Prognostic relevance of the FAB morphological criteria in chronic lymphocytic leukemia: correlations with IgV_H gene mutational status and other prognostic markers*

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Morphological examination is the routine first step in the diagnosis of hematological malignancies, including chronic lymphocytic leukemia (CLL). Atypical cell morphology according to the FAB criteria is known to herald disease progression. Several years ago, it was proposed that FAB morphology at diagnosis had a considerable prognostic impact. However, this proposal has not been widely adopted in practice. Thus we questioned the prognostic value of the morphological examination, which was performed retrospectively in 88 patients out of our 110 institutional registry patients (70 males and 40 females, median age 57 yrs) with CLL at diagnosis. We related the results to the more modern prognostic markers.

Atypical FAB morphology was shown to correlate with IgV_H gene mutation status, trisomy of chromosome 12 and deletion of 17p detected either by conventional G-banding or by fluorescence *in situ* hybridization (FISH) analysis. The correlation of FAB morphology with CD38 antigen expression or with the histopathological pattern of bone marrow infiltration was not significant. Overall survival (OS) data were available for 84 morphologically examined patients. The patients with atypical morphology (64 patients) had a significantly shorter OS (103 months) than the 20 patients presenting with typical CLL morphology (237 months; p=0.03). Only the mutation status of IgV_H genes correlated more closely with OS (p=0.002). Of note, there was no leukemia-related death within "unmutated" cases who had typical FAB morphology (p=0.14), and *vice versa*, the mutation status had a significant prognostic impact within the morphologically atypical cases (p=0.01). Thus FAB morphology and the mutation status may yield complementary prognostic information. OS was affected both by the presence of cytogenetic aberrations (p=0.03) – most adversely by deletions of 17p and 11q, and by CD38 expression (p=0.003).

We conclude that careful examination of peripheral blood smears according to FAB is a simple, cheap and valuable tool in the first-line assessment of prognosis of CLL patients and should not be overlooked even in 3rd millenium when more sophisticated prognostic markers are at hand. This ought to be confirmed in larger prospective studies with multivariate analysis of data.

Key words: chronic lymphocytic leukemia, prognostic markers, morphology, FAB classification, IgV_H gene mutation status, cytogenetics

Management of chronic lymphocytic leukemia (CLL) currently undergoes revolutionary changes. Efficient therapies are developed and the therapeutic goal gradually shifts from mere palliation to cure, at least in a subset of younger patients with adverse prognosis [1]. The clinical behavior of CLL varies greatly, from an indolent disease to an aggressive one: overall survival (OS) may be unaffected in some patients, or it may be less than 3 years in others. An array of prognostic markers is available for risk stratification of patients in order to select candidates for aggressive treatment modalities,

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which are not devoid of serious side effects. Among the prognostic markers, the mutational status of IgV_H genes (or its "surrogate" – expression of the ZAP-70 antigen) and cytogenetic aberrations such as deletions of 17p and 11q have the greatest impact. In addition, many other factors may predict prognosis, e.g. bone marrow histopathology, the lymphocyte doubling time, the extent of lymphocytosis, various biochemical markers – see the recent reviews [1, 2].

Morphological examination of peripheral blood cells is still the first-line contact of the physician with the patient's disease. The criteria elaborated in 1989 by the French-American-British (FAB) group [3] are still of value for diagnosing CLL; they are based both on precise morphological evaluation and on immunophenotypic analysis. These principles were largely adopted by the diagnostic criteria of the International Workshop on CLL (IWCLL), and later by the National Cancer Institute Working Group (NCI-WG) and more recently by the WHO classifications [4–6]. The FAB criteria [3] recognize 2 major categories of CLL: 1. the typical one with quite monotonously looking mature-like CLL cells, 2. atypical CLL, with varying percentages of larger cells ("mixed cells"). One subclass of atypical CLL, CLL/PL, has a "dimorphic" appearance with 2 subsets of cells – the larger prolymphocytes with nucleoli and the smaller typical cells. The other subclass comprises cells of variable size, from the small ones to larger pleomorphic prolymphocytoid cells lacking nucleoli. CATOVSKY has later called this subtype "pleomorphic" [7]. The original article of the FAB group [3] has noted that atypical morphology may be connected with disease progression. Other groups have shown that cell morphology may be relevant to prognosis [8] or may correlate with other prognostic markers, such as trisomy 12 [7–10]. However, the Belgic morphologically based concept of CLL as two (typical or atypical) closely related entities with divergent prognostic characteristics has never been widely adopted into practice [10]. Currently, the possible prognostic relevance of cell morphology is shadowed by other markers, such as cytogenetic aberrations, the mutational status of IgV_H genes, expression of CD38 or ZAP-70 antigens, or potentially HLA-G expression [1, 2, 11–16].

We attempted to check how far relevant is insisting on precise morphological examination of peripheral blood slides in the beginning of the 3rd millenium with the numerous prognostic markers at hand. Here we report the results of our retrospective analysis of several prognostic markers in 88 of our CLL patients. To our surprise, even cell morphology could efficiently predict their prognosis.

Patients and methods

Patients. As of June 30, 2005, altogether 110 CLL patients currently or recently followed-up at the Institute of Hematology and Blood Transfusion, Prague, with diagnosis of CLL according to the NCI-WG criteria [5], were included in our registry. Their data were retrospectively analyzed. They were 70 males (64%) and 40 females, mean age 57 years (range 36–83), diagnosed 1982–2004, with a medium follow-up period of 56 months. The mean WBC at presentation was 58.8 (7.1–569.0) x 10^9/l. Reliably documented clinical Rai stages [17] were the following: Rai 0: n=46 patients; Rai 1: n=21; Rai 2: n=7; Rai 3: n=2; Rai 4: n=7 patients. Treatment in the majority of them was basically commenced according to the NCI-WG recommendations [5] and was palliative in principle: either chlorambucil ± prednisone, the COP regimen (cyclophosphamide + oncovin + prednisone) [7], or fludarabine monotherapy [18]. A subset of patients, based on negative prognostic criteria (unmutated IgV_H genes and 11q or 17p deletions) and signs of progression, received more aggressive therapy – FluCy (fludarabine-cyclophosphamide) combination, some with rituximab in addition (the FCR protocol) [18, 19]. Four patients received an autograft and 10 patients an allograft. OS was evaluated in view of CLL-related deaths (CLL progression, direct complications of CLL or sequellae of antileukemic treatment). Patients who died of diseases unrelated to CLL (n=8) were canceled the day prior to death. All patients gave a written informed consent with the investigational procedures.

Morphological evaluation. Altogether 88 patients (55 males, 33 females, median age 57 years) had good quality peripheral blood or bone marrow slides from the time of diagnosis for morphological evaluation. Preferentially peripheral blood (83 cases) smears from untreated patients at diagnosis were retrospectively read "blindly" by one of us (D.M.). In the 5 remaining cases, bone marrow specimens were used. It was acceptable, that in 25 patients (whose slides from the date of diagnosis were either not available or were of poorer quality), slides from later follow-up examinations were used for morphological examination, provided that the patients had not received any prior therapy. Cell morphology was evaluated according to the FAB classification [3], on the basis of presence of atypical cells (larger lymphocytes, prolymphocytoid cells and prolymphocytes) – see Figures 1 & 2. The result was either typical or atypical (pleiomorphic or dimorphic) morphology.

Bone marrow histopathology. Results of a unilateral bone marrow biopsy were available for 52 patients. The histopathological pattern of bone marrow infiltration was evaluated according to ROZMAN et al [20]. Standard hematoxylin-eosin stained preparations were examined, immunohistology (CD20, CD45, CD3) was performed for discrimination of the lymphocyte subsets and for confirmation of the B cell nature of the infiltration. The result was either a diffuse or a non-diffuse (nodular, interstitial or mixed) type of CLL infiltration [20].

Immunophenotypic studies. Diagnostic immunophenotyping was performed in all 110 patients. Until the early 90's, fluorescence microscopy was used. Since then, Becton-Dickinson or Coulter Epics flow cytometry analyzers were used. All patients included in the study had to conform with the diagnostic criteria outlined by the IWCLL (CD5/CD19+,

weak surface Ig) [4]. Cases diagnosed after 1995 had the Royal Marsden Score 4–5 [21, 22]. CD38 antigen expression was analyzed in 73 patients, most of them in a CD19+ gate using flow cytometry, and was deemed positive if >30% of cells stained positive.

Cytogenetic and FISH analysis. Routine G-banding cytogenetics was performed in unstimulated bone marrow aspirates. Since mid-90's, standard interphase flurescence *in situ* hybridization (FISH) analyses has been employed in 87 of patients. FISH analyses were performed on bone marrow or peripheral blood smear preparations (or both). For detection of trisomy 12, the centromeric CEP 12 DNA probe was used. To detect chromosomal deletions, locus-specific probes were employed: LSI D13S319 for 13q14.3 band deletions, LSI p53 for 17p13.1 and LSI ATM for 11q22.3 deletions. All DNA probes were from Abbott-Vysis (Downers Grove, IL, USA). In last 2 years, the Vysis CLL Probe Panel (including 2 probe sets: Probe Set 1 LSI ATM/LSIp53 and Probe Set 2 CEP 12/LSI D13S319/LSI13q34 enabling even the diagnosis of biallelic 13q14 deletions) has been used. Two hundred nuclei were analyzed for each probe. For details, see ŠINDELÁŘOVÁ et al [23].

Mutation status of IgV_H genes. The assay was performed in 87 patients, in some of them retrospectively, as the mutation status is deemed to be stable in CLL patients [14]. Ficoll-Paq isolated mononuclears were lyzed and RNA extracted using the TriZol reagent (Invitrogen, KRD, Prague, Czech Republic). RNA was transcribed into cDNA using Superscript II (Invitrogen), which was subjected to PCR amplification with Ampli-Taq Gold polymerase (Applied Biosystems, Prague, Czech Republic) in 6 separate assays to detect clonal proliferation in the 7 families of IgV_H genes. The touch down methodology using degenerated primers is described elsewhere [24]. RT-PCR products were purified and sequenced using the Big Dye Terminator kit v. 3 and ABI Prism 310 Genetic Analyzer (both from Applied Biosystems). The sequences of IgV_H genes were compared to their germ-line configuration using the BLAST program (www.ncbi.nlm.nih.gov/igblast/; accessed September 5, 2005) and a cut-off value of 2% (i.e. 98% of homology) was set to discriminate between the mutated and unmutated genes [24].

Statistical analyses. The correlation of various prognostic parameters was analyzed in contingency tables using the chi-square test. For analysis of quantitative data (WBC counts) in 2 groups, medians were detected and a non-parametric two-tailed Mann-Whitney test was performed. Overall survival was analyzed using the Kaplan-Mayer regression method and the statistical significance was calculated using the Mantel-Haenschel log-rank test. To reveal differences in overall survival in various groups of patients according to the FISH analysis results, the log-rank test for trend was used. All analyses were performed at the 95% confidence interval and the p values were found using the GraphPad Prism version 3.03 software (GraphPad Software, San Diego, CA, USA).

Results

FAB morphology and overall survival (OS). Typical FAB morphology (see Fig. 1) was found in 25 (28.4%) and atypical morphology (see Fig. 2) in 63 (71.6%) of 88 patients. Cases with atypical morphology had inferior OS (p=0.03), the projected median OS was 103 and 237 months for patients with atypical and typical FAB morphology, respectively (Fig. 3).

Correlation of FAB morphology and WBC counts. Initial WBC counts were recorded in 82 patients. Interestingly, they were higher in patients with typical morphology (Fig. 4). In this group, median WBC was 45.0 x 10^9/l (range 13–382), whereas this value was 29.0 (7–448) in patients with atypical morphology (p=0.04). An arbitrary cut off value 30 (x 10^9/l) of WBC discriminated the patients with respect to their OS (Fig. 5): the projected median OS was 102 months and not reached in patients with WBC >30 and WBC <30 (x 10^9/l), respectively (p=0.02).

FAB morphology and the mutational status of IgV_H genes. Atypical morphology was found more often in patients with unmutated rather than with mutated IgV_H genes: 36/44 (81.8%) and 23/38 (60.5%) patients, respectively (p=0.03; Fig. 6). Inferior OS was characteristic of patients with unmutated IgV_H genes (median 99 months), compared to patients with mutated IgV_H genes (237 months; p=0.002) – see Figure 7. Interestingly, the analysis of 43 "unmutated" cases with respect to morphology has revealed some trend to inferior OS (p=0.14) in the subgroup of patients with atypical morphology (Fig. 8), the median OS being 99 months and not reached, respectively. No CLL-related death was documented among the 7 "unmutated" cases with typical FAB morphology. Vice versa, the analysis of the mutation status within the morphologically atypical group (57 patients) showed a significant difference (p=0.01) in OS: 99 months and not reached for "unmutated" and "mutated" cases, respectively (Fig. 9).

FAB morphology and cytogenetics. Two of the cytogenetic aberrations, trisomy 12 (9 patients) and deletion of 17p (7 patients), were connected purely with atypical morphology (Fig. 10). In the remaining patients, either with no aberration detected, or with deletions of 13q or 11q, atypical morphology was uniformly found in 68–71% of patients (17/25, 15/21 and 10/14 cases, respectively). There was an OS hierarchy in patients according to the results of chromosomal aberrations. Their rating was the following (from the most to the least favorable): no cytogenetic aberrations, deletion of 13q, trisomy 12, deletion of 11q and 17p (Fig. 11). This hierarchy was statistically significant when the log-rank test for trends was employed (p=0.03).

FAB morphology and CD38 antigen expression. Atypical morphology was found only slightly more frequently in CD38+ patients than in CD38- cases (Fig. 12): 17/20 (85%) *vs* 34/45 (76%) cases, respectively. This finding did not reach any statistical significance (p=0.4). Patients expressing the

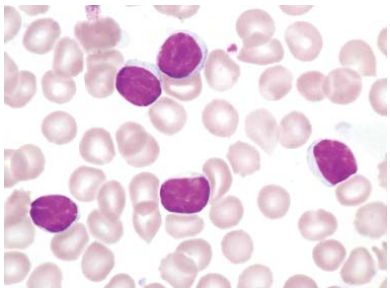

Figure 1. "Typical" CLL morphology according to FAB [3]. Small lymphocytes with monotoneous size and shape.

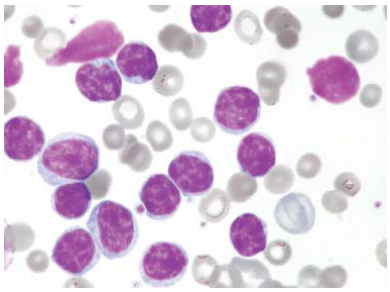

Figure 2. "Atypical" CLL morphology ("mixed cells") according to FAB [3]. Small lymphocytes predominate, but somewhat larger lymphocytes and prolymphocytoid cells with more abundant basophilic cytoplasm are depicted. This picture corresponds to the pleiomorphic subgroup.

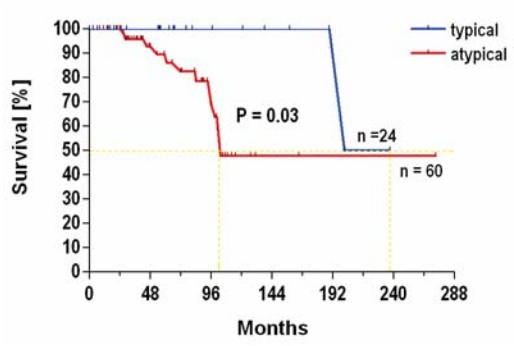

Figure 3. OS according to FAB morphology.

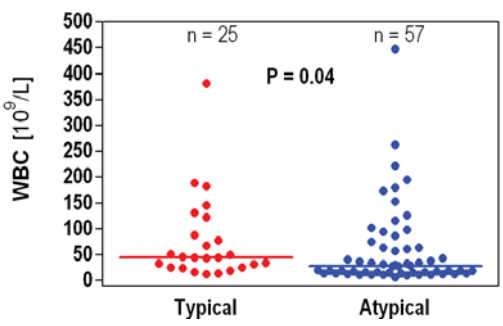

Figure 4. WBC counts and FAB morphology. Medians are depicted.

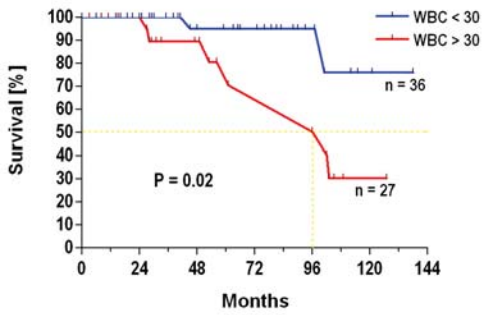

Figure 5. OS according to WBC counts. An arbitrary cut-off of WBC $30x10^9$/l was set.

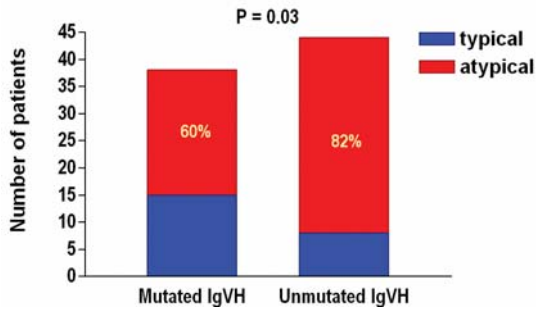

Figure 6. The mutation status of IgV_H genes and FAB morphology.

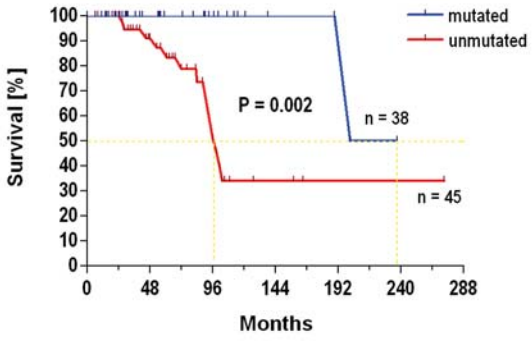

Figure 7. OS according to the mutational status of IgV_H genes.

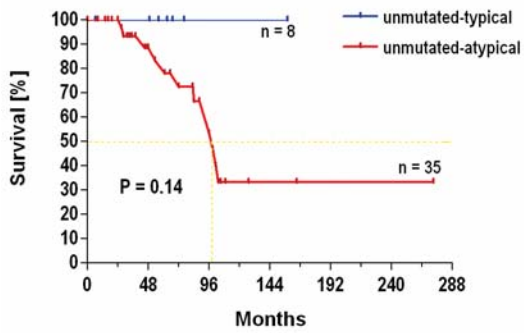

Figure 8. OS within the unmutated cases according to FAB morphology.

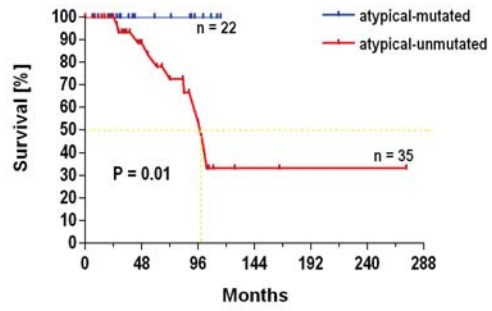

Figure 9. OS within the morphologically atypical FAB cases according to the mutational status of IgV_Hgenes.

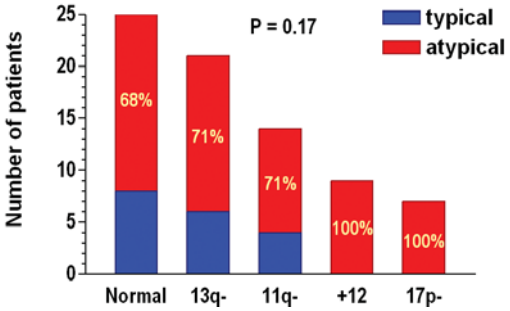

Figure 10. Cytogenetic aberrations and FAB morphology. The group with trisomy 12 and deletion 17 significantly (p=0.01) comprises of more atypical FAB cases than the group with normal cytogenetics or deletions of 13q and 11q.

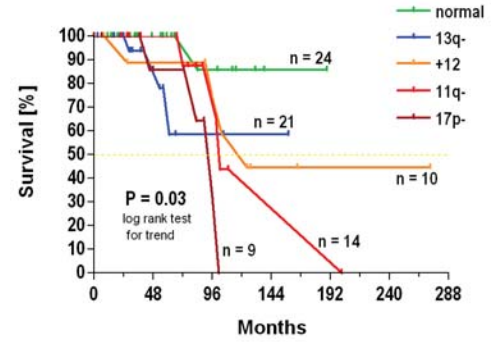

Figure 11. OS according to the cytogenetic aberrations.

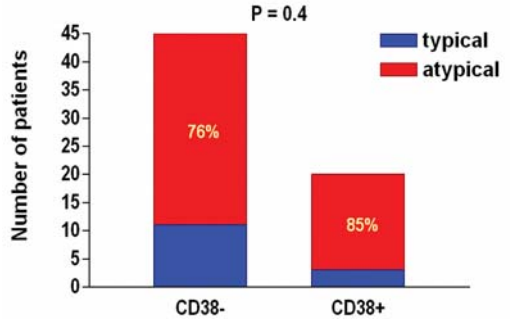

Figure 12. CD38 antigen expression and FAB morphology.

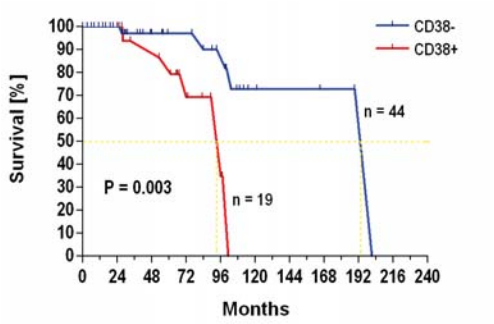

Figure 13. OS according to CD38 antigen expression.

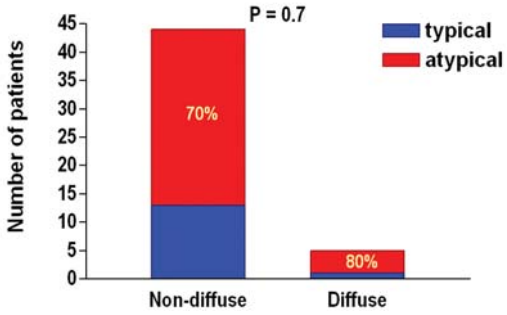

Figure 14. Histopathological pattern of bone marrow infiltration and FAB morphology. Evaluated according to ROZMAN et al [20].

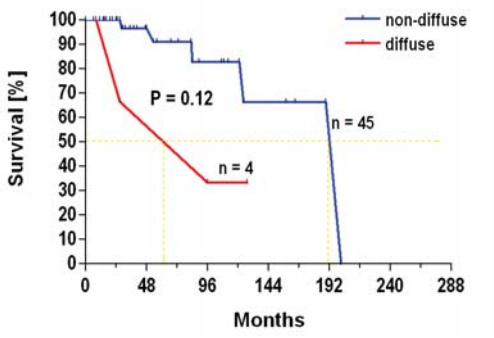

Figure 15. OS according to the histopathological pattern of bone marrow infiltration. Evaluated according to ROZMAN et al [20].

cell surface CD38 antigen had shorter OS than those lacking it (median OS was 96 and 201 months, respectively; p=0.04; Fig. 13).

FAB morphology and histopathology. Atypical morphology was found in 4/5 (80%) with diffuse and in 31/44 (70%) patients with non-diffuse histological pattern of bone marrow infiltration (p=0.7; Fig. 14). Some trend for worse OS was seen in the only 4 patients with diffuse bone marrow histopathology at diagnosis when compared with the 45 patients with a non-diffuse pattern of infiltration according to ROZMAN et al [20] (p=0.12; Fig. 15).

Discussion

Morphological analysis (preferentially of peripheral blood smears) is a first step of the routine diagnostic work-up of chronic leukemias in every laboratory. It is quick, cheap and efficient.

However, in CLL, it is not generally accepted that cell morphology as defined by the FAB criteria [3] might be of prognostic value in CLL, despite that the Belgic group has formulated a concept claiming that typical and atypical CLL according to FAB are closely related but distinct diseases with different outcomes [10]. In 1999 and 2000, studies on the prognostic impact of two important parameters, ie. the mutation status of IgV_H genes and FISH detected chromosomal aberrations, were published [11–13] and somewhat shadowed the possible prognostic potential of FAB defined morphology. Therefore we tried to check whether FAB morphology may be of any value in the 3rd millenium having the newer prognostic markers at hand. Our data strongly support the notion that FAB morphology is a reliable prognostic marker. Notably, only a minority (28.4%) of our patients had typical morphology. However, their OS was spectacular: the first leukemia-associated death was documented 201 months after the date of diagnosis. Interestingly, in the large Belgic cohort of patients, 300 of 390 (76.9%) had typical morphology. The reason of the considerable discrepancy between the Belgic and our own results is not clear. There is some selection bias at our Institute, as patients having clinical problems are preferentially seen. On the other hand, this selection is only marginally reflected by the data of the mutation status of IgV_H genes: 44 out of 82 (53.7%) evaluable patients in our study had unmutated IgV_H genes, whereas only 45–51% of unmutated cases were found in the original studies [12, 13]. It seems, after all, that the discrepancy of the percentages of the morphologically atypical cases in our and the Belgic study [8, 10] were caused by the subjective nature of reading the morphological slides. This is probably also the major reason why morphological evaluation of CLL cells is not regarded a reliable tool. However, it is interesting that despite these differences in reading the slides, both in our and the Belgic studies, prognostic relevance of the FAB classification could be still demonstrated. We feel it is an advantage if a single experienced hematologist evaluates all the specimens over a short period of time. This might have contributed to consistence of our evaluation procedure performed on the basis of the FAB classification.

Among the correlations of FAB morphology with other markers, it was somewhat peculiar that higher WBC counts were found at diagnosis of the morphologically typical cases. However, this does not necessarily imply that patients with higher WBC counts had a more progressive disease. It could be the case that patients with typical CLL are diagnosed later as their symptoms appear later. The other explanation might be that the patients with atypical morphology could have a more lymphomatous type of the disease (with a higher tumor distribution index, lower WBC counts and adverse prognosis) according to JAKSIC et al [25]. On the other hand, our study has confirmed that a WBC count >30 x 10^9/l is associated with poorer outcome.

Our results have revealed correlation of FAB morphology and the mutation status of IgV_H genes. However, discrepant cases (typical FAB-unmutated and atypical FAB-mutated) were identified. Interestingly enough, it is shown here that the mutation status of IgV_H genes could discriminate prognostic subgroups within the morphologically atypical cohort (Fig. 9) and *vice versa*, even within the unmutated cases, no patient succumbed to CLL in case he/she had typical FAB morphology (Fig. 8), although the latter finding did not reach significance (probably the number of the cases identified was too small). The mutation status of IgV_H genes has a higher statistical power than FAB morphology in discriminating OS probabilities in univariate analyses. However, the two characteristics jointly bring additional prognostic information and thus it seems that the two prognostic factors are complemetary, if not independent (this remains to be proven in future studies with multivariate analysis of data).

FAB morphology correlated with cytogenetic results. We confirmed that there is a strong association between trisomy 12 and atypical morphology [7, 8, 10]. Above that, we demonstrated the same was true for deletion 17p cases. In fact, virtually all cases with trisomy 12 and deletion 17p had atypical morphology. In contrast to the original German data [11], OS in our group with no cytogenetic aberration was superior in comparison to deletion 13q cases. Otherwise the prognostic hierarchy of the remaining aberrations was equal in the two studies.

The correlation of FAB morphology on the one hand and CD38 expression or the histopathological pattern of bone marrow infiltration on the other was not significant, probably due to lower numbers of patients examined. However, CD38 expression still had impact on OS, thus suggesting that CD38 expression and FAB morphology yield complementary (and perhaps independent) prognostic information. It has been shown by others that CD38 expression and mutation status of IgV_H genes are independent prognostic factors [2, 26].

In conclusion, our study shows that careful cytological evaluation of the peripheral blood smears of CLL patients according to the FAB classification may bring a prognostically valuable information that is easily accessible at every hematological laboratory within 10 minutes of analysis. The prognostic impact of the morphological analysis (if we compare the p values of statistical significance) is only slightly inferior to the impact of the more laborious analyses, such as the IgV_H gene mutation status or CD38 antigen expression. Our data from a modestly-sized single institution study should be confirmed in a prospective multiinstitutional study employing divergent prognostic parameters (including e.g. ZAP-70) allowing multivariate analysis. In that case, we would propose central review of the blood smears.

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