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Publisher's version / Version de l'éditeur:

<https://doi.org/10.1002/jemt.22278>

Microscopy Research and Technique, 76, 11, pp. 1147-1153, 2013-08-20

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Morphology and Expression Status Investigations of Specific Surface Markers on B-Cell Chronic Lymphocytic Leukemia Cells

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KEY WORDS B-cell chronic lymphocytic leukemia; CD5; CD19; CD20; CD38; fluorescence microscope; ImageJ

ABSTRACT The morphology of cells and expression status of specific surface markers [cluster of differentiation (CD)], such as CD5, CD19, CD20, CD38, and CD45, have long been considered as the essential indicators for the diagnosis and prognosis of B-cell chronic lymphocytic leukemia (B-CLL). Clinically, it is difficult to simultaneously obtain cell morphology and distribution of surface markers with flow cytometry, especially for some surrogate markers such as CD38. Here, as an alternative and complementary prognostic method, fluorescence microscopy and image processing method are introduced to directly visualize the cells from patients and to quantitatively determine the expression status of surface markers. In this study, the morphological parameters of B-CLL cells were measured to establish the correlation between the cellular morphology and the surface marker expression. It was clear that the CD38+ and CD38− B-CLL cells from the same CD38+ patients had hardly any size differences; however, an increase in perimeter was observed for CD38− patients. Moreover, the expression level of the receptors on the cell was independent of the cell size. There was no evidence showing that the expression intensities of CD19 and CD38 were related to each other for the CD38+ B-CLL cells. On the same cells, CD5 was more selectively expressed on the cell membrane; however, the expression patterns suggested that the cell membrane of CD38− B-CLL cells contained the least expression level of CD19. *Microsc. Res. Tech.* 76:1147–1153, 2013. © 2013 Wiley Periodicals, Inc.

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is identified as a disease that has both homogeneity and heterogeneity features (Caligaris-Cappio and Ghia, 2007; Chiorazzi et al., 2005; Deaglio et al., 2003). Its homogeneity is mainly reflected by its phenotype expression consistency (Caligaris-Cappio and Ghia, 2007; Deaglio et al., 2003; Matutes, 2007). The heterogeneous aspect of B-CLL is associated with its clinical course variations: the progress of the B-CLL can be either indolent or aggressive (Caligaris-Cappio and Ghia, 2007; Deaglio et al., 2003, 2006). As previously introduced, the cellular morphology is one of the most important diagnostic factors for B-CLL. Matutes (2007) considered cellular morphology and surface marker expression status as two first-line laboratory investigations. Several factors have been proved to have significant impacts on the determination of subtype of B-CLL, which is essential both clinically and in leukemia research, including the gender, cellular morphology, bone marrow pattern, immunophenotype, chromosomal abnormalities, and IgV_H gene mutation status (Deterre et al., 2000). Several reports had indicated that abnormal morphology was linked to the unmu-

tated IgV_H region so as to positive expression of CD38, which is associated with a more aggressive case (Deaglio et al., 2007; Reyes et al., 1997). The threshold for the diagnosis of more aggressive B-CLL is the presence of more than 15% abnormal B-CLL cells (Matutes, 2007). Although there has been no direct evidence showing any causal nexus between CD38 expression level and the unmutated status of IgV_H gene, Deaglio et al. (2006) and others (Chu et al., 2010) have suggested that the unmutated IgV_H gene associated to the positive CD38 expression (Chiorazzi et al., 2010; Deaglio et al., 2003), indicating CD38 can be a reliable prognostic marker for B-CLL patients. Hence, the expression status of CD38 is one of the most important considerations of B-CLL for both clinical and research purposes (Hus et al., 2006).

Additional Supporting Information may be found in the online version of this article.

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Received 22 June 2013; accepted in revised form 29 July 2013

Contract grant sponsor: NSERC Strategic Network for Bioplasmonic Systems (BiopSys).

DOI 10.1002/jemt.22278

Published online 20 August 2013 in Wiley Online Library (wileyonlinelibrary.com).

Fig. 1. Bright-field and fluorescence images of white blood cells: bright-field images (A) and (D); fluorescence images of FITC-labeled CD19 (B); and PE-labeled CD38 (C). The cells present in images (B) and (C) are CD19+ and CD38+ cells from Patient II, respectively. Cells coexpressing CD19 and CD38 appearing in both (B) and (C) were indicated in yellow circles. The cells labeled α , β , γ , and δ in (D)

represent four types of morphologies observed: α represents the most common white blood cell morphology observed in CD38+ B-CLL samples; β represents swelled α cells; γ represents the CD19-/CD38+ cell; and δ represents CD19-/CD38- cell. The scale bars are 20 μ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.intelibrary.com).]

Fluorescent flow cytometry is the most commonly used technique in measuring B-CLL cell surface markers. To diagnose a patient as CD38+ B-CLL, at least 20 or 30% of total B-cells should be CD38+ (Deaglio et al., 2006; Hamblin, 2003; Hus et al., 2006). However, for some patients, the CD38 expression often presents as a continuous spectrum in its fluorescence intensity on flow cytometry graphs (see Supporting Information Scheme S1). Thus, it is difficult to have a clear cutoff between positive and negative expression levels using flow cytometry alone.

Although flow cytometry analysis can provide phenotypic profile for the diagnosis of B-CLL, the heterogeneous cell morphology cannot be easily evaluated by conventional flow cytometry (Almasri et al., 1992; Cabezudo et al., 1999; Pekarsky et al., 2005). Using fluorescence microscopy combined with automated data processing to image individual cells, the B-CLL cell morphology can be directly visualized, and the characteristic expression status of surface markers can be simultaneously obtained. In this study, the expression status will be evaluated in three aspects: (1) the expression level of the surface markers, which can be calculated as the ratio of positive expressed cells to the total cells; (2) the expression intensity that can be determined by the fluorescence intensity, which reflects the amount of surface marker expressed on the cells; (3) and the expression pattern of the surface marker that mirrors its expression position selectivity on the cell surface. Furthermore, the cell size distribution is found to be a morphological factor differentiating B-CLL cells. Similar to flow cytometry diagnosis, all the above analyses are determined based on fluorescence image analysis. The purpose of these investigations is to correlate cellular morphology, expression status, and B-CLL types (less or more aggressive B-CLLs) to give complementary and improved prognosis of B-CLL.

METHODS

Sample Preparation

B-CLL cell samples were obtained from the samples used for diagnosis of the B-CLL patients in flow cytometry

analyses at Mount Sinai Hospital, Toronto, ON. Briefly, the red blood cells were removed by using the lysing buffer (Beckman Coulter TQ Prep.). The B-CLL cells were enriched and suspended in 1% fetal calf serum (FCS) in PBS buffer (FCS buffer). Cells were resuspended, and then 20 μ L of fluorophore-labeled anti-surface marker antibodies (purchased from Beckman Coulter, Canada) were added to the cell solution (see Supporting Information for details).

Cell Imaging and Image Processing

All fluorescence and bright-field images were taken on an Olympus 1X81 fluorescence microscope equipped with a high-sensitivity CCD camera (Cascade 512, Photometrics) and a 60 $\times$ 1.45 NA Plan Apochromat objective. Depending on their absorbance characteristics, fluorophores were excited by lasers of appropriate wavelengths (488 nm argon and 543 or 633 nm He-Ne lasers), and the emitted fluorescence was collected using filter sets with dichroic mirrors. More specific technical details and analysis Macro design can be found in the Supporting Information. Briefly, customized macros in ImageJ were developed and used to efficiently locate cells on each captured fluorescence image and to measure their sizes in perimeters, as well as to determine the fluorescence intensities of each surface marker on a given cell. For both macros, up to 2,000 images can be loaded at the same time. Once the analysis of one image is finished, the next uploaded image is opened and analyzed automatically. The final results including the long and short diameters, the intensity of the cell, and its quadrant intensities are summarized and saved as a text file for further analysis.

RESULTS

Cellular Morphology Analysis

One of the most remarkable functions of image processing technique is the investigation of cell morphologies. Representative bright-field and fluorescence images of typical B-CLL cells are given in Figure 1. All cells present in a given area were imaged in bright-field mode; however, cells visualized through a filter

Fig. 2. Perimeters of CD38+ B-CLL cells measured by using surface marker-specific filter sets. The average perimeters of CD38+ B-CLL cells for each patient, captured using CD19-FITC filter set, were 22.1, 25.1, and 23.4 μm , respectively; the average perimeters of the same cell samples, measured in CD38-PE- or CD38-PC5-specific filter

sets, were 22.2, 25.2 and 23.3 μm , respectively. An arbitrary error of $\pm 5\%$ was set by considering the uncertainty of $2 (\pm 1)$ pixels in diameter determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

set were only those to which particular fluorophore-labeled antibodies were bound. Most of the B-CLL cells had a smooth round shape of diameter of 6–7 μm (Fig. 1A). For this particular B-CLL sample, surface marker CD19 was bound with fluorescein (FITC)-labeled anti-CD19 antibody (CD19-FITC), and CD38 was bound with phycoerythrin (PE)-labeled anti-CD38 antibody (CD38-PE). Figures 1B and 1C showed two representative epifluorescence images recorded using FITC filter set for CD19 and PE filter for CD38. All the cells visualized in Figure 1B are CD19-positive (CD19+) cells, whereas the ones observed in Figure 1C are CD38-positive (CD38+) cells. The images shown in Figures 1B and 1C were taken from the same area of the same sample, which suggested that cells at the same position in each image were very likely the same ones. In Figures 1B and 1C, the cells coexpressing CD19 and CD38 were circled in yellow; they were termed CD19+/CD38+ cells, whereas the cells expressing only CD19+ were termed CD19+/CD38– cells. The CD19+/CD38+ coexpression ratio was calculated by using the number of CD38+ B-CLL cells divided by the overall number of B-CLL cells. The coexpression ratio calculated for Patients I–VII was 57.5, 43.0, 50.5, 8.6, 0.5, 1.8, and 0.7%, respectively.

During image processing, we have found that the morphology and size of B-CLL cells were not identical for different patients, sometimes even different for the same patient. To give an example, Figure 1D shows four cell morphologies, representative of the four most frequently observed morphologies in our experiments. They were not necessary to be seen from the same patients. The morphology of labeled cell α was the most common CD19+/CD38+ and CD19+/CD38– cells present in the diagnosed CD38+ B-CLL patients, for example, Patients I, II, and III. They had a large and condensed chromatin but a very small cytoplasm. In addition to uniform B-CLL cells, several larger cells or some elliptically shaped cells were observed. The cell labeled β in Figure 1D shows a case of B-CLL cell “swelling,” where the nucleus was as large as that of a normal B-CLL cell and the cytoplasm area was enlarged significantly. In addition, the boundary between the nucleus and the cytoplasm became clear.

In Figure 1D, morphology γ shows the most common type of CD19–/CD38+ cells, and less than 1% of the cells had morphology δ as shown in Figure 1D.

Another advantage of using an imaging technique is the ability to correlate cell morphologies with surface marker expression levels. Although the majority of B-CLL cells were roughly round in shape, B-CLL cells were better described as a bright elliptic ring on the epifluorescence images. We thus used the perimeter instead of the diameter as a size indicator. Typical cells were roughly composed of 5-pixel thick rings, and the perimeters were determined using the middle (third) pixel rings. First, the average perimeters of CD38+ B-CLL cells were measured on captured images using both CD19- and CD38-specific filter sets, respectively. Again, the CD38+ B-CLL cells were both CD19+ and CD38+. Because of the limited number of CD38+ B-CLL cells, Patients IV, V, VI, and VII were not included in this case, only cell samples from CD38+ B-CLL patients I, II, and III were measured (Fig. 2). It is worth noting that the cell perimeters measured using different fluorescence labels and filter sets provided identical results for the same patient. In addition, for different patients, the variation of cell sizes is able to be identified. Specifically, Patient I had the smallest perimeter (22.1 μm), which was 13% smaller than the perimeter of Patient II, and 7% smaller than that of Patient III.

Second, the sizes of CD38+ and CD38– B-CLL cells were investigated. The histograms of perimeters for CD38+ (left column) and CD38– (right column) B-CLL cells from all CD38+ patients I, II, and III are shown in Supporting Information Figure S2. Note that although the difference of the cell sizes measured using different filter sets for recording the images are negligible (Fig. 2), to minimize the influences, only size measurements obtained from images of CD19-FITC are compared. Consistent with the CD38+ results, the mean perimeter of CD38– B-CLL cells of Patient I was the smallest. Interestingly, the average perimeters of CD38+ B-CLL cells were 2.3% and 3.3% larger than those of CD38– B-CLL cells for Patients I and II, respectively (Supporting Information Fig. S2). On the contrary, for Patient III, the average perimeter of

Fig. 3. Histograms of perimeters of all CD38+ B-CLL cells from CD38+ patients (I–III) (A); all CD38– B-CLL cells from CD38+ patients (I–III) (B); and all CD38– B-CLL cells from CD38– patients (IV–VII) (C). The mean perimeters of CD38+ and CD38– B-CLL cells in CD38+ B-CLL patients were 22.9 and 23.3 μm , respectively, with

narrow distributions. There were two fitted peaks (23.5 and 28.2 μm) for the mean perimeters of CD38– B-CLL cells from CD38– patients. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CD38+ B-CLL cells was 1.3% smaller (Supporting Information Fig. S2).

Third, the sizes of B-CLL cells in CD38– B-CLL patients (IV–VII) were investigated (Supporting Information Fig. S3). The variation of the smallest average size to the largest one was as high as 16%, which was similar to the result obtained for CD38+ B-CLL patients. Notably, the perimeter distributions for individual patients IV, VI, and VII were relatively large, and there were several “extremely” large cells present in the same sample.

Moreover, the data were summarized based on different B-CLL cell types, as shown in Figure 3. First, for all three CD38+ patients, the perimeter difference of two sets of cells (CD38+ and CD38– cells) from the same patient were small, and thus, it was safe to conclude that there was no appreciable size difference between CD38+ and CD38– B-CLL cells in the same CD38+ patient.

Lastly, the sizes of the same types of B-CLL cells were not preserved in different CD38+ B-CLL patients. The two populations of cell sizes found in Patient V and VII might cause the two distribution peaks observed in Figure 3C. Additionally, the mean perimeter of CD38– B-CLL cells in CD38+ patients (23.3 μm) was generally smaller than those in CD38– patients (26.8 μm). As mentioned above, B-CLL, with better clinical outcome, has more than 85% of cells smaller than normal lymphocytes, and with worse clinical outcome, it has more than 85% of cells larger than normal lymphocytes (Chiorazzi et al., 2010). This is not consistent with our results, where the larger cells have been observed in better clinical outcome patient samples as well.

Fluorescence Intensity Analysis

Using imaging technique, we have analyzed the expression level of one specific surface marker, not only with regards to the number of cells expressing it

but also the amount of the surface marker on each cell. To analyze the expression status of surface markers, both the number of positively expressed cells and the amount of specific surface markers on each cell have been analyzed. First of all, there were several issues to be carefully considered. Most obviously, the emission intensities of different fluorophores labeled on antibodies were not directly comparable because of their diverse quantum efficiencies. Moreover, the status of fluorescence-labeled antibody, such as the number of fluorophore molecules per antibody or the binding ability and affinity to the surface markers, would certainly limit the direct comparisons of different fluorophore-labeled surface markers. Besides, the bleaching and quenching levels of different fluorophore labels may vary because of different excitation conditions, thus causing reduced fluorescence intensities with fluctuations. Taken all these factors into account as much as possible, we would only compare the surface markers labeled with the same fluorophores in different patients (such as CD19-FITC in Patients I, II, and III) using the same excitation and similar emission conditions (sample substrates could not be the same) for imaging. The correlation between cell size and surface marker expression level was investigated by analyzing the relationship between perimeter and fluorescence intensity of each cell. An example is illustrated in Figure 4. For both surface markers CD19 and CD38, the perimeters and intensities of the cells were nearly in a linear relationship with a flat slope. The variation of fluorescence intensities indicated that the amount of surface markers expressed on the cells varied and this amount would not change with the increasing cell size.

The intensity distributions of CD19-FITC and CD38-PE expressed on CD38+ B-CLL cells from CD38+ B-CLL patient I were measured on 175 cells,

Fig. 4. The correlation between expression intensity of surface markers and cell sizes. The measured 175 cells were all CD38+ B-CLL cells from Patient I: CD19 was labeled by FITC (A) and CD38 was labeled by PE (B). Solid lines were linear fit to the data. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and the results are summarized in Figure 5. The order was sorted with the ascending fluorescence intensities for CD19 in Figure 5A, and CD38 in Figure 5B. For majority of the cells, with increase of the CD19-FITC intensities, on the same CD38+ B-CLL cells, CD38-PE intensities were changing without any obvious associations; extremely high and extremely low readings exist in both cases. Meanwhile, for some of the cells (~30%), their CD19 intensities were found to be related to CD38 intensities.

The correlation of other surface markers such as CD5, CD19, and CD20 were important in the study of B-CLL (Almasri et al., 1992; Ginaldi et al., 1998; Insel et al., 2010; Sheikh et al., 2002). Two sets of comparisons were considered: one was between CD5-FITC and CD19-FITC in Patients IV and VII, as illustrated in Figure 6A, and the other was between CD19-phycoerythrin cyanin7 (PC7) and CD20-PC7 in Patients IV, V, VI, and VII, as shown in Figure 6B.

In Figure 6A, within the same patient, the fluorescence intensities of CD19 were about 50% higher than those of CD5; for the same surface marker in two different patients, the intensities for Patient VII were 100% larger than their intensities for Patient IV. For the sets of CD19 and CD20, the intensities of CD19 were 60% and 20% higher than CD20 for Patients IV and V, and 90% and 30% lower than CD20 for Patients VI and VII, respectively (Fig. 6B).

Surface Marker Distribution Patterns on CD38+ and CD38- B-CLL Cells

To investigate the surface marker distribution on B-CLL cells, each cell was divided into four quarters, and the fluorescence intensities of these four quadrants

Fig. 5. Fluorescence intensity correlation of CD19 and CD38 on CD38+ B-CLL cells of Patient I. The intensities of CD19 were sorted with the ascending order (A); and for the same set of cells, the intensities of CD38 were sorted with the ascending order (B). The intensities of CD38 and CD19 were drawn accordingly. All the CD19 were labeled by FITC, and the CD38 was labeled by PE. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were measured. The quadrant intensity ratio was then calculated by using the fluorescence intensity of each quadrant divided by the sum of those four quadrants. This ratio represents the distribution evenness of specific surface markers. The standard deviations of calculated quadrant intensity ratios reveal the variation of the intensity ratios. When surface markers are evenly distributed on the cell surface, the average quadrant intensity ratio of each quarter should be 0.25. Smaller standard deviations indicate a more homogeneous distribution, and larger deviations indicate an increased heterogeneous distribution of surface markers on B-CLL cells. The quadrant intensity ratios from four groups of B-CLL cells were considered: (1) CD38+ cells measured using both CD19-FITC and CD19-PC7 filter sets, respectively; (2) CD38+ B-CLL cells measured with both CD38-PE and CD38-PC5 filter sets; (3) CD38- B-CLL cells from CD38+ patients using CD19-specific filter sets; and (4) CD38- B-CLL cells from CD38- patients using CD19-specific filter sets. It is worth noting that the values measured using different filter sets can be now combined in one histogram. This is because the quadrant intensity ratio is an absolute value, which eliminates the error among different fluorophore labels. The histograms associated with four sets of B-CLL cells are shown in Supporting Information Figure S4. The quadrant intensity ratios for all sets of cells involved in Supporting Information Figure S4 were all around 0.25, and the corresponding standard deviations were 0.018, 0.022, 0.026, and 0.031, respectively.

Fig. 6. Fluorescence intensity of B-CLL cells from Patient IV to VII. The comparison of fluorescence intensities of CD5-FITC and CD19-FITC (A) and CD19-PC7 and CD20-PC7 (B) is shown. The mean intensities and their standard deviations were obtained from the histograms fitted by Gaussian distribution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Furthermore, quadrant intensity ratios of four surface markers on B-CLL cells were considered: CD5, CD19, CD20, and CD38. The corresponding histograms are shown in Supporting Information Figure S5. The mean ratios for all four surface markers were very close to 0.25; however, the standard deviation varies: CD5 had the largest standard deviation, whereas CD19 and CD20 had the lowest standard deviations, with the value for CD38 in between.

DISCUSSION

Fluorescence imaging has allowed us to directly visualize individual B-CLL cells with identified surface markers using specific labeled antibodies. The images taken from the same area of the same sample suggested that cells at the same position in each image were very likely the same cells. From the images, it is obvious that the fluorescence intensities of cells were not identical. Using the same filter set, some cells showed stronger intensity than others. Assuming the number of the same fluorescent labels is similar per antibody for all antibodies, the fluorescence intensity is then closely related to the expression status for the corresponding surface markers. Therefore, the fluorescence intensity can be correlated to the expression amount of surface markers and to the location of these labels on the cell membrane. Furthermore, using super-resolution techniques such as STED and STORM to obtain high-resolution images may provide direct insights into the expression level and correlation among surface markers.

The morphology and size of B-CLL cells were not identical even for the same patient; and the expression of surface markers could also correspond to the morphology and size variation of B-CLL cells. Two possibilities for the presence of large cells were cell swelling and the presence of different types of cells. The swelling of cells may happen when the concentration of

cytosol inside the cell becomes larger and an osmotic pressure gradient is generated. The other types of larger cells were probably not B lymphocytes. Morphology γ showed the most common type of CD19⁺ but CD38⁺ cell, which was most likely a T lymphocyte or a natural killer cell (Abousamra et al., 2009). Morphology δ was CD19⁺ and CD38⁺, and likely not lymphocytes but rather neutrophil cells.

The CD19⁺/CD38⁺ coexpression ratio can be used for the diagnosis of B-CLL and related to clinical outcome. For the samples in this study, CLL cells accounted for 90–99% of the total B-cells based on flow cytometry results. Therefore, most CD19⁺ cells were CLL cells. In our microscopy studies, we have counted the CD19⁺ cells as a total number for comparison with other marker on B-CLL cells. Based on the diagnosis threshold of 30% for CD38⁺ expression ratio (Deaglio et al., 2006), Patients I, II, and III were most likely to be CD38⁺ B-CLL patients, whereas Patients IV, V, VI, and VII were CD38⁺ B-CLL patients. These conclusions were consistent with the flow cytometry results. In addition, for the obscure flow cytometry readings, such as the one for Patient II, the coexpression ratio results obtained from cell imaging technique supply a complementary diagnosis for the physicians.

One type of fluorescence-labeled anti-surface marker antibody is believed to specifically target to corresponding surface marker, and the fluorescence signals of a given cell are quantitatively detectable. Ideally, this fluorescence signal (intensity) should be proportional to the amount of corresponding surface markers on the cell membrane. Assuming that the surface markers are homogeneously distributed on cell surfaces, the fluorescence intensity of a given surface markers should depend linearly on the cell size (or surface area). The variation of fluorescence intensities obtained in the measurements indicated that the amounts of surface markers expressed on the cells varied, and the flat slope between two parameters (Fig. 4) indicated that the amount of the surface markers might be fixed for individual cells and would not be altered with the increasing of cell membrane area.

Most of the surface markers must collaborate with other markers, proteins, enzymes, and lipids to complete cellular functions and processes. They are managed to participate in a variety of cell mechanisms by forming functional complex. Based on the assumptions, the intensities for CD19 and CD38 were assumed to be correlated to each other: the increase of CD19 or CD38 intensities were predicted to show an ascending order of CD38 or CD19 intensities, respectively. However, only 30% of the results showed significant correlations between the expression of CD19 and CD38. The fluorescence intensity of CD19 was about 50% higher than that of CD5 on the same B-CLL cell (Fig. 6A), indicating that for both Patients IV and VII, the amount of CD19 markers was expressed more than CD5 on the B-cells. As for why there were more CD19⁺ cells than CD20⁺ cells (Fig. 6B), it is well known that at later stage of differentiation, the CD20 expression is downregulated on these B-lymphocytes, whereas CD19 expression is upregulated on B-lymphocytes (Ginaldi et al., 1998; Rawstron and Hillmen, 2008).

In some images, it was obvious that some fluorescence labels were not evenly distributed on the cell

surface, which suggested that the surface markers might be clustered and expressed heterogeneously on the cell surface. In most cases, on the same cell, the expression patterns of different surface markers were not identical, which meant that different surface markers could express on different positions on the membrane. First, on the same set of B-CLL cells (e.g., CD38+ cells), the expression of CD38 was more heterogeneous than the expression of CD19. Second, within the same type of patients (i.e., CD38+ patients), CD19 expressed more homogeneously on CD38+ B-CLL cells than on CD38- B-CLL cells. Third, on the same type of B-CLL cells, such as CD38- B-CLL cells, the CD19 expressed more heterogeneous on CD38- patients than on CD38+ patients.

The standard deviation differences were most possibly caused by the surface markers distribution pattern. The homogeneity of the surface marker distribution revealed the expression preference on the cell membrane. A more heterogeneous distribution of a surface marker suggested that it expressed on specific cell membrane components selectively and sensitively. On CD38+ B-CLL cells, the CD38 was more selectively expressed on the cell membrane than CD19. The selectivity of CD19 was not identical in different B-CLL cell types and patient types. Its expression on CD38- B-CLL cells from CD38- patients was found most sensitive on the cell membrane components. The membrane components of CD38+ cells and the CD38- B-CLL cells in CD38+ patients seemed to have less CD19 preferred compositions. Similarly, for the same set of cells, small standard deviations were found for CD19 and CD20 indicating a more homogeneous distributions. The larger deviation for CD5 indicates an increased heterogeneous distribution of CD5 on B-CLL cells. The restriction on the expression position of CD5 suggested that the CD5 was position sensitive. It can only express on specific parts of the membranes. There was almost no difference on the expression of CD19 and CD20 for healthy B-cells, which indicates that the membrane positions they expressed were identical. For the CD38, it functions as a receptor for the Ca^{2+} level control once it interacts with CD19 on the lipid raft parts of the cell membrane (Deaglio et al., 2010), which explains why its express pattern was similar to that of CD19.

In summary, we have demonstrated that as an alternative and complementary prognostic method, fluorescence microscopy and automated data analysis are introduced to directly visualize cells from patients and to quantitatively determine the expression status of surface markers. The expression status can be evaluated in three ways: (1) the expression level of the surface markers, which can be calculated as the ratio of positive expressed cells to the total cells; (2) the expression intensity that can be determined by the fluorescence intensity, which reflects the amount of surface marker expressed on the cells; (3) and the expression pattern of the surface marker that mirrors its expression position selectivity on the cell surface. To correlate cellular morphology, expression status, and B-CLL types, this imaging and processing method will provide new insights in the prognosis of B-CLL

subtypes (Caligaris-Cappio, 1996; Deaglio et al., 2006; Hamblin, 2007; Hus et al., 2006; Shanafelt et al., 2006).

REFERENCES

- Abousamra NK, El-Din MS, Azmy E. 2009. T-cell CD38 expression in B-chronic lymphocytic leukaemia. *Hematol Oncol* 27:82-89.
- Almasri NM, Duque RE, Iturraspe J, Everett E, Braylan RC. 1992. Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukemia. *Am J Hematol* 40:259-263.
- Cabezudo E, Carrara P, Morilla R, Matutes E. 1999. Quantitative analysis of CD79b, CD5 and CD19 in mature B-cell lymphoproliferative disorders. *Haematologica* 84:413-418.
- Caligaris-Cappio F. 1996. B-chronic lymphocytic leukemia: A malignancy of anti-self B cells. *Blood* 87:2615-2620.
- Caligaris-Cappio F, Ghia P. 2007. The normal counterpart to the chronic lymphocytic leukemia B cell. *Best Pract Res Clin Haematol* 20:385-397.
- Chiorazzi N, Allen SL, Ferrarini M. 2005. Clinical and laboratory parameters that define clinically relevant B-CLL subgroups. *Curr Top Microbiol Immunol* 294:109-133.
- Chiorazzi N, Damle RN, Wasil T. 2010. CD38 as a prognostic indicator in B cell chronic lymphocytic leukemia. U.S. Patent 7842461-B2.
- Chu CC, Cattera R, Zhang L, Didier S, Agagnina BM, Damle RN, Kaufman MS, Kolitz JE, Allen SL, Rai KR, Chiorazzi N. 2010. Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: Implications for patient outcome and cell of origin. *Blood* 115:3907-3915.
- Deaglio S, Capobianco A, Bergui L, Durig J, Morabito F, Dührsen U, Malavasi F. 2003. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood* 102:2146-2155.
- Deaglio S, Vaisitti T, Aydin S, Ferrero E, Malavasi F. 2006. In-tandem insight from basic science combined with clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. *Blood* 108:1135-1144.
- Deaglio S, Vaisitti T, Billington R, Bergui L, Omede P, Genazzani AA, Malavasi F. 2007. CD38/CD19: A lipid raft-dependent signaling complex in human B cells. *Blood* 109:5390-5398.
- Deaglio S, Aydin S, Grand MM, Vaisitti T, Bergui L, D'Arena G, Chiorino G, Malavasi F. 2010. CD38/CD31 interactions activate genetic pathways leading to proliferation and migration in chronic lymphocytic leukemia cells. *Mol Med* 16:87-91.
- Deterre P, Berthelot V, Bauvois B, Dallou A, Schuber F, Lund F. 2000. CD38 in T- and B-cell functions. *Chem Immunol* 75:146-168.
- Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Catovsky D. 1998. Levels of expression of CD19 and CD20 in chronic B cell leukaemia. *J Clin Pathol* 51:364-369.
- Hamblin TJ. 2003. CD38: What is it there for? *Blood* 102:1939-1940.
- Hamblin TJ. 2007. Prognostic markers in chronic lymphocytic leukaemia. *Best Pract Res Clin Haematol* 20:455-468.
- Hus I, Podhorecka M, Bojarska-Junak A, Rolinski J, Schmitt M, Sieklucka M, Wasik-Szczepanek E, Dmoszynska A. 2006. The clinical significance of ZAP-70 and CD38 expression in B-cell chronic lymphocytic leukaemia. *Ann Oncol* 17:683-690.
- Insel PA, Zhang L, Murray F, Kanter JR, Kipps T, Rassenti L. 2010. Diagnosis and treatment of chronic lymphocytic leukemia (CLL). U.S. Patent 7846664-B2.
- Matutes E. 2007. Differential diagnosis in chronic lymphocytic leukaemia. *Best Pract Res Clin Haematol* 20:367-384.
- Pekarsky Y, Calin GA, Aqeilan R, Croce CM. 2005. Chronic lymphocytic leukemia: Molecular genetics and animal models. *Curr Top Microbiol Immunol* 294:51-70.
- Rawstron AC, Hillmen P. 2008. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia reply. *New Engl J Med* 359:2066.
- Reyes E, Prieto A, Carrion F, Garcia-Suarez J, Esquivel F, Alvarez-Mon M. 1997. Morphological variants of leukemic cells in B-chronic lymphocytic leukemia are associated with different T cell and NK cell abnormalities. *Am J Hematol* 55:175-182.
- Shanafelt TD, Byrd JC, Call TG, Zent CS, Kay NE. 2006. Narrative review: Initial management of newly diagnosed, early-stage chronic lymphocytic leukemia. *Ann Int Med* 145:435-447.
- Sheikh SS, Kallakury BVS, Al-Kuraya KA, Meck J, Hartmann DP, Bagg A. 2002. CD5-negative, CD10-negative small B-cell leukemia: Variant of chronic lymphocytic leukemia or a distinct entity? *Am J Hematol* 71:306-310.