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Integration of automated morphological features resolves a distinct group of atypical chronic lymphocytic leukemias with chromosomal aberrations

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ABSTRACT

Automated morphological assessment of peripheral blood slides has become an important modality facilitating characterization and quantification of cells in a uniform, fast and robust manner. In this study, we evaluated the morphological diversity in peripheral blood films of 94 chronic lymphocytic leukemia (CLL) patients using the DM1200 CellaVision automated microscopy system. Aberrant lymphocytes and smudge cells were enumerated and correlated with CLL immunophenotype, chromosomal aberrations and prognostic parameters. Herein, we show that the percentages of aberrant and smudge cells was highly variable between patients and did not correlate with each other. Increased aberrant lymphocytes and fewer smudge cells were associated with an atypical immunophenotype including low expression of CD23, higher levels of FMC7 and bright surface levels of CD20. High fraction of aberrant lymphocytes also was associated with trisomy 12. These cells were predominantly of small/medium size, sometimes with cleft nuclei. No correlation was noted between aberrant or smudge cells and clinical stage, CD38, ZA70 or time to first treatment. Taken together, automated morphological analysis of peripheral blood leukocytes emerged as a powerful and robust tool for the quantitative morphological stratification of CLL. Integration of the automated morphological features discriminates between different CLL phenotypes and distinct chromosomal aberrations.

1. Introduction

The diagnosis of chronic lymphocytic leukemia (CLL) is based on a set of diagnostic criteria for morphological and immunophenotyping features compatible with the disease [1]. However, in some patients due to a considerable heterogeneity in morphological and immunological phenotype, CLL presents a diagnostic challenge [2,3]. In particular, identification and definition of atypical CLL is important, since it has been demonstrated to correlate with recurrent chromosomal aberrations and poorer outcome [4,5].

While the assessment of cytogenetic abnormalities and immunophenotyping of CLL has improved considerably with the introduction of a panel of fluorescent in situ hybridization (FISH) probes and multi-parametric flow cytometry [6–10], morphological analysis remains problematic. This is due to a dependence on the ability of experts to classify, quantitate and integrate data obtained from bright field microscopy of peripheral blood films (PBFs). Further, the lack of a comprehensive, systematic and quantitative assessment of morphological features (apart from the enumeration of aberrant lymphocytes in PBF) may result in loss of data that could have supported a more informative morphological analysis. For example, smudge or basket cells are a well-known characteristic of PBFs in CLL [11]. Their formation has traditionally been considered a laboratory artifact caused by specific biological properties of CLL cells [12,13]. However, smudge cells have been demonstrated to be associated with various histochemical features of CLL cells and to carry a prognostic value [12–14]. For example, fewer smudge cells have been reported to associate with CD38 and ZAP70 positivity and a poorer clinical outcome [12–14].

In recent years, automated morphological assessment of PBFs has become a powerful modality that allows for a uniform, fast and robust characterization and quantification of cells. Conventionally,

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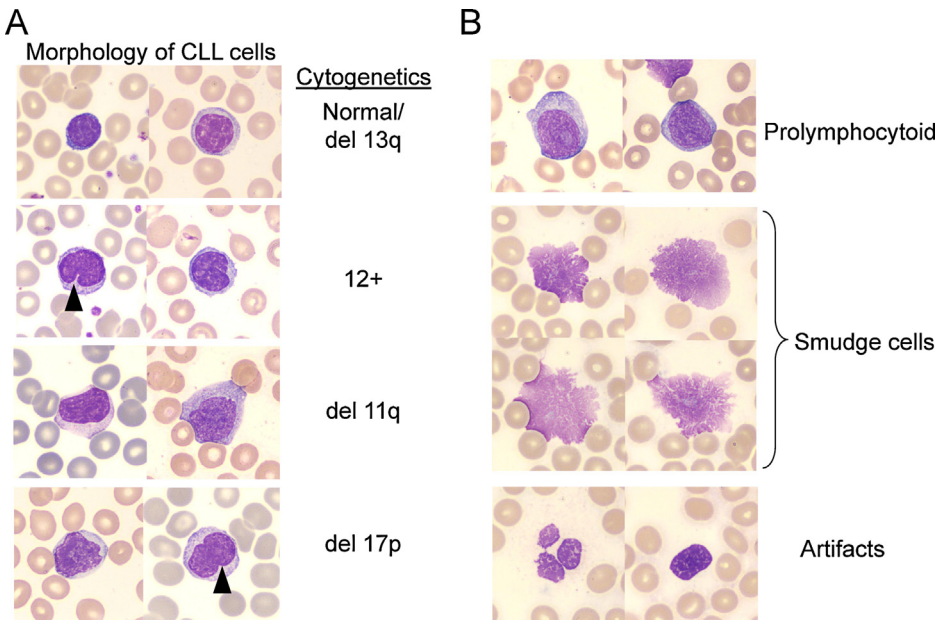

Fig. 1. Morphological features of peripheral blood CLL films. Leukocyte images were captured and classified by the CellaVision DM1200 automated microscopy system. (A) Lymphocytes with normal morphology (top row) and aberrant lymphocytes (three bottom lines) were classified separately. Major morphological abnormalities included size variations, irregular or cleft nuclei (arrowheads) and the presence of nucleoli.Then, morphological aberrations were classified according to their appearance in samples with cytogenetic abnormalities. (B) Prolymphocytoid cells (top) and smudge cells (middle) were enumerated by the CellaVision DM1200 system. Smudged neutrophils, eosinophils and other cells, as well as isolated nuclei with no remnants of cytoplasm (bottom) were excluded from this category.

this method has been applied to the analysis of routine blood tests. However, it could just as easily be applied to the analysis of highly abnormal blood films, such as the ones seen in lymphoproliferative disorders (LPD). This study evaluated the morphological diversity in peripheral blood films of 94 CLL patients using the DM1200 CellaVision automated microscopy system, and examined the association of morphologically distinct cells, such as aberrant lymphocytes and smudge cells, with immunophenotype, patient characteristics, prognostic parameters, and clinical outcome.

2. Materials and methods

2.1. Patient sample collection and staining

We examined routine peripheral blood samples from 94 patients with CLL and nine healthy controls. Blood samples were applied on laboratory glass slides (Beckman Coulter, Brea, CA, USA) by automated slide maker (LH, Beckman Coulter, Nyon, Switzerland), and stained by automated slide stainer (LH, Beckman Coulter, Nyon, Switzerland) according to the following protocol: Fixation with methanol (Merck, Darmstadt, Germany), 4 min; staining with May-Grunwald (Beckman Coulter, Brea, CA, USA), 4 min; wash with water, 1 min; staining with Giemsa (Beckman Coulter, Brea, CA, USA) in buffer $Na_2HPO_4{\cdot}2H_2O/KH_2PO_4$ pH = 6.8 (Merck, Darmstadt, Germany), 10 min; wash with water, 1 min; dry. Patients had a diagnosis of CLL in accordance with the 1996 National Cancer Institute working group criteria [1,15]. Clinical data and prognostic factors, including ZAP-70 and FISH were retrieved from the clinical records of the patients. β_2 microglobulin levels were measured by enzyme immunoassay (Orgentec Diagnostika, Mainz, Germany). The study was approved by the institutional Helsinki committee.

2.2. Morphological analysis of peripheral blood films

The morphological diversity in PBFs was analyzed by the CellaVision DM1200 automated microscopy system (CellaVision, Lund, Sweden). The stained slides were applied to the DM1200 analyzer. Two hundred leukocytes were captured from each slide and classified automatically. Slides were further reviewed by clinical laboratory specialists. Following classification, the percentage of aberrant lymphocytes (Fig. 1A) from total PBF lymphocytes was established, and the amount of smudge cells of all PBF leukocytes (Fig. 1B) and of all PBF lymphocytes was determined (correlations of the latter two parameters with the outcome variables yielded similar results that were somewhat better for the smudge cells/total-leukocytes, and therefore this parameter is presented in this paper). Of note, in some cases the number of smudge cells exceeded that of leukocytes resulting in percentages of smudge cells greater than 100%. Damaged non-lymphoid cells (primarily neutrophils) and isolated cell nuclei without cytoplasmic remnants were classified as artifacts and excluded from the 'smudge cell' category (Fig. 1B).

Typical CLL cells were defined as small/medium-sized lymphocytes with clumped chromatin and scanty cytoplasm [11] (Fig. 1A, top row). Lymphocytes with an atypical morphology consisted of three major groups: The first was characterized by lymphocytes with irregular nuclei including cells with cleft nuclei (Fig. 1A, second line from top); the second included relatively large lymphocytes some with nucleoli, in most cases with irregular nucleus (Fig. 1A, two bottom lines); and the third group contained a variety of morphological abnormalities (e.g. small cells with mature nuclei with nucleoli, not shown). Prolymphocytes were enumerated separately (Fig. 1B, top). Smudge cells were classified as a distinct group by the DM1200 system (Fig. 1B, middle). We excluded samples from patients with prolymphocytic leukemia (PLL) or Richter's syndrome, according to the previously established morphological criteria [11]. In cases of atypical CLL, the diagnosis of other lymphoma subtypes and in particular mantle cell leukemia with leukemic phase was excluded by bone marrow or lymph nodes biopsies (e.g. negative immunohistochemical staining for cyclin D1) and/or by the absence of t(11;14)(q13;q32) determined by peripheral blood FISH.

2.3. Flow cytometric analysis

CLL cells (1×10^6) were incubated with the designated antibodies for 30 min at 4° in the dark, washed and treated with FACS Lysing Solution for whole blood for 12 min in room temperature. Cells were subsequently washed with PBS. We acquired 3×10^4 events from each sample by FACS Calibur or FACS CantoII, and analyzed them using the CellQuest or the DIVA software respectively (Becton Dickinson, San Jose, CA, USA). The following anti-human antibodies were utilized: CD45-PerCP (Clone 2D1), CD19-APC (Clone SJ25C1), CD19-FITC (Clone 4G7), CD20-FITC (Clone L27), CD23-PE (Clone EBVCS-5), FMC7-FITC (Clone FMC7) and FACS Lysing Solution were from BD Biosciences (San Jose, CA, USA). Anti-human CD5-PE (Clone BL1a) was from Beckman Coulter (Fullerton, CA, USA). Cell staining and FACS analysis of ZAP-70 expression were performed similar to the method described by Crespo et al. [16]. Results for CD20, CD23 and FMC7 expression levels were further subgrouped based on the published literature: CD23 was dichotomized to negative/low or high expression subgroups using a cut-off of 50% positive CLL cells [17–19]; FMC7 was classified into negative/low or high expression subgroups using a cut-off of 40% positive CLL cells [11]. CD20 and immunoglobulin light chain expression levels were subclasiffied into low, intermediate and high flouresence intensity CLL cells.

2.4. Fluorescence in situ hybridization analysis

Interphase FISH analysis was performed from the PBF unstimulated cell culture preparations fixed in methanol and acetic acid (3:1). The DNA probe sets included: *MYB*(6q23.3), *ATM* (11q22.3), D12Z3 (CEP 12), D13S319 (13q14.3) and