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Original article

# New insights into smudge cell percentage in chronic lymphocytic Leukemia: A novel prognostic indicator of disease burden

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ABSTRACT

*Background:* Percentage of smudge cells in CLL patients has recently been reported as a novel prognostic factor.
*Objectives:* To investigate the impact of smudge cells percentage on the clinicolaboratory data of CLL patients and to evaluate the relationship between it and other prognostic factors in CLL.
*Methods:* Ninety adults with CLL were enrolled. Smudge cells percentage was calculated by microscopic evaluation of blood smears. Testing of CD38 expression was done by immunophenotyping and detection of ATM, P53 deletions and trisomy 12 were performed using fluorescent in situ hybridization (FISH)
*Results:* Lower smear cells percentage (<30%) was significantly correlated with age, lymphadenopathy, organomegaly and advanced staging. It was also associated with high TLC, low hemoglobin and platelets count and high absolute and atypical lymphocytic count. Correlation studies with other prognostic factors revealed an association between low smear cells percentage and CD38 expression, short LDT, P53 and ATM deletions. Logistic regression analysis was also done to provide complementary prognostic information identifying the significant independent factors that predict low smear cell percentage.
*Conclusion:* low percentage of smudge cells (<30%) could be considered as an adverse prognostic predictor being associated with high risk markers in CLL.
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## 1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) results from accumulation of small mature B lymphocytes that have undergone monoclonal expansion in blood, bone marrow and lymphoid organs which is mainly due to inhibition of apoptosis rather than enhanced cell proliferation [1].

The clinical course of CLL is heterogenous in different patients, some patients die within 2–3 years with refractory disease, whereas others live for decades after diagnosis without need for a therapy [2]. To address this heterogeneity and predict the prognosis of patients, several prognostic markers based on genetic phenotypic or molecular characteristics of CLL B cells have been discovered [3].

Prognostic markers such as expression of specific proteins in or on CLL cells (ie, CD38, Zap70 or CD 49d), cytogenetic abnormalities (del 13q, del 11q, del 17p and trisomy 12) quantified by fluorescent in situ hybridization (FISH) and immunoglobulin heavy chain (IGHV) gene mutation have all been very useful [2].

Despite this progress, many patients have limited access to these laboratory procedures, which require highly sophisticated instruments and a high degree of technical expertise and are costly to perform. Therefore, less expensive prognostic markers are needed. The percentage of smudge cells on routine blood smears has recently been reported as a prognostic test available to patients with CLL especially those in developing countries. It is simple, accessible and inexpensive [4].

Smudge cells are ruptured CLL B cells seen on routine blood smears of virtually all CLL patients. For nearly a century, smudge cells were thought to be an artifact of slide preparation. Many investigators recently discovered that smudge formation is related to the content of the cytoskeletal protein vimentin present in leukemic cells [5].

Smudge cell formation has been demonstrated to be linked to a reduced expression of vimentin in CLL lymphocytes. High vimentin expression (low percentage of smudge cells) has been shown to be associated with poor prognosis and a shortened time to first treatment [6].

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## 2. Aim of the study

To highlight the role of smudge cells percentage as a novel prognostic marker in CLL, in addition to investigate the impact of smudge cells percentage on the clinicolaboratory data of CLL patients and to evaluate the relationship between it and other prognostic factors in CLL.

## 3. Subjects and methods

This prospective study was carried out on 90 newly diagnosed adults with CLL recruited from hematology and Oncology unit at Ain Shams University hospital. Their ages ranged from 33 to 85 years (Mean 58. 83 ± 11. 79 years). 51 were males and 39 were females with a male to female ratio of 1. 2: 1. An informed consent was obtained from each patient before participation in the study. The procedures applied in this study were approved by the Ethical Committee of Human Experimentation of Ain Shams University, and are in accordance with the Helsinki Declaration of 1975.

Patients were diagnosed on the basis of: i) complete history taking and through clinical examination; ii) laboratory investigations including: complete blood count (CBC) using LH 750 (Bechman Coulter), examination of Leishman stained peripheral blood (PB) films laying stress on the percentage of Smudge cells (ratio of smudged to intact cells plus smudged lymphocytes) according to *Johnston* et al. [7] who considered 30% of Smudged cells as a cut off level to differentiate between low and high risk group. The same cut off value was used in two studies by *Nowakowski* et al. [5,8]. In addition to bone marrow (BM) examination, flowcytometric immunophenotyping was performed using EPICS XL Coulter flowcytometer. FISH analysis using locus-specific identifier (LSI) probes for detection of ataxia telangiectasia mutated (ATM) and protein 53(P53) genes deletions. Centromeric enumeration probe (CEP) for trisomy 12 detection was also used. Two age-matched healthy volunteers were used as controls; to check the intensity of signals of the used probes.

Staging of the patients was done according to the Rai staging system [9]

### 3.1. Sample collection

PB and BM samples were collected on ethylene diaminetetraacetic acid (EDTA) (1. 2 mg/ml) for morphological and immunophenotyping. BM aspirates were collected in sterile preservative-free lithium heparin coated vacutainer tubes for cytogenetic analysis.

### 3.2. FISH technique

The FISH analysis was performed on BM aspirates using LSI for detection of ATM and P53genes deletions as well as CEP for trisomy 12. At least 100 interphase nuclei and/or 20 metaphases were scanned under fluorescence microscope for the detection of ATM, P53 deletions and trisomy 12. In monoallelic ATM deletion, one red signal was reported in >10% of examined cells and biallelic deletion was reported by absence of red signals in at least 10% of examined interphase cells. P53 deletion was reported if 1 red signal was noticed in >10% of cells. Trisomy 12 was reported if 3 red signals were observed in at least >10% of interphase cells and/or >2% of metaphase.

### 3.3. Statistical analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS$^{TM}$) version 20. Qualitative data were presented as numbers and percentages while quantitative data were entered into Kolmogorov-Smirnov test of normality and parametric distribution data were presented as mean, standard deviations and ranges while non parametric distribution data were presented as median with interquartile range (IQR). In order to compare parametric quantitative variables between two groups, Student *t*-test was applied. For comparison of non-parametric quantitative variables between two groups, Mann-Whitney test was used. The comparison between two groups with qualitative data was done using Chi-square test. Logistic regression analysis was employed to determine variables affecting low smear cell percentage.

## 4. Results

Descriptive and laboratory data of the studied patients are shown in Table 1. The prognostic markers described in this study to predict the disease progression and to assess the tumor burden are listed in Table 2. The impact of the studied cytogenetic abnormalities on demographic, clinical and laboratory data of the patients was shown in Tables 3.The relation between high risk cytogenetic abnormalities and other studied prognostic factors are demonstrated in Table 4.

**Table 1**
Demographic, clinical and laboratory data of all the studied patients.

| Parameter | Patient (n = 90) |
|---|---|
| Age (years),<br>Mean ± SD<br>Range | 58. 83 ± 11. 79<br>33–85 |
| Sex, n (%)<br>Female<br>Male | 39 (43. 3%)<br>51 (56. 7%) |
| Lymphadenopathy, n(%) | 36 (40. 0%) |
| Splenomegaly, n(%) | 54 (60. 0%) |
| Hepatomegaly, n (%) | 51 (56. 7%) |
| Staging, n(%)<br>Low grade(I,II)<br>High grade(III,IV) | 21 (23. 3%)<br>69 (76. 7%) |
| TLC(x$10^9$/L),<br>Median (IQR)<br>Range | 36. 3 (22. 5–105)<br>11. 2–270 |
| Hemoglobin (g/dL),<br>Mean ± SD<br>Range | 10. 76 ± 2. 82<br>5. 6–18. 5 |
| Platelets (×$10^9$/L),<br>Mean ± SD<br>Range | 188. 27 ± 92. 81<br>48–382 |
| PB Lymphocytes (×$10^9$/L),<br>Median (IQR)<br>Range | 31. 9 (19. 5–96. 6)<br>9. 5–231. 1 |
| Atypical lymphocytes (%),<br>Median (IQR)<br>Range | 9 (7–10)<br>5–36 |
| Prolymphocytes (%),<br>Mean ± SD<br>Range | 3. 03 ± 1. 44<br>1–6 |
| BM Lymphocytes (%),<br>Median (IQR)<br>Range | 66 (35–75)<br>16–96 |
| Immunophenotyping score, n(%)<br>Atypical<br>Typical | 15 (16. 7%)<br>75 (83. 3%) |

TLC: total leucocytic count; PB: peripheral blood; BM: Bone marrow, IQR: interquartile range, SD: standard deviation.

**Table 2**
Prognostic markers in all the studied patients.

| Prognostic marker | Patient (n = 90) |
|---|---|
| CD38 expression, n(%) | 44(48. 9) |
| ATM delation, n(%) | 15(16. 7) |
| P53 deletion, n(%) | 21(23. 3) |
| Trisomy 12, n(%) | 18(20) |
| LDT (months), n(%) | 53(58. 9) |
| >12 months | 37(41. 1) |
| <12 months | |
| Smear cell percentage | 54(60) |
| >30% | 36(40) |
| <30% | |

CD:cluster of differentiation, ATM:ataxia telangiectasia mutated, P53:protein 53,LDT:lymphocyte doubling time

### 4.1. Clinical and laboratory characteristics of CLL patients in relation to smear cell percentage

A lower percentage of smear cell was significantly predominant in older age (p = .046). Low Smear cell percentage (<30%) showed a significantly higher incidence of lymphadenopathy, organomegaly and advanced staging (p < .05) (Table 5). Additionally, it was associated with a high total leucocytic count, low hemoglobin and platelet count, high absolute lymphocytic counts and a high percentage of atypical lymphocyte (p < .05). However, there was no significant difference regarding prolymphocytic percentage, BM lymphocytic infiltration and immunophenotyping scoring in high versus low smear cell percentage in CLL studied patients (Table 5).

### 4.2. Correlation between smear cell percentage and studied prognostic factors

Correlation studies with other prognostic factors revealed that lower Smear cell percentage was highly significantly associated with CD38 expression, short lymphocyte doubling time (LDT), the presence of cytogenetic abnormalities, ATM and P53 deletions (p < .05). However, no correlation was reported with trisomy 12 (P > .05) (table 6).

### 4.3. Logistic regression analysis for predictors of low smear cell percentage

Using logistic regression analysis, we showed the effect of demographic, clinical, and laboratory variables on the low per-

**Table 3**
The impact of the studied cytogenetic abnormalities on demographic, clinical and laboratory data:

| | Non cytogenetic abnormality n = 66 | Cytogenetic abnormality n = 24 | Test value | P-value |
|---|---|---|---|---|
| Age (years) | 58.55 ± 13.20 | 59.63 ± 5.73 | −0.387• | 0.700 |
| Mean ± SD | 33–85 | 52–67 | | |
| Range | | | | |
| Sex, n(%) | 30 (45.5%) | 9 (37.5%) | 0.454* | 0.501 |
| Female | 36 (54.5%) | 15 (62.5%) | | |
| Male | | | | |
| Lymphadenopathy, n(%) | 18 (27.3%) | 18 (75.0%) | 16.705* | 0.000 |
| Splenomegaly, n(%) | 30 (45.5%) | 24 (100.0%) | 21.818* | 0.000 |
| Hepatomegaly, n(%) | 30 (45.5%) | 21 (87.5%) | 12.671* | 0.000 |
| Staging, n(%) | 18 (27.3%) | 3 (12.5%) | 2.147* | 0.142 |
| Low grade(I,II) | 48 (72.7%) | 21 (875%) | | |
| High grade(III,IV) | | | | |
| TLC($\times 10^9$/L), | 31 (16–69) | 176.5 (61–226) | −4.481≠ | 0.000 |
| Median (IQR) | 11–270 | 28–267 | | |
| Range | | | | |
| Hemoglobin (g/dL), | 11.05 ± 2.97 | 10.13 ± 2.13 | 1.392• | 0.168 |
| Mean ± SD | 6–18 | 8–14 | | |
| Range | | | | |
| Platelets ($\times 10^9$/L), | 201.32 ± 98.98 | 152.38 ± 55.34 | 2.290• | 0.024 |
| Mean ± SD | 48–382 | 57–208 | | |
| Range | | | | |
| PB Lymphocytes ($\times 10^9$/L), | 28.5 (14–58) | 115 (46.5–184) | −4.235≠ | 0.000 |
| Median (IQR) | 10–230 | 20–231 | | |
| Range | | | | |
| Atypical lymphocytes (%), | 7.5 (5–10) | 21.5 (17–27) | −6.229≠ | 0.000 |
| Median (IQR) | 5–10 | 7–36 | | |
| Range | | | | |
| Prolymphocytes(%), | 3.55 ± 1.99 | 2.75 ± 0.99 | 1.875• | 0.064 |
| Mean ± SD | 1–7 | 1–4 | | |
| Range | | | | |
| BM Lymphocytes(%), | 64 (35–70) | 74 (45–85) | −2.101≠ | 0.036 |
| Median (IQR) | 16–96 | 32–90 | | |
| Range | | | | |
| Immunophenotyping score, n (%) | 15 (22.7%) | 0 (0.0%) | 6.545* | 0.011 |
| Atypical | 51 (77.3%) | 24 (100.0%) | | |
| Typical | | | | |

TLC: total leucocytic count; PB: peripheral blood; BM: bone marrow, IQR:interquartile range.
* Chi-square test; Independent *t*-test.
≠ Mann-Whitney test.

**Table 4**
Relation between Cytogenetic abnormality and other studied prognostic factor.

| | Non Cytogenetic abnormality n = 66 | Cytogenetic abnormality n = 24 | Chi-square test | |
|---|---|---|---|---|
| | | | $X^2$ | P-value |
| CD38 expression, n(%)Positive | 25 (37.9%) | 19 (79.2%) | 10.411 | 0.001 |
| LDT, n(%) | 46 (69.7%) | 7 (29.2%) | 10.327 | 0.001 |
| LDT > 12 months | 20 (30.3%) | 17 (70.8%) | | |
| LDT < 12 months | | | | |
| Smear cell, n(%) | 48 (72.7%) | 6 (25.0%) | 14.775 | 0.000 |
| Smear cell > 30% | 18 (27.3%) | 18 (75.0%) | | |
| Smear cell < 30% | | | | |

CD: Cluster of differentiation; LDT: lymphocyte doubling time.

**Table 5**
Impact of Smear cell percentage on demographic, clinical and laboratory data.

| | Smear cell > 30% | Smear cell < 30% | Test value | P-value |
|---|---|---|---|---|
| | n = 54 | n = 36 | | |
| Age (years), | 56. 83 ± 11. 6933–73 | 61. 83 ± 11. 10 | −2. 027• | 0. 046 |
| Mean ± SD | | 50–85 | | |
| Range | | | | |
| Sex, n(%) | 24 (44. 4%) | 15 (41. 7%) | 0. 068* | 0. 794 |
| Female | 30 (55. 6%) | 21 (58. 3%) | | |
| Male | | | | |
| Lymphadenopathy, n(%) | 12 (22. 2%) | 24 (66. 7%) | 17. 778* | 0. 000 |
| Splenomegaly, n(%) | 18 (33. 3%) | 36 (100. 0%) | 40. 00* | 0. 000 |
| Hepatomegaly, n(%) | 21 (38. 9%) | 30 (83. 3%) | 17. 376* | 0. 000 |
| Staging, n(%) | 18 (33. 3%) | 3 (8. 3%) | 7. 547* | 0. 006 |
| Low grade(I,II) | 36 (66. 7%) | 33 (91. 7%) | | |
| High grade(III,IV) | | | | |
| TLC(x$10^9$/L), | 25. 15 (15. 5–36. 3) | 176. 65 (60. 7–249) | −6. 418≠ | 0. 000 |
| Median (IQR) | 11. 2–105 | 28. 2–270 | | |
| Range | | | | |
| Hemoglobin (g/dL), | 11. 46 ± 3. 06 | 9. 73 ± 1. 95 | 3. 007• | 0. 003 |
| Mean ± SD | 5. 6–18. 5 | 6. 3–13. 5 | | |
| Range | | | | |
| Platelets (×$10^9$/L), | 220. 56 ± 93. 66 | 139. 83 ± 64. 08 | 4. 512• | 0. 000 |
| Mean ± SD | 93–382 | 48–230 | | |
| Range | | | | |
| PB Lymphocytes (×$10^9$/L), | 22. 6 (13. 2–31. 2) | 114. 85 (46. 7–220. 1) | 6. 230≠ | 0. 000 |
| Median (IQR) | 9. 5–96. 6 | 19. 7–231. 3 | | |
| Range | | | | |
| Atypical lymphocytes (%), | 7 (5–9) | 17 (9. 5–25. 5) | −5. 510≠ | 0. 000 |
| Median (IQR) | 5–10 | 5–36 | | |
| Range | | | | |
| Prolymphocytes(%), | 2. 87 ± 1. 61 | 3. 18 ± 1. 27 | 0. 971• | 0. 334 |
| Mean ± SD | 1–6 | 2–6 | | |
| Range | | | | |
| BM Lymphocytes(%), | 64 (35–70) | 67. 5 (45–80) | −1. 636≠ | 0. 102 |
| Median (IQR) | 16–96 | 32–90 | | |
| Range | | | | |
| Immunophenotyping score, n (%) | 6 (11. 1%) | 9 (25. 0%) | 3. 000* | 0. 083 |
| Atypical | 48 (88. 9%) | 27 (75. 0%) | | |
| Typical | | | | |

TLC: total leucocytic count, PB:peripheral bone,BM:bone marrow, IQR:interquartile range.
* Chi-square test.
• Independent t-test.
≠ Mann-Whitney test.

centage of smear cells (<30%) as a poor prognostic marker. We revealed that advanced stage (Rai III & IV), lymphadenopathy, hepatomegaly had a significant effect on low smudge cells percentage. As regards laboratory data, total leucocytic count (>35 x$10^9$/L), hemoglobin level (<9. 2 g/dl), platelets count (<120 x$10^9$/L), absolute lymphocytic count (>31.2 × $10^9$/L), and atypical lymphocytic count (>10%) together with LDT (<12 months) and presence of high risk cytogenetic abnormalities were the significant independent factors that predict low smear cell percentage (<30%) ($p < .05$) (Table 7).

## 5. Discussion

Smudge cells, or so-called Gumprecht shadow, are the nuclear remnant of ruptured leukocytes (usually lymphocytes) during the slide preparation of peripheral blood film (PBF) [10].

The appearance of smudge cells on a PBF is a characteristic feature of CLL, with virtually all patients exhibiting at least some degree of smudging [5].

The story of smudge cells is very long and interesting. Firstly, it has been suggested as an artifact in PBF due to the mechanical fra-

**Table 6**
Relation between Smear cell and other studied prognostic factors.

| Prognostic parameter | Smear cell > 30% n = 54 | Smear cell < 30% n = 36 | Chi-square test | |
|---|---|---|---|---|
| | | | $X^2$ | P-value |
| CD38 expression, n(%) | 11(20. 4) | 33(91. 7) | 43. 940 | 0. 000 |
| LDT (months), n(%) | 43(79. 6) | 10(27. 8) | 23. 988 | 0. 000 |
| >12 months | 11(20. 4) | 26(72. 2) | | |
| <12 months | | | | |
| Cytogenetic abnormality, n(%) | 6(11.1) | 18(50) | 16.705 | 0. 000 |
| ATM deletion, n(%) | 0(0) | 15(41. 7) | 27. 000 | 0. 000 |
| P53 deletion, n(%) | 0(0) | 21(58. 3) | 41. 087 | 0. 000 |
| Trisomy 12, n(%) | 9(16. 7) | 9(25) | 0. 937 | 0. 330 |

CD: cluster of differentiation; LDT:lymphocyte doubling time; ATM:ataxia telangiectasia mutated, P53:protein 53.

**Table 7**
Logistic regression analysis for predictors of smear cell < 30%:

| | Odds ratio (OR) | 95% C. I. for OR | | P-value |
|---|---|---|---|---|
| | | Lower | Upper | |
| Age > 57 years | 2.2 | 0.932 | 5.195 | 0.072 |
| Advanced stage | 5.5 | 1.483 | 20.391 | 0.011 |
| Lymphadenopathy | 7 | 1.364 | 35.929 | 0.02 |
| Hepatomegaly | 7.857 | 1.312 | 47.044 | 0.024 |
| TLC (>35 $\times$ $10^9$/L) | 28. 6 | 7.614 | 107.431 | 0.000 |
| Hemoglobin ($\leq$9.2 g/dL) | 7 | 2.64 | 18.564 | 0.000 |
| Platelets ($\leq$120 $\times$ $10^9$/L) | 8 | 2.741 | 23.347 | 0.000 |
| PB lymphocytes (>31.2 $\times$ $10^9$/L) | 38.5 | 10.033 | 147.74 | 0.000 |
| Atypical lymphocytes (>10%) | 74.2 | 9.211 | 597.73 | 0.000 |
| LDT < 12 months | 10.164 | 3.795 | 27.219 | 0.000 |
| High risk cytogenetic abnormalities | 25 | 3.522 | 177.477 | 0.001 |

TLC: total leucocytic count; PB: peripheral bone; LDT: lymphocyte doubling time; C.I:confidence interval.

gility of the cells [11]. Some authors have thought that they represent an early senescent change in the protein structure of the cells. Others believe that the expanded number of these cells results from disease, chemotherapy, sepsis or necrotic cell lysis, or that as a rule they corresponded to high leucocytic count [12,13]. In *2002, Constantino*[14] showed that the exceptional attributes of CLL lymphocytes (small cytoplasmic rim, abnormal cytoskeleton structure and cell membrane, and increased accumulation of malignant cells that fail to die) predispose them to smearing during film handling.

Further investigation revealed that smudge cell formation has been connected to reduced expression of the cytoskeletal protein vimentin in CLL lymphocytes. It is an intermediate filament protein important for lymphocyte rigidity and integrity. The physiologic role of vimentin may extend beyond maintaining cell integrity. Rearrangement of vimentin fibers was shown to participate in cell activation and signal transduction. High vimentin expression (low percentage of smudge cells) has been appeared to be related to poor outcome and an abbreviated time to first treatment [6].

In the present study, we have calculated the percentage of smudge cells on blood smears at diagnosis and we also investigated the relationship of this percentage and other prognostic factors. We found that 40% of the patients had a low percentage of smear cells (<30%). Similarly, *Gogia* et al. [15]; Brown et al. [16] found 37% and 32% of their patients with low smudge cell percentage.

In the current work, lower smear cell percentage was associated with older age. Conversely, Johansson et al. [17] reported that age was not associated with smear cell percentage. As regards gender, we could not establish a relation between it and smear cell percentage. The same finding was reported by Sall et al. [4].

Our results showed a significant association between lower smear cell percentage and lymphadenopathy and organomegaly. However, in a recent study published by Gogia et al. [15], no correlation of proportion of smudge cells with organomegaly was reported.

In this study, we reported an association of smudge cell percentage with advanced Rai staging. In agreement with Sall et al. [4] *and* Gogia et al. [15], who previously reported a strong correlation between low numbers of smudge cell percentage (<30%) and advanced stage disease at diagnosis.

As regards laboratory parameters, lower smudge cell percentage was correlated with high total leucocytic count, high absolute and atypical lymphocytic count, low hemoglobin level and low platelets count. Since these parameters reflect a high tumor mass and poor outcome, it is possible that patients with lower smudge cell percentage have an increased tumor burden. Also, Sall et al. [4] concluded that a percentage of smudge cells <30% was associated with a high lymphocytic count. On the other hand, Gogia et al. [15], found no correlation of proportion of smudge cells with the lymphocytic count.

We reported that lower smudge cell percentage was corresponded with the high atypical lymphocytic count. This finding was previously clarified by Constantino [14] who studied the factors associated with smudge cell formation in the blood film. He stated that the cytoplasmic volume of the cells is inversely related to the quantity of smudge cells formed. Therefore, large lymphocytes with abundant cytoplasm (atypical lymphocyte) are correlated with a lower number of smudge cells formed.

We also studied the correlation of smudge cell percentage with other prognostic markers including CD38 expression, LDT and cytogenetic aberrations. We revealed an inverse relation between smudge cell percentage and CD38 expression. This finding may be attributed to that the unruptured cells have CD38 expression. This was also reported in previous studies by Nowakowski et al. [5]; Johansson et al. [17]; Sall et al. [4].

Leukemic clones with higher numbers of CD38 + cells are more responsive to B-cell receptor (BCR) signaling and are characterized

by enhanced migration. In vitro activation through CD38 drives CLL proliferation and chemotaxis via the signaling pathway that includes ZAP-70. Consequently, CD38 appears to be a global molecular bridge to the environment, promoting survival/proliferation over apoptosis [18].

Therefore, a high CD38 expression has been shown as a poor prognostic indicator and patients with high CD38 positivity experience a more aggressive clinical course with respect to treatment requirement [19]. Therefore, the high CD38 expression by the unruptured cells may explain the link between the lower percentage of smudge cells and worse clinical outcome in CLL cases [20].

The LDT, defined as the length of time it takes the absolute lymphocyte count to double from diagnosis, has also been found to have prognostic value in CLL patients. Patients with LDT of longer than 12 months have a better prognosis than those with LDT < 12 months [21]. In our study, we reported that most of the patients with < 30% smear cell percentage had shorter LDT being < 12 mon ths. LDT requires serial blood measurements over time, in addition, the absolute lymphocytic count can vary as a result of other events unrelated to the malignancy. Therefore, this prognostic factor has a significant limitation [21].

Acquired genetic aberrations have an important role in CLL pathogenesis. The most frequent chromosomal abnormalities are partial losses of one affected chromosome, such as deletions on 11q22, 17p13 or 13q14; gains of entire chromosomes, such as trisomy 12, are less frequent [22].

Loss of chromosome 11q22 is considered a poor prognostic cytogenetic event. This location harbors the ATM gene. The ATM protein, a member of phosphatidylinositol 3-kinase-related kinases (PIKK) family, is the main integrator of cellular response after DNA double strand breaks and is responsible for the phosphorylation and stabilization of p53 leading to cell cycle arrest and apoptosis induced by ionizing radiation [22]. These findings have considerable clinical implication because ATM mutations may be important in predicting potential treatment failure [23].

Patients with 17p13 deletion have always been included into the highest risk prognostic category. This finding can be explained not only because of the cell cycle deregulation caused by loss of tumor protein 53 (TP 53) but also the usual requirement of chemotherapy [24]. At the cellular level, TP 53-disruptive CLL exhibits a complete absence of DNA damage-induced apoptosis in vitro, whereas ATM-disruptive CLL retains a capacity for apoptosis after in vitro-induced DNA damage, though at a reduced level [22].

On the other hand, trisomy 12 was considered to be an intermediate risk marker. However, this category still remains quite controversial. Some authors hypothesize that it carries a bad prognostic impact when it is associated with NOTCH1 mutation as well as CD38, ZAP 70 and the integrins (CD11a and CD49d) [25].

The NOTCH receptor genes encode a family of heterodimeric transmembrane proteins (NOTCH1 to NOTCH4) that function both as cell surface receptor and transcription regulators. NOTCH1 mutation results in impaired degradation and accumulation of active NOTCH1 isoform sustaining deregulated signaling. NOTCH1 alterations, potentially inducing upregulation of the expression of critical genes located on chromosome 12, might cooperate with trisomy 12 to drive leukemia [26]. Moreover, the coexistence of NOTCH1 mutations downregulates integrins expression which may allow escape from immune surveillance indicating a novel interaction that may be of a potential importance in aggressive poor risk CLL [27].

To prove the poor prognostic impact of the previous cytogenetic markers in CLL patients, we combined these high risk cytogenetic markers together and analyzed their effect on the clinicolaboratory data of our patients. Our results confirmed that patients with these cytogenetic abnormalities were characterized by unfavorable clinical conditions and laboratory parameters. We also studied the relationship between these markers and other studied prognostic indicators. The strong significant association with CD38 expression, short LDT and lower smudge cell percentage provided an additional evidence to the prognostically poor impact of these cytogenetic abnormalities on the disease progression.

Our study evaluated the impact of each one of the studied cytogenetic anomalies on smudge cell percentage. We did not observe any significant difference in the percentage of smudge cells based on trisomy 12 presence. However, it was significantly lower in high risk patients with 11q22 and 17p13 deletions.

A recent study by Guarini et al. [23] concluded that the majority of patients with ATM mutations showed poor prognostic biological features, i. e unmutated IGHV, and ZAP-70 and CD38 expression. In an attempt to explain this phenomenon Kalla et al. [28] showed that leukemic cells carrying the 11q22. 23 deletion, showed down-modulation of the genes involved in the apoptosis machinery and DNA repair, and mapping to the 11q23 region, thus pointing to a gene dosage effect survival among prognostic markers reviewed. This finding together with our results raise the question: why these cells are unruptured? Are the rigid unruptured cells in CLL cause more malignant clone and resistant disease?

It is known that vimentin not only serves as a cytoskeleton protein, but also plays a key role in the development and progression of cancer. Although its expression is more emphasized in the epithelial mesenchymal transition process, it is equally possible that tumorigenic events including tumor migration and invasion are a consequence of vimentin over-expression in these cells. However, these events are a result of fine- tuning occurring in the cancer cells and vimentin might be acting as a scaffolding protein during signal transduction and promoting tumorigenic events in association with other tumor-promoting oncogenes [29]. Thus, it may be not surprising that the high vimentin expression (low percentage of smudge cells) may explain the rigidity of the malignant clone.

On the contrary, two studies by Nowakowski et al. [5] *and* Johansson et al. [17] previously observed no significant difference in the percentage of smear cells regarding high risk or low risk cytogenetic abnormalities. One possible explanation for this discrepancy could be the divergence of race-ethnicity.

In the current study, we evaluated how the studied parameters affect smudge cell percentage. Our investigation reported that age does not impact the smear cell percentage. However, lymphadenopathy, hepatomegaly and advanced staging demonstrated a significant effect on smudge cell percentage being <30%. Regarding laboratory variables we also reported that total leucocytic count (>35 $\times$ $10^9$/L), hemoglobin level ($\leq$9.2 g/dL), platelets count ($\leq$120 $\times$ $10^9$/L), absolute lymphocytic count (>31.2 $\times$ $10^9$/L), atypical lymphocytic count (>10%) together with LDT (>12 month) and cytogenetic abnormalities were the significant independent factors that affect smudge cells percentage and predict low percentage b eing < 30%. This may provide an insight into the role of smudge cell percentage as a surrogate marker of disease burden that may reflect biologic characteristics of the leukemic cells.

Smudge cell percentage was found to be an independent predictor of overall survival both in multivariate and univariate analyses and authors suggested the smudge cell percentage for risk stratification in addition to the other biologic parameters. Moreover, they showed that the highest percentage of smudge cells was found to be the most important indicator of the survival of the case and death of the malignant lymphocyte [5,17,15]. Although this finding needs validation in other studies, now this is a very important and practical point for clinicians and also scientists who would like to study the biology of CLL [20].

The smudge cell percentage has two potential advantages over other recently identified prognostic markers. First, it is nearly universally accessible because the microscopic evaluation of a blood

smear is typically available even to patients in countries with limited resources. Second, the smudge cell percentage can be retrospectively determined for patients participating in completed or ongoing studies based on the review of archived slides even if no other biologic samples were stored [8].

In Conclusion, our data indicate that low percentage of smudge cells (<30%) could be considered as an adverse prognostic predictor being associated with high risk prognostic markers. Because minimal technical resources are required, the estimation of smudge cells percentage is a potential, universally available, and applicable prognostic marker. It should be considered in an optimal prognostic stratification of CLL patients. Future studies directed towards elucidating the role of vimentin in various signaling pathways would open up new approaches for the biology of this disease and the development of new therapeutic targets.

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