



The relevance of multidrug resistance-associated P-glycoprotein expression in the treatment response of B-cell chronic lymphocytic leukemia

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ABSTRACT

Background and Objectives. The aim of this study was to determine whether expression of P-glycoprotein (Pgp) is an intrinsic feature of B-lymphocytes in B-cell chronic lymphocytic leukemia (B-CLL) and how it correlates with hematologic indices and tumor load in the disease. Furthermore, the change of Pgp expression under cytotoxic treatment and its correlation to treatment outcome were studied.

Design and Methods. In 42 B-CLL patients, of whom 13 were sequentially monitored, expression of extracellular (MRK-16) and intracellular (C-219) Pgp epitopes on peripheral blood lymphocytes was determined by flow cytometry analysis and quantified by ratio of the mean fluorescence (RMF) in flow cytometry analysis.

Results. Median RMF values in B-CLL patients were higher than in age-matched controls. Pgp expression did not correlate with any of the hematologic features or clinical stage of the disease. Patients who received some type of cytoreductive treatment prior to the study had lower Pgp values for both measured epitopes (median RMF for C-219 and MRK-16 of 1.99 and 2.03 in comparison to those of non-treated patients: 3.11 and 2.88, respectively). In 13 patients monitored during treatment the decrease in RMF was noted after treatment with chlorambucil, with RMF values for both Pgp epitopes decreasing in responders. This was in contrast to unchanged or even increased RMF values in those patients who did not respond to therapy.

Interpretation and Conclusions. Our study confirms the importance of quantitative evaluation of Pgp expression by flow cytometry. At the clinical level, cross-sectional, single test evaluation of Pgp is of limited value whereas sequential follow-up values correlate with treatment response.

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Key words: multidrug resistance, Pgp, B-CLL

Chronic lymphocytic leukemia (CLL) usually presents as a slowly progressive lymphoproliferative disease. However, aggressive forms, especially in younger patients, can pose a serious therapeutic problem. Treatment strategies include alkylating agents such as chlorambucil (Clb), corticosteroids, anthracyclines, vinca alkaloids and the more recently introduced purine analogs fludarabine monophosphate (FAMP) and 2-chloro-deoxy-adenosine phosphosphate (2-CDA). However, regardless of the type of initial treatment and usually favorable therapeutic response, regrowth of tumor occurs. Selection of alternative drugs or reinstitution of the original induction treatment is then justified.¹⁻³ Eventually, however, the disease, complicated by progressive marrow failure, immunosuppression with recurring infections, and increasing resistance to chemotherapy, is fatal. In this context, the question of multidrug resistance (MDR) in B-CLL has attracted considerable attention in recent years.^{4,5} One of the best known MDR-mechanisms is linked to the overexpression of membrane P-glycoprotein (Pgp),^{6,7} which plays a physiologic role as a pump catalyzing the rapid efflux of cytotoxic drugs from the cell and may act as an anti-apoptotic molecule by reducing chemotherapy-induced apoptosis.^{8,9} Development of monoclonal antibodies (mAbs) against Pgp has increased the specificity of detection of this protein.^{10,11} Some of these mAbs recognize a well-conserved cytoplasmic region of Pgp,¹²⁻¹⁴ whereas others react with epitopes exposed on the cell surface.^{15,16} As the normal cell counterpart of the B-CLL lymphocyte also expresses Pgp molecules at a low level,¹⁷ it has become evident that when the cell Pgp phenotype is studied, not only the percentage of the positive cell population but also the level of intensity of its expression should be taken into account.

The aim of this study was to investigate whether Pgp, measured in a cohort of B-CLL

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Table 1. Clinical characteristics of the patients studied.

| N | B-CLL | | Treatment | | | | Clinical parameters (before and after treatment) | | | | | | | |
|----|---------|------------------|-----------|---------|------------------|----|--|---|------|------|-------|------|--------|------|
| | Age/sex | dur ¹ | prev | current | dur ² | R | mod RAI | | TTM | | C-219 | | MRK-16 | |
| 1 | 62/ M | 0 | 0 | Clb | 8 | R | 2 | 2 | 12 | 7.5 | 2.0 | 0.95 | 1.97 | 1.05 |
| 2 | 70/ M | 0 | 0 | Clb | 8 | R | 3 | 3 | 26 | 2 | 2.58 | 1.00 | 4.11 | 2.54 |
| 3 | 59/ F | 0 | 0 | Clb | 8 | R | 3 | 1 | 13 | 4.7 | 10.0 | ND | 3.91 | 1.40 |
| 4 | 60/ F | 0 | 0 | Clb | 4 | R | 3 | 2 | 30.8 | 7 | 9.95 | 6.57 | 2.88 | 1.35 |
| 5 | 60/ M | 0 | 0 | Clb | 4 | NR | 3 | 3 | 13.1 | 12.9 | 5.1 | 9.4 | 3.5 | 10.3 |
| 6 | 56/ F | 0 | 0 | Clb | 3 | R | 3 | 1 | 29.4 | 2.2 | 3.58 | 6.7 | 2.83 | 0.85 |
| 7 | 79/ M | 40 | 0 | FAMP | 8 | R | 3 | 3 | 11.3 | 9 | 11.5 | 2.43 | 2.61 | 1.11 |
| 8 | 64/ M | 3 | 0 | FAMP | 4 | NR | 3 | 3 | 8 | 12 | 3.1 | 3.7 | 2.1 | 1.0 |
| 9 | 65/ F | 20 | 0 | FAMP | 3 | R | 2 | 2 | 11 | 2 | 12.2 | 5.57 | 3.11 | 0.96 |
| 10 | 50/ F | 46 | Clb | Clb | 2 | NR | 3 | 3 | 19 | 11 | 2.3 | 2.4 | 4.1 | 4.1 |
| 11 | 75/ F | 126 | Clb | Clb | 8 | R | 1 | 1 | 10 | 1.4 | 1.6 | 5.5 | 1.4 | 1.4 |
| 12 | 73/ F | 23 | Clb | CHOP | 5 | NR | 3 | 3 | 3 | 9.1 | 2.5 | 2.9 | 2.6 | 2.6 |
| 13 | 48/ M | 75 | FAMP | CHOP | 4 | NR | 3 | 3 | 17 | 15 | 1.61 | 3.3 | 1.3 | 1.3 |

M/F= male/female, dur¹ = duration of B-CLL from diagnosis (months), prev = previous treatment, dur² = duration of treatment (months), Clb = chlorambucil, FAMP = fludarabine monophosphate, CHOP = cyclophosphamide + doxorubicin + vincristine + prednisone, R = response to treatment, NR= no response, mod RAI = modified Rai staging, TTM = total tumor mass score.

patients, correlated with any hematologic or clinical aspects of the disease. Pgp expression was also evaluated in the respective cells of healthy controls. Furthermore, a small group of patients was followed sequentially with the idea of getting insight into intra-individual behavior of Pgp mechanisms under the influence of therapy.

Design and Methods

Study population

Forty-two B-CLL patients (25 males and 17 females, median age 60 years) referred to the Departments of Hematology, Merkur Clinical Hospital and Sv. Duh General Hospital, were included in the study. The diagnosis of CLL was made according to standard morphologic and immunophenotypic criteria.¹⁸ Peripheral blood (PB) samples were obtained from the patients after informed consent.

Control samples were collected from 36 healthy volunteers (15 males and 21 females), of whom 25 (12 males and 13 females) were age-matched to the patients to serve as comparison to the CLL cases.

Clinical staging and hematologic laboratory parameters

Two CLL clinical staging systems were used to classify the patients: the modified Rai (mod RAI) staging system for low (RAI 0), intermediate (RAI I and II) and high risk (RAI III and IV) disease, and total tumor mass score (TTM) quantitatively measuring tumor load.^{19,20} Blood counts including hemoglobin, lactic dehydrogenase (LDH) levels in serum and serum immunoglobulins were correlated to Pgp data.

Chemotherapy

At entry to the study, 31 patients were untreated and 11 patients had been treated and evaluated upon completion of intensive lymphoreductive therapy or in the low-intensity maintenance therapy phase. In the second part of the study, treatment for high tumor load was given to 11 patients: these patients were analyzed prior to and after therapy. Two more patients (#12 and 13 in Table 1) were evaluated after receiving alternative treatment regimens for resistant disease. Baseline level of Pgp and other study parameters, including standard hematologic data and staging, were measured and then remeasured upon drug discontinuation. Therapy protocols have been described in detail elsewhere.^{20,21}

Evaluation of MDR status

Monoclonal antibodies

EDTA-whole fresh PB samples were labeled by an indirect method using murine primary MRK-16 and C-219 IgG2a mAbs and secondary goat anti-mouse FITC-conjugated immunoglobulin (GAM-FITC, IgG2a). MRK-16 mAb (Syrinx Diagnostica GmbH, Germany) reacts with extracellular epitopes of the Pgp molecule.¹⁵ C-219 mAb (P-glycoCHEK, Centocor Diagnostic, Malvern, USA) is directed to internal Pgp epitopes,¹³ so it is necessary to modify the labeling procedure by including membrane permeabilization. The samples were also stained for B-cell associated antigen CD19 and T-cell associated antigen CD3 (Becton Dickinson GmbH, Heidelberg, Germany).

Pgp determination

C-219 mAb. Cell membrane permeabilization was achieved by a commercially available lysing

solution (Becton Dickinson) in the dark.²² Peripheral blood 50 μ L aliquots were incubated for 10 min in 2 mL of diluted (1:10) lysing solution, washed three times in phosphate-buffered saline (PBS) containing 1% BSA, incubated in 10% human AB serum (15 min at 37°C), immunostained with C-219 (120 min at 4°C), washed twice in PBS, and treated with GAM-FITC for 30 min. Control PB samples were incubated with 10 μ L of FITC P-glycoCHEK negative antibody (Centacor Diagnostic).

MRK-16 mAb. Immunostaining of 100 μ L PB was performed at room temperature with 10 μ g/mL of MRK-16 for 60 min according to the Memphis Workshop recommendation.²³ After labeling, the cells were washed three times in PBS with 1% BSA and stained with 50 μ L GAM-FITC for 30 min at 4°C. Negative controls were produced by incubating cells with normal mouse serum instead of MRK-16 mAb. To check the Pgp expression on lymphocyte subpopulations, 100 μ L blood aliquots were incubated first with MRK-16 for 60 min, treated with GAM-FITC for 30 min, and then with CD3-PE and CD19 Per-CP for 60 min.

Labeled samples were assayed on a flow cytometer (FACScalibur, Becton Dickinson). For each sample, 20,000 events were analyzed using the FACScalibur CellQUEST software. Data were analyzed on linear scale dot plot histogram regions (FSC, 50-250 and SSC 10-50 height) from gates set around small lymphocytes and prolymphocytes. The unfixed cells incubated with C-219 or MRK-16 mAb were compared to that region of the cells incubated with the isotypic control, i.e. the percentage of positive cells and mean green fluorescence (MF) intensity of the lymphocytes with anti-Pgp mAbs were compared to those of lymphocytes with isotype control (Figure 1). Furthermore, to check Pgp expression on healthy PB subpopulations, MRK-16 was analyzed on gated CD3⁺ and CD19⁺ lymphocytes.

A crucial point in the analysis of flow cytometric data for Pgp expression of leukemic samples is definition of positivity. Even using the same detection method it is difficult to compare Pgp expression in cell lines and clinical samples from different studies because of different thresholds for positivity of different mAbs. Laboratories of the *French Drug Resistance Network*²⁴ established a reference method of immunofluorescent detection of Pgp. According to those recommendations we expressed the Pgp data as the ratio of mean fluorescence (RMF) of total gated cells positive for anti-Pgp mAb and isotypic control. An RMF ≥ 1.5 is considered positive.²⁵ Moreover, in order to overcome possible insensitivities of RMF in measuring Pgp positivity, the

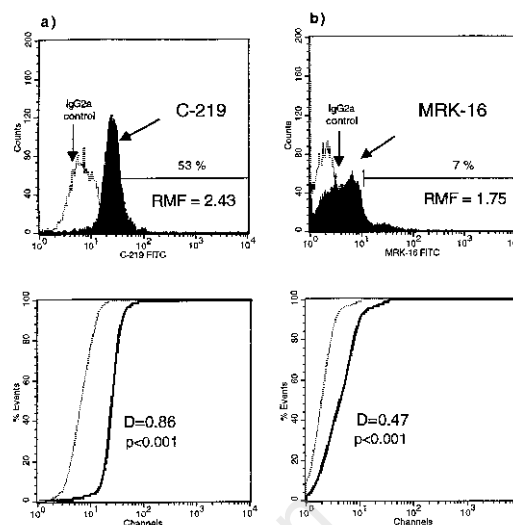


Figure 1. Representative histograms of peripheral blood of one of the 42 B-CLL patients studied. Before the flow cytometry analysis the cells were stained with mAbs C-219 (a) and MRK-16 (b) directed against different Pgp epitopes, as described in the Methods section. The results of the analyses are expressed as fluorescence intensities of anti-Pgp (solid histograms) and control Abs (dotted lines). In the first row Pgp expression is depicted as a percentage of Pgp-positive cells and ratio of mean fluorescence (RMF) and in the second row using Kolmogorov-Smirnov fluorescence distribution difference (D) analysis.

Kolmogorov-Smirnov two-sample test was also applied. This test calculates the difference (D) between fluorescence intensity distributions of Pgp⁺ and Pgp⁻ samples and tests its significance.²⁶

Statistics

Results obtained in B-CLL patients and control subjects were compared by the non-parametric Mann-Whitney U test ($p < 0.05$ was considered statistically significant). A computer statistical package (STATISTICA™ v 5.0, StatSoft, Inc, Tulsa, OK, USA) was used for the statistical analyses.

Results

Evaluation of Pgp expression

We evaluated Pgp expression on peripheral blood lymphocytes of 36 healthy volunteers. The percentage of C-219⁺ and MRK-16⁺ cells within the lymphocyte gate varied greatly. (2-86% for C-219⁺, and 5-49% for MRK-16⁺). As shown in Figure 2, the percentage of MRK-16⁺ lymphocytes increased significantly with age ($p < 0.001$, $r = 0.56$). Based on this information we set our Pgp reference values in 25 healthy individuals matching our patient population by age (between 37 and 71 years).

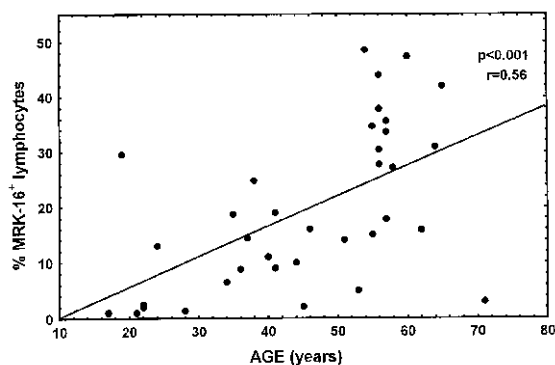


Figure 2. Correlation between the percentage of MRK-16⁺ peripheral blood lymphocytes and age in a group of 36 healthy volunteers.

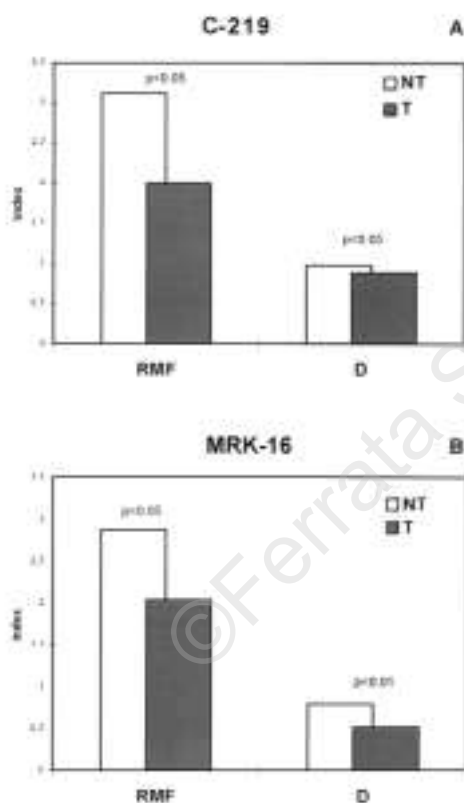


Figure 3. Median RMF values and D indices for C-219 (A) and MRK-16 (B) stained Pgp epitopes in 31 non-treated (NT) and 11 treated (T) CLL patients.

To investigate the distribution of Pgp among the circulating T- and B-lymphocyte subpopulations in normal PB, a 3-color labeling procedure was performed and the percentage of MRK-16⁺ was analyzed on gated CD3⁺ and CD19⁺ lym-

phocytes. In normal PB Pgp expression was higher among B-cells than among T-cells (median values 35 and 10, respectively) and Pgp subpopulations among T- and B-lymphocytes were almost equal (data not shown).

In B-CLL, since the baseline expression for both Pgp epitopes was relatively high and variable, the determination of the percentage of Pgp⁺ cells *per se* was an unreliable parameter. Therefore, the ratio of mean fluorescence (RMF) and Kolmogorov-Smirnov D values were introduced. An RMF above 1.5 and D values above 0.5 are considered positive. Although the RMF values for both epitopes varied greatly (0.3-13.5 for C-219, and 0.9-15.0 for MRK-16), Pgp was still significantly overexpressed in the PB lymphocytes of the majority of B-CLL patients ($p < 0.05$ and $p < 0.01$); the median RMF was 2.6 for C-219 and 2.8 for MRK-16. No correlation was found between Pgp values and basic hematologic laboratory data, LDH, serum immunoglobulin and TTM or Rai scores.

The effect of chemotherapy on Pgp expression in B-CLL

At the onset of the study, 31 B-CLL patients were untreated and 11 received therapy. As shown in Figure 3, initial chemotherapy with Clb, a Pgp-independent drug, decreased Pgp expression. In addition, in the treated patients RMF values for both Pgp epitopes were significantly lower (C-219=1.9, $p=0.05$, and MRK-16=2.0, $p=0.01$) than in untreated patients (3.1 and 2.8, respectively). The fluorescence distribution differences (D) obtained by the Kolmogorov-Smirnov test were also decreased.

In a separate group of nine previously untreated CLL patients requiring chemotherapy, Pgp values were measured before and after stopping the cytoreductive therapy with Clb or FAMP. In four other patients, previously treated with Pgp-independent drugs, active disease required reinstitution of therapy and these patients were followed through their treatment (see Table 1). Details on the design of chemotherapy regimens, drug dosages and response evaluation criteria have been reported previously.^{13,14} Eleven CLL patients responded to therapy with Clb or FAMP. Two patients who did not respond to Pgp-independent drugs, received the Pgp-dependent CHOP combined regimen. Eight out of the eleven patients treated with Clb responded favorably. Those responding favorably had significantly decreased RMF values for both Pgp epitopes. However, five out of thirteen patients showed a poor response or did not respond, their Pgp values remaining unchanged or even increased (non-responders). Three of them eventually died of progressive disease (Figure 4).

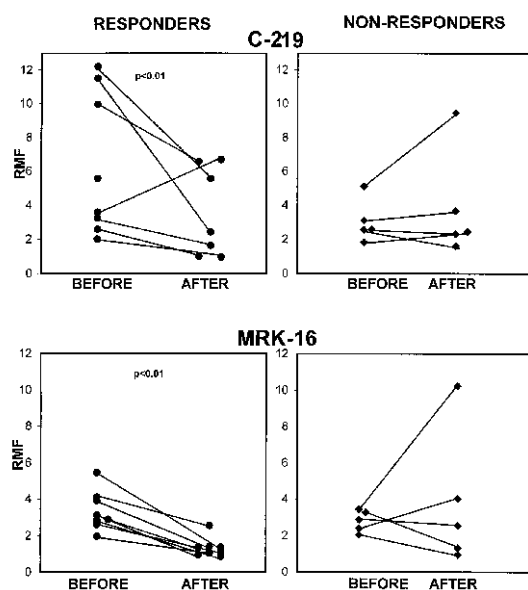


Figure 4. RMF values for C-219 and MRK-16 Pgp epitopes in responders and non-responders before and after various chemotherapy regimens (see Table 1).

Discussion

The question of multidrug resistance in CLL has been addressed in a number of studies of various biological factors including Pgp and multidrug resistance protein (MRP) at protein and genomic levels.²⁷⁻²⁹ Somewhat conflicting results on the relationship of the level of expression of MDR marker and previous treatment with Pgp-transportable drugs left the question of its clinical importance unresolved. Only recently, by taking another approach of combining the percentage of positive cells with the degree of intensity of positive reaction, was it suggested that Pgp and MRP are involved in drug resistance but only in patients treated with Pgp-transportable drugs. In an attempt to contribute to this still controversial topic we insisted on defining the appropriate method for the measurement of Pgp molecules that would allow a relevant interpretation of experimental results.

Many factors appear to influence the low and heterogeneous detection of Pgp on leukemic lymphocytes, such as reagents with variable specificity (different epitopes and avidity) and differences in sample preparation and analysis. As referred by Thiebaut and Tsuruo, even with extracellular epitopes, the fluorescence intensity may be too weak to be detected.³⁰ In accordance with the recommendations of the *Multidrug Resistance Detection Methods Workshop*^{23,31} to use two or more

vendor-standardized anti-Pgp mAbs, we selected mAbs reacting with extracellular (MRK-16) and intracellular (C-219) epitopes. The latter mAb demonstrated higher expression, probably related to the staining procedure (longer incubation time and membrane permeabilization) or its cross-reactivity.¹⁴ Another problem is definition of positivity. We followed the recommendations of the *French Drug Resistance Network*²⁵ and express Pgp data as the ratio of mean fluorescence (RMF) of total gated cells positive to anti-Pgp mAb and isotopic control and the Kolmogorov-Smirnov D value.

Methodologically precise definition of Pgp-positive leukemic B-cells is additionally complicated by the fact that normal lymphocytes present in tumor samples also express Pgp. Furthermore, when we looked at the expression of Pgp on peripheral blood cells of healthy volunteers, a significant ($p < 0.001$) age-dependent increase in the percentage of Pgp⁺ lymphocytes was found. The age-related increase of Pgp percentages was also found by Gupta.^{32,33} However, in spite of drawbacks in defining lymphocyte subpopulations in tumor samples, our CLL patients, when compared with age-matched controls, still had significantly ($p < 0.05$) higher RMF values.

Sparow *et al.*³⁴ found a small subpopulation of cells expressing Pgp among normal CD19⁺ CD5⁺ B-cells suggesting that these cells might be potential precursors of leukemic cells. In addition, by measuring ¹²³rhodamine efflux, Ludescher *et al.*³⁵ reached a similar conclusion and suggested that CLL might be a malignancy arising from normal B-cells expressing the MDR1 gene. The untreated CLL patients expressed higher levels of both Pgp epitopes than treated ones, suggesting that Pgp is over-expressed in the majority of, if not all, B-CLL patients. Thus Pgp over-expression is probably the inherent characteristic of the leukemic cells, regardless of the stage and duration of the disease.³⁶ This finding was further analyzed in a subsequent follow-up study of 13 patients monitored before and after receiving cytotoxic drugs. The group was clinically heterogeneous with *de novo* and previously treated cases, but this reflects a real-life situation which, we believe, is important. Chemotherapy reduced the level of Pgp in the majority of the patients and the reduction correlated with a favorable clinical response.

There is one report on serial analysis of MDR in B-CLL patients in which ¹²³rhodamine efflux was measured at a 7-10 months' interval in 12 patients.³⁵ Four patients in that study were treated during the observation period. The percentage of cells with rhodamine efflux (transported by the Pgp pump) was significantly lower in untreated patients than in those treated with at

least one MDR-associated drug. In another study³⁷ very low or undetectable Pgp expression was found in 24 untreated B-CLL patients, but, interestingly, expression of Pgp on granulocytes was significantly higher. Similar results on the influence of chemotherapy were reported by Wulf *et al.* in 40 B-CLL patients.³⁸ Using C-219 mAb they found elevated Pgp expression in patients who had received previous therapy regimens containing MDR-related drugs. Recently, Maynadie *et al.*³⁹ found no significant difference in the number of Pgp molecules with respect to treatment response, but a higher number was found in non-responders. This observation might reflect induction of Pgp expression and/or selection of MDR-expressing cells during the prolonged chemotherapy. In another study in 51 CLL patients, Pgp expression was evaluated using C-494 and JSB-1 mAbs. There was no correlation between high or low Pgp levels and Rai stage of disease, nor with *ex vivo* drug sensitivity or patient survival.⁴⁰ The same results were obtained by Michieli *et al.*²⁹ They investigated 63 B-CLL patients using the same combination of mAbs as we did (C-219 and MRK-16) and found no significant difference in the proportion of positive cells related to prior treatment, time from diagnosis, absolute lymphocyte count and clinical stage.

In conclusion, we report several observations related to the biological significance of Pgp-mediated multidrug resistance in CLL. Technically, the study further confirms the importance of quantitative methods of measuring Pgp molecules by flow cytometry. Our experience is that the use of an extracellular epitope-detecting antibody e.g. MRK-16 is preferable and that its reactivity should be expressed by the RMF index. Moreover, because overexpression of Pgp may affect chemotherapy differently, serial analyses of Pgp are clinically important in CLL. Although overexpressed Pgp can be downregulated by Pgp-independent drugs in initial treatment in a number of CLL patients and this is associated with a favorable clinical response, in other patients, despite administration of Pgp-independent or Pgp-dependent drugs, Pgp overexpression remains unchanged or even increases with a frequently fatal outcome. Thus, successful treatment reduces neoplastic B-cells, but relevant outcome of therapy correlates with P-glycoprotein expression.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript Processing

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Potential implications for clinical practice

- ◆ sequential quantitative evaluation of MDR might be useful in monitoring treatment efficacy in B-CLL patients.

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