patients were excluded. Treatment decisions were not based on molecular and/or immunohistochemical features. Patients were treated according to standard therapeutic protocols, including polychemotherapy (CHOP or third-generation regimes, depending on IPI and clinical stage) and radiotherapy or surgery for localized tumours. A combination of these treatments was used for certain patients.

Age, clinical stage, performance status, LDH and the number of extranodal sites of the disease were used, as determined by the International Prognostic Index (IPI). IPI was grouped as 0–1, 2, 3 and 4–5. Complete remission (CR) was defined as the absence of clinical and radiological evidence of disease for a minimum of 4 weeks. Other degrees of response were considered to represent treatment failure. Patients who died of causes unrelated to the disease were censored at the time of death. OS was measured as the interval between the beginning of treatment and the death of the patient or the date of the last follow-up revision. Patients known to have died after finishing the treatment due to causes unrelated to the lymphoma (one colorectal cancer diagnosed shortly after the diagnosis of lymphoma, one acute myocardial infarct and one elderly patient with Alzheimer's disease) were considered as censored for the purposes of survival analysis. DFS was estimated for patients having CR, by measuring the interval between the end of the treatment and relapse or death attributable to the tumour, or the date of the last follow-up revision in patients who had no relapse. All data were checked for validity and consistency.

Antibodies

$p27^{KIP1}$ protein was detected with a monoclonal antibody (mAb) from Transduction Laboratories, K25020 (1:1000 dilution), generated against the full-length mouse KIP1 protein. This antibody exclusively recognizes a 27 kDa protein, as previously shown by Mantel et al (1996). Ki-67, a nuclear protein expressed in the G1, S and G2/M phases, was detected by the MIB1 mAb (Novocastra) (1:50 dilution). It was also used as the control of antigenic preservation and successful antigenic retrieval.

Immunostaining techniques

All immunostaining techniques were performed in paraffin-embedded tissue. A previous step of heat-induced antigen retrieval technique was used for all four antibodies. This took the form of pressure cooker heating in a solution of 0.01 M sodium citrate prior to incubation with the antibody (Ab). Following incubation with the primary Ab, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labelled streptavidine (LSAB-DAKO) and with diaminobencidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device.

Incubation omitting the specific Ab, as well as with unrelated Abs, was used as a control of the technique. Internal control

provided by benign reactive T-cells, macrophages, epithelial or endothelial cells was used to evaluate p27^{KIP1} and MIB1.

Quantitative studies

All scoring and interpretations of immunohistochemical results were made by two pathologists (JFG and AIS), without knowledge of the clinical outcome or other clinical variables of tumour markers.

High magnification fields were chosen for the evaluation of p27^{KIP1}, focusing on tumoural areas, counting up to 200 cells, excluding small T-cells or other non-tumoural cells. All immunoreactive cells were considered positive. The percentage of p27^{KIP1}-positive tumoural cells was expressed as a ratio of positive cells to 100 cells. A manual procedure of counting p27^{KIP1} cells was used, in order to exclude all non-tumoural sub-populations (most of them expressing p27^{KIP1}), using the morphology of the cells as criterion. A cut-off of 5% of tumoural cells was used for p27^{KIP1} expression, since this threshold roughly separates tumours with null or isolated p27^{KIP1} expression from those characterized by an intermediate or high level of expression, as determined by semi-quantitative analysis. This threshold divides the series into groups of similar size (p27 ≤ 5%: 57.9% cases; p27 > 5%: 42.1% cases), and this and closer cut-off points were found to be statistically highly significant. Moreover, the cut-off points at 4 and 6% were statistically significant, although they had the disadvantage of being less easily reproducible.

The Quantitative Proliferation Index application of the CAS 200 was used for the analysis of MIB1, since this measure of the tumoural growth fraction has been shown to correlate significantly with the clinical outcome in DLBCL (Sanchez-Bezto et al, 1997). This program determines a value expressing the growth fraction of the tumour, related to the number of proliferating cells. Representative fields down to a minimum of 30 000 μ^2 were selected (approximately five fields with 45× objective and 10× ocular lenses). Sectional analysis was carried out, focusing on tumoural areas. A cut-off point of 20% of positive cells was used, as a previous study showed that this point discriminated between groups presenting different clinical behaviour. Categories were therefore classified as equal to or less than 20%, or greater than 20% (Sánchez et al, 1998).

Statistical study

The relationship between p27^{KIP1} and the proliferative index

The distributions of p27^{KIP1} and MIB-1 were compared in order to establish any possible associations, using the Spearman correlation coefficient for doubly ordered data, and Kruskal–Wallis test for single ordered data. Differences were considered significant if $P < 0.05$.

Survival: univariate analysis

The clinical variables analysed for survival studies were those included in the IPI (measured as 0–1, 2, 3, 4–5), which are: age (≤ 60 vs > 60), gender (female vs male), clinical stage (I + II vs III + IV), LDH (normal vs > normal). Actuarial survival curves (OS and DFS) were plotted using the Kaplan and Meier method (Kaplan and Meier, 1958). The statistical significance of associations between individual variables, OS and DFS was determined by using the log-rank test (Mantel, 1966). Cox's proportional

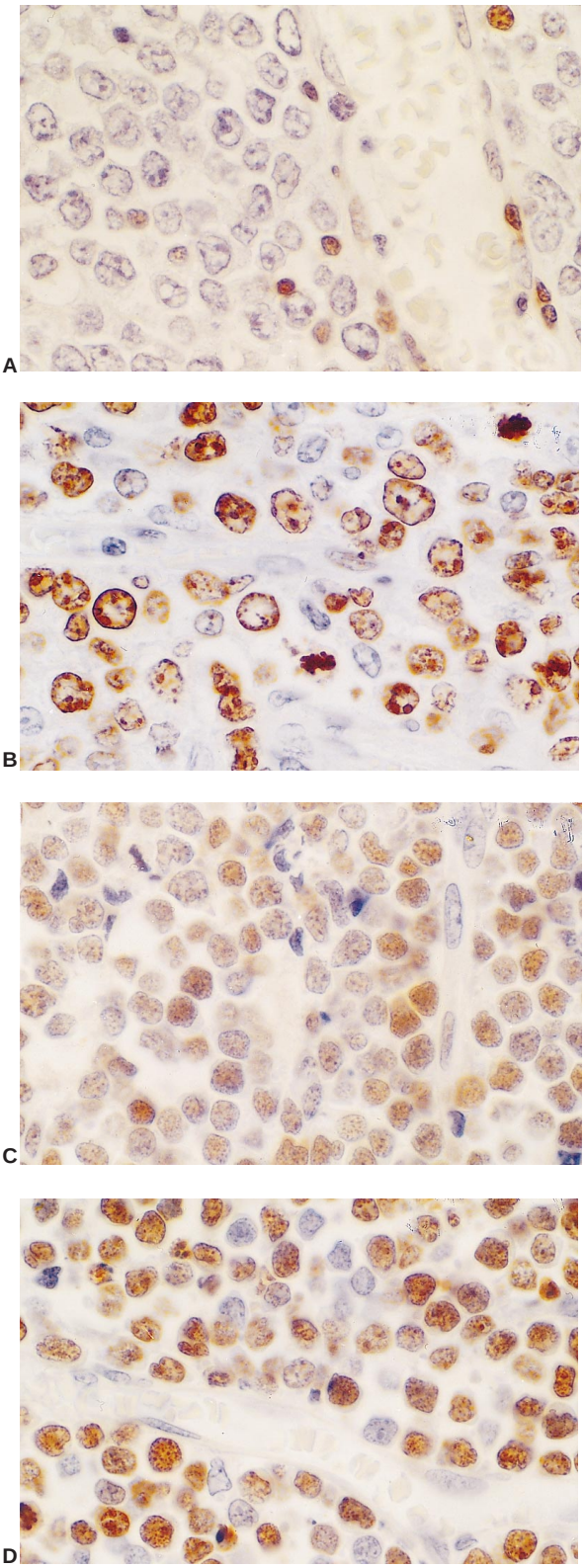

Figure 1 Immunohistochemical expression of p27^{KIP1} and MIB1 in two different cases of the series. Case 65 was characterized by low p27^{KIP1} expression (1%), mainly restricted to small lymphocytes and endothelial cells (**A**), while most tumoural cells show strong MIB1 staining (**B**), read by the image analyser as 44.16%. Case 112 shows distinct p27^{KIP1} staining of a majority of tumoural cells (90%) (**C**) and relatively similar MIB1 staining (**D**) (43.94%)

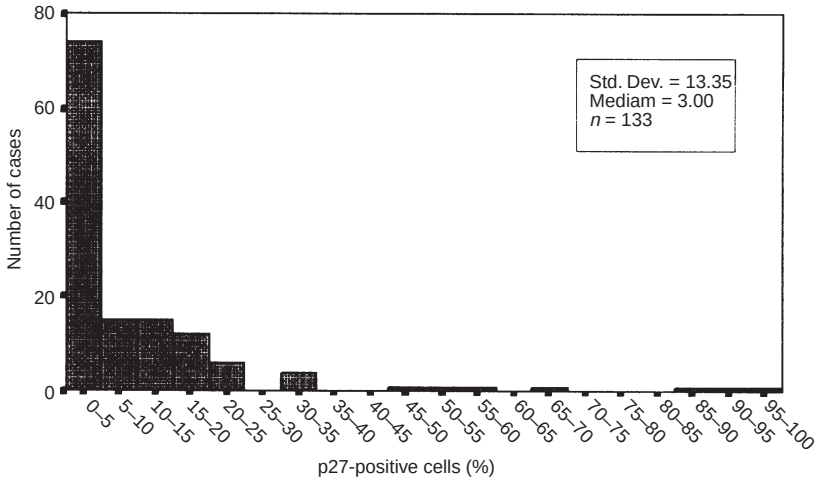

Figure 2 Histogram of $p27^{KIP1}$ expression in the DLBCL series

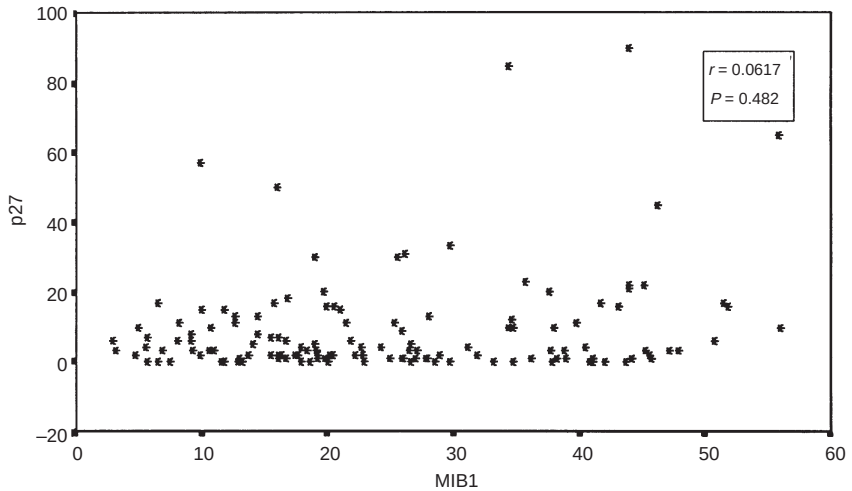

Figure 3 Analysis of lineal correlation between $p27^{KIP1}$ and MIB1. There is not a significant correlation between these two parameters

hazard univariate analysis was also performed independently for each variable, calculating the relative risk (RR) of each variable in terms of survival and confidence interval (CI) (Cox, 1972).

Survival: multivariate analysis

In order to identify the factors which might be of independent significance in influencing survival (OS and DFS), a Cox backward proportional hazard model was run (Cox, 1972). Variables included in the maximum models were: IPI (0–1, 2, 3 or 4–5), MIB1 (≤ 20% or > 20%), $p27^{KIP1}$ (≤ 5% or > 5%). The lower values of IPI, MIB1 and $p27^{KIP1}$, which had been found to be associated with longer survival probability, were used as reference levels (RL). All *P*-values were two-sided, and values of 0.05 or less were considered to indicate statistical significance.

The SPSS software package was used for all statistical analysis.

RESULTS

Characteristics of the patients

The clinical characteristics of the 133 patients included in this analysis have already been published (Sánchez et al, 1998).

Immunohistochemical study

$p27^{KIP1}$ nuclear protein expression was observed in tumoural cells with a signal range of from 0.00 to 95.00 and with a median of 3.00. Null $p27^{KIP1}$ staining was seen in 21 cases. $p27^{KIP1}$ staining was found most frequently in a low to intermediate proportion of the tumoural cells. For survival analysis, a cut-off point of 5% of $p27^{KIP1}$-positive cells was used, which divides the series into two groups, corresponding approximately to cases found by semi-quantitative analysis as negative or low-expression versus those with intermediate or high levels of expression ($p27 \leq 5\%$: 77 cases; $p27 > 5\%$: 56 cases). A smaller group of seven cases (corresponding to percentile 95) showed a very high intensity of staining of most tumoural cells (Figure 1). A histogram of the overall distribution of $p27^{KIP1}$ reactivity is shown in Figure 2.

MIB1 expression was observed in all cases, with a positive percentage of cell staining varying from 2.912 to 55.94 (median: 22.030) (Figure 1). In 73 cases (54.9%) the growth fraction was greater than 20%, this threshold having been found to be clinically relevant in previous studies (Sánchez et al, 1998). No significant correlation was found between $p27^{KIP1}$ and MIB-1 expression,

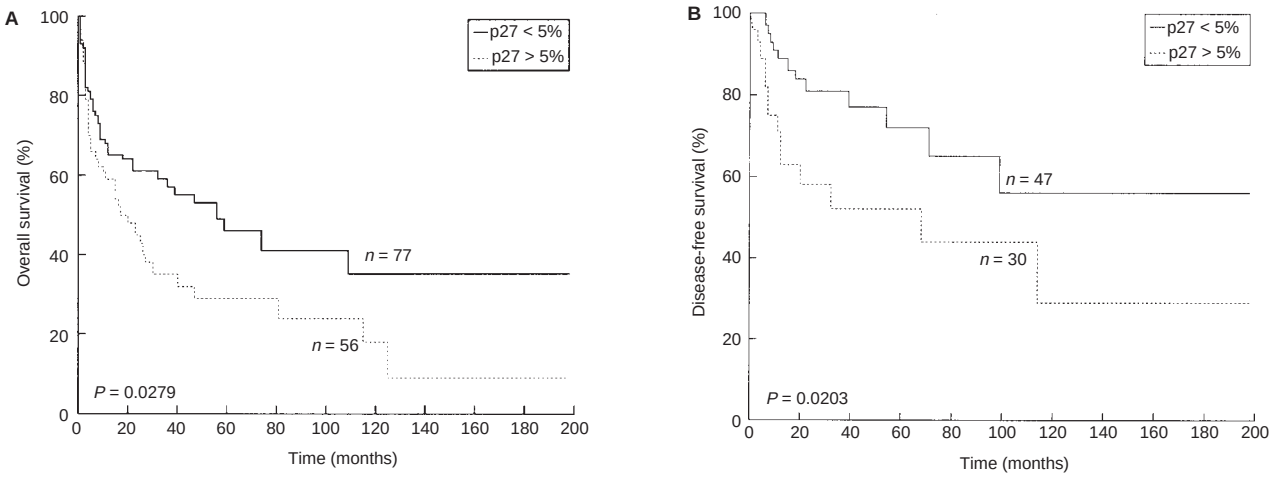

Figure 4 Overall survival (**A**) and disease-free survival (**B**). Kaplan–Meier analysis of the series according to p27KIP1 expression. Both OS and DFS were found to be significantly shortened in cases of DLBCL with higher p27KIP1 expression

using the Spearman correlation coefficient ($r = 0.0617$, $P = 0.482$) or the Kruskal–Wallis test ($\chi^2 = 0.0741$, $P = 0.7854$) (Figure 3).

Survival analysis

Univariate and multivariate survival analyses were performed to analyse the prognostic significance of p27KIP1 expression. MIB1 expression and the clinical parameters included in the IPI were included as references.

Univariate study

Investigation of OS by Cox's analysis showed that high IPI ($P = 0.0004$), MIB1 > 20% ($P = 0.0199$) and p27KIP1 > 5% ($P = 0.0320$) were significantly associated with shorter OS. The study of DFS showed that a high expression of p27KIP1 ($P = 0.0412$) is significantly associated with shorter DFS. No significant relationship was found for high MIB1 or IPI (Table 1). Figure 4A shows the plots for OS, while Figure 4B shows the plot for DFS by Kaplan–Meier analysis and log-rank test.

Multivariate study

Only low p27KIP1 expression was found to be a parameter associated with prolonged OS ($P = 0.0145$), together with the clinical variables included within the IPI ($P = 0.0010$). The significance of MIB1, as found in univariate analysis, was absent from this series. When analysing DFS, only a high p27KIP1 value was found to be significantly associated with a shorter DFS time ($P = 0.0424$) (Table 1).

DISCUSSION

This study confirms that there is a group of DLBCL characterized by an anomalous p27KIP1 expression, showing that in the DLBCL group as a whole the inverse relationship found in normal lymphoid

Table 1 Univariate and multivariate analysis of the prognostic significance of p27KIP1 and MIB1 expression in relation with the clinical parameters included within the IPI (Cox's regression model)

		Univariate study			Multivariate study		
	RL	RR	95% CI	*P*	RR	95% CI	*P*
P27	≤5%	1.6491	1.0438–2.6053	0.0320	1.7811	1.1210–2.8300	0.0145
MIB1	≤20%	1.7554	1.0931–2.8190	0.0199	1.5514	0.9420–2.5550	0.0845
IPI				0.0004			0.0010
IPI(2)	IPI(0–1)	2.7338	1.2761–5.8568	0.0097	2.7513	1.2796–5.9156	0.0096
IPI(3)	IPI(0–1)	3.5224	1.6555–7.4945	0.0011	3.6285	1.7024–7.7337	0.0008
IPI(4–5)	IPI(0–1)	4.8971	2.3176–10.3474	0.0000	4.5606	2.1337–9.7479	0.0001
Disease-free survival							
P27	≤5%	2.2732	1.0334–5.0005	0.0409	2.2899	1.0349–5.0669	0.0409
MIB1	≤20%	1.1531	0.5180–2.5668	0.7271	1.4448	0.6060–3.4446	0.4065
IPI				0.1475			0.1148
IPI(2)	IPI(0–1)	2.6184	0.9204–7.4491	0.0712	2.6280	0.9133–7.5620	0.0732
IPI(3)	IPI(0–1)	3.1793	1.1018–9.1740	0.0324	3.3917	1.1662–9.8646	0.0250
IPI(4–5)	IPI(0–1)	1.4543	0.2931–7.2165	0.6468	1.1496	0.2169–6.0924	0.8698

Chi-square: 28.949, $P < 0.0001$ for overall survival; Chi-square: 10.736, $P = 0.0569$ for disease-free survival. RL: reference level; RR: risk ratio; CI: confidence interval.

tissues between $p27^{KIP1}$ and MIB1 expression is lacking. This anomalous $p27^{KIP1}$ expression was also associated with an adverse clinical outcome in univariate as well as multivariate analysis.

This finding contradicts observations in reactive lymphoid tissue, where $p27^{KIP1}$ expression is always associated with quiescent status, as shown by immunohistochemistry of reactive lymph nodes and tonsils, flow cytometry and Western blot studies of phytohaemaglutinin-stimulated peripheral blood lymphocytes. These show that the $p27^{KIP1}$ protein is mainly found in MIB-1 negative quiescent lymphocytes, its expression being down-regulated along cell cycle progression (Sánchez-Beato et al, 1997). Thus benign centroblasts, the proliferating B-cell subset of the germinal centre and the supposed normal counterpart of most DLBCL, usually show a MIB1+, p27– phenotype, which is lost to a certain extent in DLBCL. This had already been noticed in a previous study of NHL by our group, in which the existence of a small group of cases of Burkitt's lymphoma and DLBCL with this anomalous nuclear $p27^{KIP1}$ accumulation was described (Sánchez-Beato et al, 1997); these results were confirmed by further studies by Quintanilla-Martínez et al (1998) and Erlanson et al (1998). A more detailed study was therefore performed. This analysed a larger series of cases using a more sensitive technique and quantified the results with a more analytical procedure, including statistical analysis of the relationship between $p27^{KIP1}$, MIB1 and survival probability. This made it possible to confirm and expand earlier observations, indicating that a gradient of $p27^{KIP1}$ expression may be seen in DLBCL. Contrary to what was expected on the basis of the findings in non-tumoural tissues and other tumours, $p27^{KIP1}$ expression appears to be associated with an unfavourable clinical course, thereby supporting the hypothesis that this abnormal accumulation of $p27^{KIP1}$ protein is deregulated (Sgambato et al, 1997). To exclude the possibility that the adverse effect of $p27^{KIP1}$ expression on survival could be attributed exclusively to the small group of cases with very high $p27^{KIP1}$ expression, we repeated the analysis excluding the cases with values of p27 > 25%, the same findings being repeated in this group (data not shown). This same series of patients was the subject of a recent report showing that high growth fraction is an adverse prognostic factor in multivariate analysis (Sánchez et al, 1997). Now, and once $p27^{KIP1}$ is included in the analysis, the predictive value of MIB1 is lost in favour of that of $p27^{KIP1}$.

The techniques used here for the quantitative analysis of $p27^{KIP1}$ and MIB1 reflect the difficulties arising in estimating these in DLBCL. As is the case for most reactive T-cells, endothelial cells and histiocytes are $p27^{KIP1}$-positive. We therefore decided to count the cells manually, thereby selecting tumoural cells exclusively on the basis of their morphology. This made it possible to recognize and estimate the presence of a minority p27-positive tumoural cell subpopulation in a significant group of DLBCL. This finding was also described in the other analysis published to date of $p27^{KIP1}$ expression in DLBCL, thereby underlining the consistency of these techniques and the reproducibility of the results described. Thus, the studies of Quintanilla-Martínez et al (1998) and Erlanson et al (1998) both describe the presence of anomalous $p27^{KIP1}$ expression in a subset of DLBCL cases, together with an associated loss of the normal p27/MIB1 ratio found in normal cells.

Nevertheless, software specifically designed for the recognition of the growth fraction of tumours was used for MIB1 analysis, since most positive cells in DLBCL are members of the tumoural cell population, which makes it possible to use an image analysis program. A relatively low percentage of proliferating cells was found in this study, depending of the characteristics of the software used, and this must be taken into consideration when comparing these values with those corresponding to other series.

Although the loss of the inverse relationship between $p27^{KIP1}$ and MIB-1 expression has been described previously in different studies of breast and colorectal cancer specimens and derived cell lines (Catzavelos et al, 1997; Fredersdorf et al, 1997; Lodu et al, 1997; Sgambato et al, 1997), the existence of an adverse clinical significance for increased expression of $p27^{KIP1}$ in a specific tumoural type has only been rarely noticed. Indeed, earlier reports emphasized that a more favourable clinical outcome is usually associated with a high level of $p27^{KIP1}$ expression, as seen in non-small-cell lung cancer (Esposito et al, 1997), breast cancer (Catzavelos et al, 1997; Porter et al, 1997), colorectal cancer (Loda et al, 1997). A recent report by Erlanson et al (1998) found that, when all histological types of NHL are taken into consideration, high $p27^{KIP1}$ values are associated with longer survival probabilities, and therefore probably reflect the fact that the study simultaneously includes different entities, while it does not analyse the specific meaning of $p27^{KIP1}$ expression in each histological type. Nevertheless, when considering a specific entity like B-CLL, a recent analysis by Vrhovac et al (1998) illustrates the same phenomenon found here, i.e. high $p27^{KIP1}$ levels associated with a poorer overall prognosis, which appeared to be related to the association between high $p27^{KIP1}$ and apoptosis resistance. These results overlap those of other reports in epithelial tumours, in which an anomalous deregulated $p27^{KIP1}$ expression in some tumoural cell types has been described (Sgambato et al, 1997; Yatube et al, 1998).

It has been proposed that $p27^{KIP1}$ overexpression is involved in the drug resistance displayed by solid tumours, and the suggestion has even been made that tumour-targeted $p27^{KIP1}$ antagonists could be used in conjunction with conventional anticancer therapy (Croix et al, 1996). In B-CLL it has been shown that an increased $p27^{KIP1}$ level protects cells against apoptosis, this being reverted after fludarabin exposure (Vrhorac et al, 1998). These experimental data could explain the shorter survival in the patients analysed here.

This abnormally accumulated $p27^{KIP1}$ protein seems to lack the capacity for inhibiting Cyclin E–CDK2 complexes, since these aggressive B-cell lymphomas have a high proliferative index and an unfavourable clinical outcome. This situation has also been observed in human breast cancer cell lines, where an increase in $p27^{KIP1}$ expression was paralleled by an increase in the level of Cyclin D1 and Cyclin E. As $p27^{KIP1}$ expression is mainly regulated at a post-transcriptional level, it has therefore been suggested that there is a homeostatic mechanism for the up-regulation of $p27^{KIP1}$, induced by overexpression of Cyclin D1 or Cyclin E (Sgambato et al, 1997). Additional works by Blain et al (1997), Cheng et al (1998), Reynisdottir and Massague (1997) and Soos et al (1996) have shown that a high level of $p27^{KIP1}$ expression may be found in growing cells, confirming that this negative regulator of the cell cycle is kept inactive by adhesion to Cyclin D–CDK4 complexes. This could also be an hypothetical explanation for the anomalous $p27^{KIP1}$ expression in this group of DLBCL.

It is still not known if the adverse clinical meaning of $p27^{KIP1}$ expression in DLBCL is the direct consequence of the accumulation of this protein, or whether it is merely a reflection of a deeper alteration of cell cycle regulator genes leading to this anomalous $p27^{KIP1}$ expression.

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