

## Cell cycle regulatory proteins and apoptosis in B-cell chronic lymphocytic leukemia

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**Background and Objectives.** The pathogeny of B-cell chronic lymphocytic leukemia (B-CLL) involves both deregulated proliferation and inhibition of cell death. A particular role in the regulation of these phenomena is played by proteins involved in early G<sub>1</sub> phase regulation: pRb kinases: cyclin-dependent kinases (cdk): cdk4 and cdk6 activated by cyclins D, and universal cdk inhibitor p27<sup>Kip1</sup>.

**Design and Methods.** We determined by flow cytometry the expression of p27<sup>Kip1</sup> and cyclins D (D2 and D3) in populations of peripheral blood lymphocytes obtained from 59 (for p27<sup>Kip1</sup>) and 31 (for cyclins D) previously untreated patients with B-CLL, and compared them with cell cycle parameters, cell viability and apoptosis in 72-hour cultures in medium only. As a control we determined the expression of p27<sup>Kip1</sup>, cyclin D2 and D3 in peripheral blood CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes from 15 healthy donors.

**Results.** p27<sup>Kip1</sup> was present in nearly 100% of lymphocytes in all B-CLL populations tested. Its cellular content estimated semiquantitatively by specific mean fluorescence intensity was higher than in normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, p27<sup>Kip1</sup> was inversely correlated with patients' age and not correlated with other clinical variables, cell cycle or apoptosis rate. Cyclin D2 was detectable in 25 out of 31, and cyclin D3 in all B-CLL lymphocytes populations studied. In contrast to p27<sup>Kip1</sup> present in all CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, both cyclins were detected only in a subset of neoplastic cells: 27.5 to 87% (mean 51.2) for cyclin D2 and 20.3 to 98% (mean 76.5) for cyclin D3. In cyclin D2- and D3-positive normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes and B-CLL cell populations, cyclin D3 was expressed in a higher percentage of cells than cyclin D2. Both cyclin D2- and cyclin D3-positive fractions of B-CLL cells were, on average, larger than corresponding fractions of normal CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes.

**Interpretation and Conclusions.** Our results indicate that cyclin D3 plays an important role in the regulation of normal and neoplastic CD5<sup>+</sup>/CD19<sup>+</sup> cells, and point to the possibility of the exit of a number of CLL lymphocytes from quiescence.

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Key words: chronic lymphocytic leukemia, cyclins D, p27<sup>Kip1</sup>, cell proliferation, apoptosis.

Tumor progression in B-cell chronic lymphocytic leukemia (B-CLL), the most frequent form of leukemia in Western countries, is thought to result from the gradual accumulation of clonal B-lymphocytes in early phases (G<sub>0</sub>/G<sub>1</sub>) of the cell cycle. This accumulation is probably due to the breakdown of the equilibrium between cell death inhibited by the accumulation of the anti-apoptotic protein Bcl2, and cell proliferation.<sup>1,2</sup> Cell cycle progression in mammalian cells is governed by interactions between cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors. Individual cyclins act at different phases of the cell cycle by binding to suitable cdks and allowing the subsequent activation of the cyclin-cdk complexes.<sup>3</sup> The D-type cyclins (D1, D2 and D3) regulate the G<sub>1</sub> phase progression by binding to and stimulating the activities of their catalytic partners: cdk4 or cdk6. Kinases cdk4 and cdk6 phosphorylate the retinoblastoma protein (pRb).<sup>4,5</sup> This process leads to inactivation of pRb and release of a transcription factor E2F, which activates transcription of the components of the DNA replication machinery, thereby committing the cell to S phase of the cell cycle.<sup>6</sup> In human normal T lymphocytes the gene for the cyclin D1 is quiescent, but the lymphocytes express cyclins D2 and D3 in early and in late G<sub>1</sub> phase, respectively.<sup>7,8</sup>

Proper execution of later phases of the cell cycle requires the subsequent activation of other cyclin-cdk complexes: cdk2-cyclin E, cdk2-cyclin A, cdk1-cyclin A and cdk1-cyclin B. The inhibitory effect on the cdks or cdk-cyclin complexes is exerted by a family of proteins termed cdk inhibitors. Two classes of these inhibitors have been described so far. The first one (p16<sup>INK4a/CDKN2A</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>) is specific for pRb kinases, i.e. cdk4 and cdk6, the second one (p21<sup>WAF1/CIP1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>) has a broader range of activity and inhibits most of the known cdk-cyclin complexes.<sup>9</sup> Expression of some of these cell cycle regulatory factors, e.g. cdk4, cyclin E and cyclin D2, as well as some other cell-cycle dependent factors, such as nucleoside diphosphate kinase NM23-F2 in B-CLL lymphocytes led to the hypothesis that they are actually not quiescent but are recruited to the cell cycle and blocked in its early G<sub>1</sub> phase. This observation points to the possibility that the accumulation of B-CLL lymphocytes is due not only to defective apoptosis but also to an abnormality of the regulation of cell proliferation.<sup>10,11,12</sup>

Cyclins D and cdk inhibitor p27<sup>Kip1</sup> seem to play a particular role in the regulation of both apoptosis and proliferation of B-CLL cells. High expression of p27<sup>Kip1</sup> in B-CLL lymphocytes not only prevents them from progressing to S phase and mitosis, but may also protect them from apoptosis when cultured *in vitro*. Contrarily to the case of most solid tumors and other lymphoproliferative disorders, in which downregulation of p27<sup>Kip1</sup> is associated with aggressive disease and worse clinical course,<sup>13</sup> high expression of p27<sup>Kip1</sup> in B-CLL lymphocytes is associated with faster lymphocyte-doubling time and poor prognosis.<sup>14</sup> There are few published studies so far on the expression of cyclins D2 and D3 in B-CLL, and the results of these studies are discordant. Cyclin D2 mRNA was found to be expressed in the most B-CLL cases (29 out of 34 in the study by Delmer *et al.*)<sup>10</sup> consistent with the hypothesis of cell-cycle involvement of B-CLL cells. As to cyclin D3, currently available data are discordant. Cyclin D3 mRNA was not detected in any case studied by Delmer *et al.*<sup>10</sup>, while Suzuki *et al.*<sup>15</sup> observed the expression of cyclin D3 mRNA in 10 of 11 B-CLL cases. In a subset of aggressive B-cell lymphomas an anomalously high p27<sup>Kip1</sup> expression was found to be associated with cyclin D3 overexpression. The co-localization of both proteins in tumor cells might indicate that cyclin D3 sequesters p27<sup>Kip1</sup> and prevents it from exerting an inhibitory effect towards cdk2.<sup>16</sup> The important role played by both

p27<sup>Kip1</sup> and cyclins D in the regulation of the proliferative activity of B-CLL, as well as discordant results of previously published studies on the expression of the D-cyclin family in B-CLL cells prompted us to study the expression of p27<sup>Kip1</sup>, cyclin D2 and cyclin D3 in relation to proliferation and cell death in B-CLL lymphocytes.

## Design and Methods

### Patients

Peripheral blood samples from 59 previously untreated patients with B-CLL aged from 33 to 80 years (mean 64.6, SD 10.5) were studied. The diagnosis was based on appropriate clinical and morphologic features and was confirmed by the co-expression of CD5 and CD19 and the expression of CD23 antigen on malignant cells. The stage of the disease, defined according to the Rai classification, was assessed using standard procedures and was established as 0 in 37 patients, 1 in 10, 2 in 7, 3 in 3 and 4 in the remaining two cases. Leukocyte count ranged from 11.4 to 173×10<sup>9</sup>/L (mean 36.0, SD 32.5), lymphocyte count: from 5.8 to 156×10<sup>9</sup>/L (mean 29.9, SD 31.5), hemoglobin level: from 9.5 to 15.9 g% (mean 13.9, SD 1.35) and platelet count: from 20 to 309×10<sup>9</sup>/L (mean: 177.0, SD 57.9). As a control population, CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes obtained from 15 healthy subjects were studied.

The study of the expression of cyclin D2 and D3 was performed in 31 patients with B-CLL in active phase of the disease (stages 0-III) aged between 42 and 80 years (mean 66.8) and 15 age- and sex-matched healthy subjects. Studies were carried out with local ethical committee approval.

### Immunostaining and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation on Gradisol L (AQUA-MEDICA, Poland) from freshly drawn peripheral venous blood, washed three times with phosphate buffered saline (PBS). The expression of p27 protein and cyclins D2 and D3 in CD5<sup>+</sup>/CD19<sup>+</sup> cells was estimated by a triple color immunofluorescence method.

### Immunostaining of p27 protein

P27<sup>Kip1</sup> protein expression was studied in PBMCs of all 59 patients with B-CLL and in CD5<sup>+</sup>/CD19<sup>+</sup> cells from 15 healthy subjects. The cells were incubated for 30 min at 4°C with 2% AB serum and monoclonal anti-CD19 and anti-CD5 antibody conjugated with PerCP and PE, respectively (Becton Dickinson, Becton Dickinson Company, USA). The cells were then washed in PBS containing 1%

BSA (bovine serum albumin; Sigma, Germany). The cells were fixed and permeabilized according to the method described by Farahat *et al.*,<sup>17</sup> Anderson *et al.*<sup>18</sup> and Frydecka *et al.*<sup>19</sup> The cells were then washed twice using PBS containing 0.5% Tween-20 (Sigma, Germany) and incubated for 1h 30 min at 4°C with PBS containing 1% BSA and MoAb to p27 (PharMingen, Becton Dickinson Company, USA). Next, the cells were washed twice with 0.5% Tween-20 and incubated for 30 min at 4°C with 1% BSA in PBS and FITC-conjugated rabbit-anti-mouse immunoglobulins (DAKO, Denmark). The cells were then washed again, resuspended in PBS and analyzed by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson). Negative control were always used and treated as described above except that mouse immunoglobulins of the same isotype as monoclonal antibodies conjugated with PerCP, PE and FITC were added (DAKO and Becton Dickinson, Becton Dickinson Company).

#### *Immunostaining of cyclin D2 and D3*

This was carried out in PBMCs of 31 patients with B-CLL and in CD5<sup>+</sup>/CD19<sup>+</sup> cells from 15 healthy persons. For B-CLL cells, PBMCs were incubated for 30 min at 4°C with 2% AB serum and monoclonal antibody to CD19 and CD5 conjugated with PerCP and PE, respectively (Becton Dickinson, Becton Dickinson Company, USA). The cells were then washed twice in PBS, permeabilized and fixed according to the method described by Gong *et al.*<sup>8</sup> and incubated overnight at 4°C in the presence of PBS containing 1% BSA and FITC-anti-cyclin D2 or FITC-anti-cyclin D3 antibodies (PharMingen, Becton Dickinson Company, USA). Cells were then washed again, resuspended in PBS and analyzed by flow cytometry using a FACScalibur flow cytometer. Negative controls were always used by omitting the monoclonal antibodies (MoAbs) as well as incubating the cells with mouse Ig of the same isotype as MoAbs conjugated with PerCP, PE or FITC (PharMingen and Becton Dickinson, Becton Dickinson Company, USA).

#### *Data analysis*

Data were analyzed by Cell Quest software. The results were expressed as the proportion of CD5<sup>+</sup>/CD19<sup>+</sup> cells expressing p27 protein, cyclin D2 or cyclin D3. Moreover, for p27<sup>Kip1</sup>, a mean intensity of fluorescence (MIF) was calculated for each sample as a semiquantitative measure of mean p27 cellular protein content and expressed in arbitrary units (AU). At least 10,000 events per sample were analyzed in triple staining analysis. To ensure the reproductibility of results, each staining and analy-

sis were performed in duplicate and a mean value was used for statistical analysis.

#### *Cell cycle analysis*

Cells were incubated for 30 min at 4°C with AB serum and monoclonal antibody anti-CD19-FITC (Becton Dickinson, Becton Dickinson Company, USA). The cells were then washed twice in PBS and suspended in 70% ethanol at -20°C for 2h or overnight. Ethanol-fixed cells were stained with propidium iodide (Sigma, Germany) after ribonuclease A III (Sigma, Germany) treatment. Because of the possible overlap between PE- and propidium iodide-derived fluorescence we could not perform triple anti-CD5, anti-CD19 and propidium iodide staining. Stained cells were analyzed on a FACScalibur flow cytometer. The percentages of CD19<sup>+</sup> cells in G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases of cell cycle were calculated using the Modfit 2.0 program.

#### *Cell viability and apoptosis*

The percentages of viable B-CLL cells and apoptosis were determined before and after 72-hour culture in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine 2 mM and gentamicin 0.16mg/mL at a final concentration of 1×10<sup>6</sup> cells/mL. Cell viability was determined by the trypan blue dye exclusion test. Apoptosis was assessed by immunocytochemistry using an *in situ* cell death detection kit (Boehringer Mannheim, Germany). Briefly, fresh cells were fixed with 4% formaldehyde solution (Fluka, Germany) for 15 min in room temperature before being spun down on slides. Slides were incubated with ethanol at -20°C for 5 min, then with 0.25% Triton X (Fluka, Germany) in TBS at room temperature for 5 min and TUNNEL reaction mixture was added to the sample according to the manufacturer's instruction (*in situ* cell death detection kit, Boehringer Mannheim, Germany). Readings were made with alkaline phosphatase substrate solution (NBT/BCIP ready-to-use tablets, Roche, Germany). The percentage of apoptotic cells was determined on 500 cells.

## **Results**

#### *Cell cycle analysis*

The proliferation level, estimated by DNA content of CD19<sup>+</sup> lymphocytes populations from both B-CLL patients and healthy subjects, was low. The percentage of cells in S+G<sub>2</sub>/M cell-cycle fraction ranged from 0.17 to 5.85 (mean: 1.68, SD 1.28) and from 0.86 to 4.31 (mean: 1.98, SD 1.14), respectively. The difference between normal and leukemic lymphocytes was not statistically significant (Mann-Whitney test).

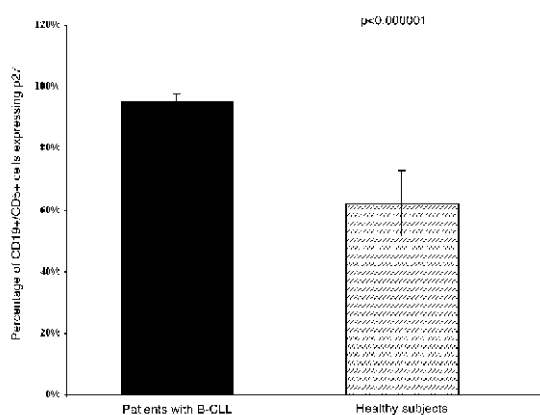


Figure 1. Mean percentage of p27<sup>Kip1</sup>-positive B-CLL cells and normal CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes.

#### p27<sup>Kip1</sup> expression in B-CLL and normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes

We found p27<sup>Kip1</sup> expression in almost all cells of each B-CLL cell population studied, since the signal was always detected in more than 90% of the cells. The signal intensity (MIF) was, however, very variable, ranging from 58 to 896 AU (mean: 262.4, SD 167.0). As expected, the percentage of CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes in healthy subjects was very low, ranging from 1.13 to 5.09 (mean: 2.73, SD 1.32)% of all mononuclear cells. In these cellular populations p27<sup>Kip1</sup> was detectable in a significantly lower percentage of cells (32-90, mean 62, SD 16.9,  $p < 0.000001$ , Mann-Whitney test) (Figure 1). In these cells p27-related MIF was also significantly lower, ranging from 29.3 to 125.2 AU (mean: 63.5; SD: 35.6). We did not find any correlation between MIF of the P27<sup>Kip1</sup>-related signal and the following clinical and laboratory data: Rai stage, hemoglobin level, leukocyte, lymphocyte and platelet counts, and cell cycle parameters. We found a negative correlation between p27<sup>Kip1</sup>-related MIF and patients' age (Pearson's correlation coefficient  $r: -0.45$ ,  $p = 0.001$ , Figure 2).

#### Cyclin D2 and D3 expression in B-CLL and normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes

Cyclin D2 expression was found in leukemic cells of 25 out of 31 patients and in 10 out of 15 normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocyte populations. Cyclin D3 was detected in all leukemic cells and in 11 out of 15 normal CD5<sup>+</sup>/CD19<sup>+</sup> populations studied. In contrast to P27<sup>Kip1</sup> found in all CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, both cyclins were detectable only in a subset of neoplastic cells. In cases expressing cyclin

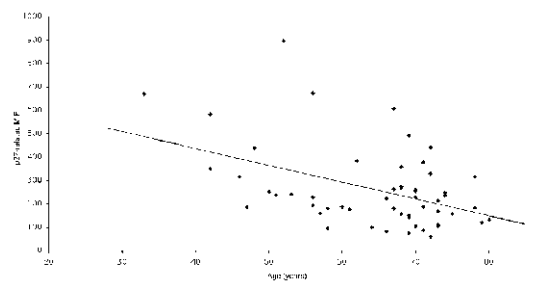


Figure 2. A negative correlation between p27<sup>Kip1</sup> content in B-CLL cells estimated by p27<sup>Kip1</sup>-related mean fluorescence intensity and patient's age.

D2, it was found in a proportion ranging from 27.5 to 87% (mean 51.2; SD 18.7) of all CD19<sup>+</sup>/CD5<sup>+</sup> cells. Cyclin D3 was detected in a subset of leukemic cells ranging from 20.3 to 98% (mean: 76.5, SD: 17.4) of all CD19<sup>+</sup>/CD5<sup>+</sup> cells. This proportion was significantly higher than in normal cyclin D2- and cyclin D3-expressing CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes populations, where cyclin D2 was present in 3-29% of cells (mean 15.9; SD 8.6;  $p = 0.0002$ , Mann-Whitney test), and cyclin D3 in 13-79% of cells (mean 36.9, SD 18.8,  $p = 0.000001$ , Mann-Whitney test). (Figure 3). The mean percentage of cyclin D3-positive CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes was significantly higher than cyclin D2-positive ones both in leukemic and in normal cell populations (sign test). Both cyclin D2- and cyclin D3-related MIF was comparable in leukemic and normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes. Neither cyclin D2 nor cyclin D3 was correlated with any clinical or hematologic feature or with cell cycle parameters.

#### Cell viability and apoptosis

After 72-hour culture, the rate of spontaneous cell death as assessed by the trypan blue dye exclusion test, ranged from 10 to 82% (mean 30.0 SD 17.0). At the same time point, apoptosis was detected in 19-97% cells (mean 51.1, SD 22.2). There was a significant correlation between the percentage of viable cells and apoptosis ratio ( $p = 0.0003$ , Spearman's test, Figure 4). Except for 3 cases, the apoptosis ratio was always higher than the percentage of cells estimated as dead in the trypan blue dye exclusion test. Neither cell viability nor apoptosis ratio was correlated with hematologic or clinical variables or expression of P27<sup>Kip1</sup> (Figure 5).

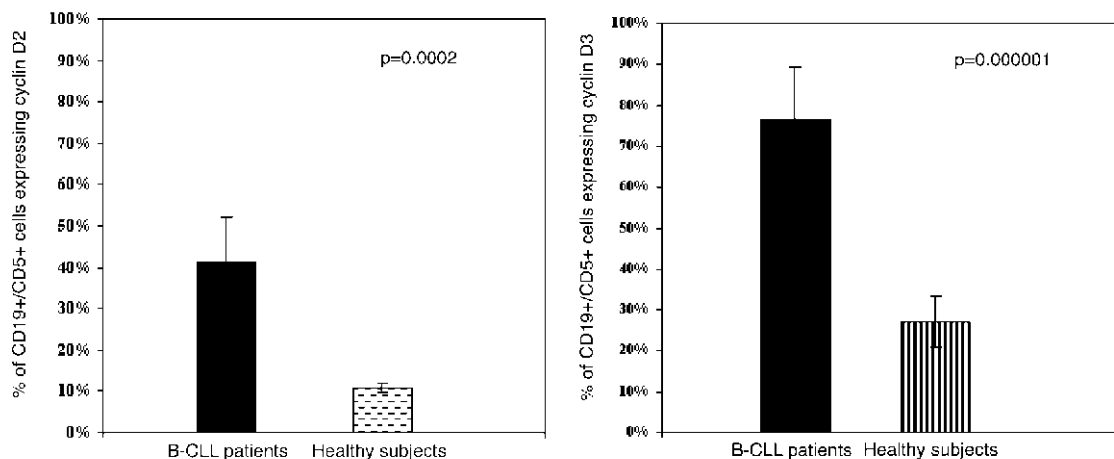


Figure 3. Mean percentage of cyclin D2- and cyclin D3-positive B-CLL cells and normal CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes.

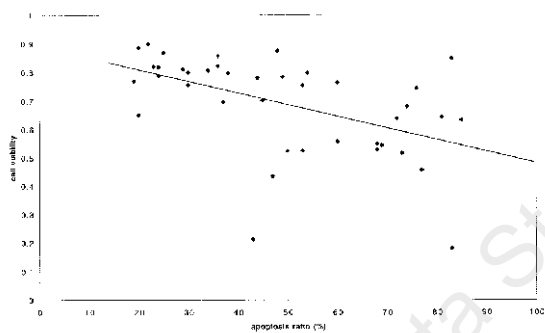


Figure 4. A negative correlation between cell viability determined by the trypan blue dye exclusion test and percentage of cells undergoing apoptosis assessed by immunocytochemistry (TUNNEL method) of B-CLL lymphocytes after 72-hour culture.

### Discussion

The accumulation of CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes in B-peripheral blood lymphocytes results from aberrant regulation of early stages of the cell cycle and inhibition of apoptosis. Both phenomena are regulated mainly by a family of pRb kinases and antiproliferative agents, particularly by p27<sup>Kip1</sup>, a universal inhibitor of cyclin-cdk complexes keeping them inactivated in quiescent cells.<sup>9</sup> In addition to its role as an inhibitor of cell proliferation, p27<sup>Kip1</sup> has been postulated to be involved in the regulation of apoptosis, although different effects were noted depending on the cells studied and experimental conditions used.<sup>20-24</sup> Decreased expression of p27<sup>Kip1</sup> protein due to its impaired stability is a

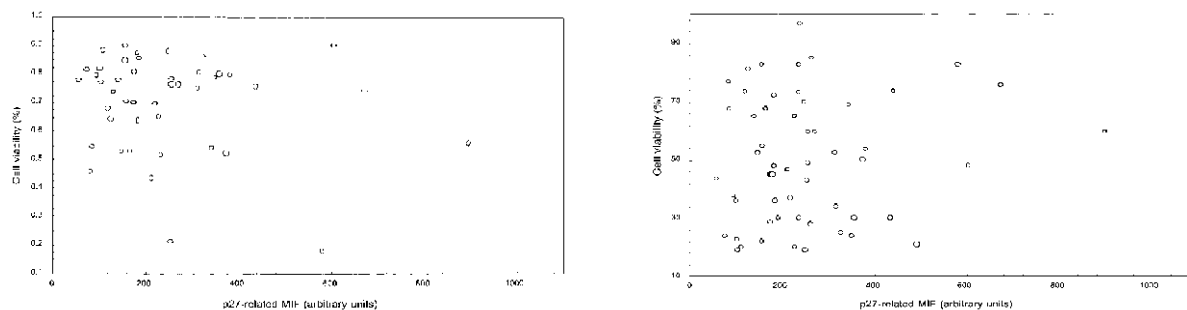


Figure 5. A: lack of correlation between p27<sup>Kip1</sup> expression and cell viability; B: apoptosis assessed by immunocytochemistry (TUNNEL method) (B) of B-CLL lymphocytes after 72-hour culture.

common feature of various cancers of different origin and bears a negative prognostic significance. Nevertheless, the biological significance of this reduction is not clear since it is not always followed by an increase of the proliferative activity of cancer cells.<sup>13</sup> B-CLL is particular in this regard in that the CD5<sup>+</sup>/CD19<sup>+</sup> CLL lymphocytes display high expression of p27<sup>Kip1</sup> mRNA and protein, and this high expression is associated with poor overall prognosis.<sup>14</sup> High p27<sup>Kip1</sup> expression in B-CLL lymphocytes seems to be associated with impairment of apoptosis, since B-CLL lymphocyte populations expressing a high level of p27<sup>Kip1</sup> have a lower spontaneous cell death ratio in culture,<sup>14</sup> and this expression is correlated with the level of anti-apoptotic protein Bcl-2.<sup>25</sup> As to the relationship between the p27<sup>Kip1</sup> expression of tumor cells and their proliferative activity, the numerous studies published so far have yielded conflicting results since, despite the well-established role of this protein as an antiproliferative agent, its downregulation is not always accompanied by an increase of cell cycle parameters. Such an inverse correlation between p27<sup>Kip1</sup> and proliferative index was found in non-Hodgkin's lymphomas<sup>26,27</sup> with the exception of mantle cell lymphomas and aggressive B-cell lymphomas, in which this relationship is abrogated.<sup>16,27</sup> A link between expression of p27<sup>Kip1</sup> and proliferative activity of B-CLL lymphocytes has not yet been established.

In our study we have confirmed previously found high although heterogeneous expression of p27<sup>Kip1</sup>. Moreover, using cytofluorometry we were able to make an original observation that this protein, whose expression is confined to a proportion of normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, is detectable in virtually all circulating *leukemic* CD5<sup>+</sup>/CD19<sup>+</sup> cells (almost all previously published studies used Western or Northern blot and, for this reason, could not determine it). B-CLL circulating lymphocytes also seem, as judged by the specific MIF, to express the p27 protein more intensively than their normal counterparts, i.e. CD5<sup>+</sup>/CD19<sup>+</sup> peripheral blood lymphocytes. The hyperexpression of p27<sup>Kip1</sup> protein seems then to be a constant feature of B-CLL peripheral blood lymphocytes. The interpretation of our finding must take into account a publication by Erlanson *et al.*<sup>26</sup> who determined, by immunohistochemistry, that this protein was present in only a subset (mean: 20%) of the cells in lymph nodes of CLL patients. Although the discrepancy between their results and ours could be, at least partially, ascribed to different sensitivities of the methods used, it seems plausible that only p27<sup>Kip1</sup>-positive

B-CLL lymphocytes are released to peripheral blood. As it was found that downregulation of p27<sup>Kip1</sup> can inhibit cell adhesion,<sup>28</sup> a relationship between the expression of this protein and cell-to-cell contact deserves to be re-evaluated with respect to B-CLL lymphocytes. We could not find a correlation between p27<sup>Kip1</sup>-related MIF and cell cycle parameters. It is possible that conventional kinetic studies are irrelevant in B-CLL since the great majority of circulating cells are in G<sub>0</sub> or G<sub>1</sub> phases which are indistinguishable from one another by flow cytometry determination of DNA content. Nevertheless, a link between cell kinetics of B-CLL lymphocytes and p27<sup>Kip1</sup> expression may actually exist, since an immunohistochemical study of lymph nodes of B-CLL patients showed that most cells in the so-called pseudofollicles containing proliferating cells are p27<sup>Kip1</sup> negative.<sup>26</sup>

The second aspect of our study of p27 expression in B-CLL lymphocytes concerned its association with cell death. In this regard there is a divergence between our results and those of a study by Vrhovac *et al.*<sup>14</sup> These latter authors found that cells expressing a high level of p27<sup>Kip1</sup>, as measured semiquantitatively by densitometry of Western blot signals, survived longer in culture in medium alone (without adding apoptosis-modulating agents) than cells with low p27<sup>Kip1</sup> expression. We did not find a correlation between p27<sup>Kip1</sup>-related MIF before culture in medium alone and cell viability or apoptosis assessed by TUNNEL reaction after culture. Nonetheless it must be noted that our experimental conditions were not identical to those of Vrhovac *et al.*, since we measured both cell death and apoptosis after 72 hours and not after 120 hours of culture. As can be seen in the graph in Vrhovac's paper, the difference of cell viability between high- and low p27<sup>Kip1</sup> expressors was less pronounced after 72 hours than 120 hours of culture. Moreover, we cannot exclude that this divergence is, at least partially, attributable to different distributions of clinical stages of disease in our patient population and that studied by Vrhovac. An interesting finding is that p27<sup>Kip1</sup> expression is inversely correlated with age. A similar observation has been made on a group of non-Hodgkin's lymphomas by Erlanson *et al.*<sup>26</sup> It is possible that the decrease of p27<sup>Kip1</sup> expression in elderly patients is related to some additional mechanisms downregulating the synthesis of this protein or facilitating its degradation.

The family of cyclins is one of the most important factors involved in the regulation of the cell cycle of both normal and malignant cells, but the

role of each member of this family is different. Cyclin D1 is known to play no role in the regulation of the proliferation of normal lymphoid cells and the majority of lymphoid malignancies. Indeed, its gene is silent both in normal lymphocytes and in almost all B-CLL cell populations studied so far. As the scope of our study was restricted to some proteins participating in the cell cycle regulation of B-CLL cells, we did not examine its expression. As far as concerns cyclins D2 and D3, their expression in B-CLL lymphocytes and the biological significance of this expression are matter of controversy. It has been well established that these two members of the cyclin D family play distinct and non-redundant roles in the regulation of proliferation of normal lymphocytes. After recruitment of normal quiescent T-lymphocytes to the cell cycle the gene for cyclin D2 is activated several hours earlier than that for cyclin D3, indicating their roles on distinct stages of early G<sub>1</sub> phase.<sup>7,15</sup> Moreover, several lines of evidence indicate that cyclin D2, in contrast to cyclin D3, may play a role in a carcinogenesis, as its amplification has been found in several malignancies (colorectal cancer, B-cell lymphoma, B-CLL).<sup>29-31</sup> Discordant results were obtained concerning the frequency of expression of their genes in B-CLL. Delmer *et al.*<sup>10</sup> showed that cyclin D2 gene (CCND2) was overexpressed in 29 out of 34 B-CLL samples, but cyclin D3 gene (CCND3) transcript was never detected. Similar results were obtained by Werner *et al.*<sup>31</sup> In contrast, Suzuki *et al.* detected CCND2 mRNA in 5 out of 11 B-CLL populations tested, and CCND3 mRNA in 10 out of 11 samples.<sup>15</sup> However, to our knowledge, protein expression of both cyclins at the level of individual cells has not yet been studied on a larger cohort of B-CLL patients. We, therefore, determined the expression of cyclin D2 and D3 on the protein level and found that both were detectable in a majority (25 out of 31 for cyclin D2) or all (for cyclin D3) B-CLL cases. In contrast to p27<sup>Kip1</sup> found in almost all CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, both cyclins were detectable only in a proportion of the neoplastic cell population: 27.5 to 87% (mean 51.2) for cyclin D2 and 20.3 to 98% (mean 76.5) for cyclin D3. Our results indicate that the presence of both cyclins is a common feature of B-CLL lymphocytes, but that their expression is confined to a proportion of leukemic cells. The percentage of B-CLL cells positive for both cyclins was significantly higher than the corresponding percentage of normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes. Previous studies showed that cyclin D2 mRNA or protein is detectable in normal B-lymphocytes at a low level.<sup>10,15,32,33,34</sup> As to cyclin D3 expression in

normal B-cells, published studies yielded discordant results. Delmer *et al.*<sup>10</sup> did not find cyclin D3 mRNA in 3 samples of normal resting B-lymphocytes, but recently Suzuki *et al.*<sup>15</sup> detected cyclin D3 transcript in B-cells and at a higher level than in T-cells. Cyclin D3 protein was also clearly, albeit at a low level, detectable in human tonsillar B-cells. Moreover, cyclin D3- but not cyclin D2-containing immune complexes elicited a strong and sustained kinase activity against pRb substrate in interleukin (IL)4-stimulated B-cells. A rise of cyclin D3 level was also observed after stimulation of B-cells with IL2 and IL10.<sup>33</sup> Those observations gave rise to the hypothesis that cyclin D3, rather than D2, plays a crucial role in the regulation of human B-cell proliferation. Our results indicate that this may be also true for CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes, since in normal CD5<sup>+</sup>/CD19<sup>+</sup> cell populations expressing both cyclins, cyclin D3 was detectable in a significantly higher proportion of cells than cyclin D2 (mean value 15.9 and 36.9%, respectively).

In this context our finding concerning differences between the percentages of cells expressing cyclins D2 and D3 in normal versus leukemic lymphocytes may reflect different usage of both cyclins in normal CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes and leukemic CD5<sup>+</sup>/CD19<sup>+</sup> cells and point to an important role of cyclin D2, and especially D3, in the regulation of the proliferation of B-CLL cells. We do not believe that it results from a true hyperexpression of these cyclins in the sense of increased cellular protein content, since cyclin D2- and D3-related MIF was not significantly different from that in control CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes. It seems possible that previously reported hyperexpression of cyclin D2 in B-CLL versus normal B-cells, estimated by the intensity of Northern blot signal,<sup>15</sup> reflected a higher percentage of positive cells rather than higher cellular cyclin D2 mRNA content. Discordance between our results indicating the presence of both cyclins in the majority of B-CLL cases tested and above-mentioned observations showing the lack of corresponding transcripts in a substantial number of B-CLL lymphocyte populations may result from the insufficient sensitivity of methods of detection of a low quantity of mRNA present in a small proportion of lymphocytes. Higher percentages of leukemic cells expressing both cyclins as compared to their normal counterparts might, thus, be interpreted as the exit of a number of cells from quiescence, in line with information in previous publications reporting the expression in B-PBL cells of some proteins normally present only in cycling cells.<sup>11,12</sup>

In summary we found that three pivotal G<sub>0</sub>/G<sub>1</sub> phase regulatory proteins: cyclin D2, cyclin D3 and p27<sup>Kip1</sup>, are expressed in all (p27<sup>Kip1</sup> and cyclin D3) or a majority (cyclin D2) of B-CLL cases examined. Our finding that the cyclin D2- and D3-positive fraction of leukemic CD5<sup>+</sup>/CD19<sup>+</sup> lymphocytes is larger than the corresponding fraction of normal CD5<sup>+</sup>/CD19<sup>+</sup> ones may provide support to the hypothesis of a substantial number of B-CLL lymphocytes exiting from quiescence and being recruited into the cell cycle.

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*DW: leading position in the study, conception and design of the study, analysis and interpretation of the data, writing the submitted version of the paper. LC: conception and design of the study, analysis and interpretation of the data, contribution to the writing of the submitted version of the paper. DB, AK, IF, KK contributed to the design of the study, interpretation and analysis of the data, and they critically read and approved the submitted manuscript.*

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#### Potential implications for clinical practice

Our findings do not have a direct impact on clinical practice, but further studies on larger cohort of patients and longer follow-up may contribute to define a possible prognostic value of the expression of the proteins studied in B-CLL.

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