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**IMMUNOPHENOTYPE OF LEUKEMIC CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH NOTCH1 AND SF3B1 GENE MUTATIONS**

**Background.** The typical chronic lymphocytic leukemia (CLL) immunophenotype is vital for diagnosis, but the expression of some antigens varies and has prognostic value. There are data that reduced CD20 expression is associated with NOTCH1 and SF3B1 gene mutations. **Aim.** To determine a high-risk group of CLL patients for prediction of unfavorable NOTCH1 and SF3B1 gene mutations based on immunophenotyping of leukemic cells. **Materials and Methods.** Flow cytometric and molecular-genetic analysis (mutations of NOTCH1, SF3B1, and TP53 genes using the polymerase chain reaction followed by direct sequencing) was performed in a group of 86 previously untreated CLL patients. **Results.** The immunophenotype of leukemic cells of all examined patients met the criteria of CLL diagnosis. NOTCH1 gene mutations were found in 21 patients (24.4%), and SF3B1 gene mutations — in 7 patients (8.1%). There were no TP53 gene mutations among the examined patients. A decreased number of CD20+CD5+ cells and a downward trend in the relative index of mean fluorescence intensity (iMFI) of CD20+ cells were found in patients with NOTCH1 and SF3B1 gene mutations. Based on the iMFI level (higher and/or lower than 3.0) and the number of CD20+CD5+ cells among all B-cells (higher and/or lower than 50%), we distinguished CLL cases with low and relatively high levels of CD20 antigen expression. Using ROC analysis and the parameter of low CD20 antigen expression, we could predict the presence of NOTCH1 and SF3B1 gene mutations in 73.3 ± 0.06% of patients (p = 0.001). The risk of NOTCH1 and SF3B1 gene mutations in cases with low CD20 antigen expression was 6.96 (95% CI = 2.53—19.18; p = 0.0001). The revealed regularities were statistically significant for patients in whom the diagnosis was established in all Binet — Rai stages except A0—AI. **Conclusion.** Our data confirmed a reduced CD20 expression in CLL patients with NOTCH1 and SF3B1 mutations. In addition, an approach was proposed to identify high-risk CLL patients for prediction of such mutations: previously untreated CLL patients at advanced Binet — Rai stages (BII, CIII, CIV) with a reduced number of double-positive CD20+CD5+ cells in peripheral blood and/or low iMFI of CD20+ cells.

**Keywords:** chronic lymphocytic leukemia, NOTCH1 and SF3B1 gene mutations, CD20+CD5+ cells.
Chronic lymphocytic leukemia (CLL) of B-cell origin is one of the most common oncohematological diseases in the adult population. CLL leukemic cells are characterized by the expression of CD5 and CD23 antigens, reduced expression of B-cell markers (CD20, CD79b, CD79a, FMC7, and surface immunoglobulins), antigen CD81 and common leukocyte antigen CD45 (compared to other lymphoproliferative diseases), a high expression of CD43 and CD200 antigens, a negative reaction with monoclonal antibodies (MoAbs) against CD10, CD103, and CD123 antigens as well as T-cell (except CD5) and myeloid markers [1]. However, certain immunophenotypic features of CLL are known. In particular, a high expression of ZAP70, CD38, and CD49d antigens and a low expression of CD21 antigen are associated with an unfavorable course of the disease [2—5]. In the last years, significant progress has been made in the treatment of CLL and stratification of patients into risk groups, which predicted the time to the first therapy and the choice of treatment approaches. A risk stratification is based on several factors: the age of patients, the stage of the disease and the related clinical and hematological indicators, serum beta-2 microglobulin level, the mutational status of immunoglobulin heavy chain (IGHV) genes, and del17p by fluorescence in situ hybridization or TP53 mutation [6, 7]. More recently, some specific mutations such as NOTCH1 and SF3B1 further refined the current prognostication system in CLL [8]. At the same time, a significantly reduced CD20 antigen expression was found on leukemic cells with the NOTCH1 and SF3B1 gene mutations [9, 10]. The aim of this work was to determine a high-risk group of CLL patients for the prediction of unfavorable NOTCH1 and SF3B1 gene mutations based on the immunophenotyping of leukemic cells.

Materials and Methods

The data of the immunophenotyping of peripheral blood samples of 86 CLL patients (67 men, 19 women) aged 56.7 ± 1.88 years (median 57.5 years), which was carried out simultaneously with obtaining samples for molecular genetic studies, were analyzed. The patients were treated at the National Research Center for Radiation Medicine, Kyiv, Ukraine. The study was approved by the local Medical Ethics Committee, and all patients gave their informed consent prior to participation in it. The diagnosis of CLL was based on the clinical history, lymphocyte morphology, and immunophenotypic criteria. A stage of the disease was established according to Rai [11, 12] and Binet [13]. Patients were examined at diagnosis (71 patients, 82.5% of all examined) or before first-line therapy (15 patients, 17.5%).

Immunophenotyping of peripheral blood cells was performed using a direct immunofluorescence test and the following combination of MoAbs: CD45/CD14; CD10/CD19; CD20/CD5; CD22/CD3; CD7/CD33; HLA-DR/CD13; CD4/CD16+CD56. The MoAbs were conjugated with FITC (fluorescein isothiocyanate) or PE (phycoerythrin). The study was performed on a FACSCalibur laser flow cytometer (Becton Dickinson, USA). The percentage of positive cells and the relative index of mean fluorescence intensity of cells (iMFI) were evaluated. The latter was calculated as a ratio of the MFI of cells reacting with a certain antibody to the MFI of the unstained cells.

The mutational status of IGHV genes, mutations of NOTCH1 and SF3B1 genes as well as TP53 were determined as described previously [14, 15].

Statistical analyses were performed with SPSS software (version 17.0; SPSS, Inc., Chicago, IL). All statistical tests were two-sided and considered statistically significant at $p < 0.05$.

Results and Discussion

The NOTCH1 gene mutations were found in 21 patients (24.4%), and the SF3B1 gene mutations — in 7 patients (8.1%). All identified
**Fig. 1.** Binet — Rai stages of examined CLL patients

NOTCH1 gene mutations were represented by typical two base pair deletion c.7544_7545delCT (P2514fs*4), all SF3B1 gene mutations were heterozygous missense substitutions. Only two patients harbored mutations in both NOTCH1 and SF3B1 genes. CLL patients without the NOTCH1 and SF3B1 gene mutations (59 cases, 68.6%) were included in group I, and 27 (31.4%) patients with the NOTCH1 and/or SF3B1 gene mutations were included in group II. There were no TP53 gene mutations among the examined patients.

The frequency of NOTCH1 and SF3B1 gene mutations did not differ among patients examined at diagnosis (23 of 71 patients, 32.4%) or before first-line therapy (4 of 15 patients, 26.6%), \( p = 0.664 \).

The distribution of patients by the Binet — Rai stage of disease was as follows: 48 patients were at the A0—AI stage (55.8%), 29 patients had stage BII (33.7%), and 9 patients were diagnosed at the CIII—CIV stage (10.5%). The mutations of both genes were more common among patients who were diagnosed at Binet stages B and C (Fig. 1).

Patients with NOTCH1 gene mutations, in contrast to the patients of group I, had a higher initial white blood cell count, WBC (73.04 ± 17.6 · 10^9/L vs. 35.56 ± 4.75 · 10^9/L, \( p = 0.001 \)), and a higher WBC at the time of examination (78.97 ± 17.6 · 10^9/L vs. 35.74 ± 6.21 · 10^9/L, \( p = 0.013 \)). WBC at diagnosis (38.62 ± 13.75 · 10^9/L) and at the time of examination (39.14 ± 13.75 · 10^9/L) in patients with the SF3B1 gene mutations did not differ from these in patients of group I (\( p = 0.856 \) and \( p = 0.852 \), respectively).

36 patients had mutated (M, 41.9%), and 50 patients had unmutated IGHV genes (UM, 58.1%). Most patients with M IGHV genes (35 cases) were from group I. On the contrary, only one patient from group II had M IGHV genes (\( p = 0.0001 \)).

The immunophenotype of leukemic cells of all examined patients met the flow-cytometric criteria of CLL diagnosis. The cells expressed CD19 antigens (65.78 ± 2.84%; iMFI 4.38 ± 0.32), CD22 (61.77 ± 2.27%; iMFI 6.02 ± 1.13), CD20 (58.81 ± 2.49%; iMFI 5.66 ± 0.62), CD5 (77.08 ± 2.34%; iMFI 7.57 ± 0.85), HLA-DR (74.89 ± 1.6%; iMFI 9.38 ± 1.16), CD23 (73.57 ± 1.85%; iMFI 7.57 ± 0.83). The mean of the CD3-positive T-lymphocytes was 8.09 ± 0.76%.

The CD38 antigen expression varied from 0.08% to 88.1% (mean 21.41 ± 2.68%). The cases with an expression level higher than 20% were considered as CD38-positive, according to the recommendations [16]. A high level of CD38 antigen expression (≥ 20 %) was found in 27 cases (31.4%): 18.75% among patients at the A0—AI stage, 37.9% at the BII stage, and 77.8% at the CIII—CIV stage (\( p = 0.001 \)). Kriston et al. [17] showed that low CD23 antigen expression correlated with high CD38 antigen expression and the presence of trisomy 12 [17]. In the observed CLL patients, we also found an inverse correlation between the number of CD23 and CD38-positive cells (\( r = -0.274; \ p = 0.03 \)). The average number of CD23-positive lymphocytes was 76.91 ± 1.93% among the CD38-positive cases and 66.89 ± 3.64% among the CD38-negative cases, \( p = 0.01 \). However, iMFI of CD23 expression depending on CD38 expression did not differ: 7.52 ± 1.02 and 7.62 ± 1.56, respectively, \( p = 0.927 \).

The flow cytometric data in patients with M and UM IGHV genes were similar, except
a higher number of CD38-positive cases: 19.4% and 44%, respectively, \( p = 0.021 \).

The total number of B-lymphocytes (their content was evaluated as the largest percent of cells reacting with one of the B-cell antigens, CD22 or CD19) did not differ in patients of group I and group II, however, leukemic B-cells differed in the expression of some antigens.

A decreased number of double-positive CD20\(^+\)CD5\(^+\) cells, a number of CD20\(^+\)CD5\(^+\) cells among all B-cells, and a downward trend in iMFI of CD20-positive cells were found in patients of group II (Table 1). A percentage of CD20\(^+\)CD5\(^+\) cells below 50% among all B-cells was detected only in 8 patients (13.6%) of group I compared to 12 cases (44.4%) in group II (\( p = 0.0016 \)). Similarly, iMFI of CD20\(^+\) cells below 3.0 was determined in 8 patients of group I (13.6%) and 12 patients (44.4%) with the \( \text{NOTCH1} \) and \( \text{SF3B1} \) gene mutations (\( p = 0.0016 \)).

Based on the iMFI level (higher and/or lower than 3.0) and the number of CD20\(^+\)CD5\(^+\) cells among all B-cells (higher and/or lower than 50%), we distinguished CLL cases with low and high levels of CD20 antigen expression. 15 patients (25.4%) of group I had low CD20 antigen expression (7 cases with low iMFI, 7 cases with low CD20\(^+\)CD5\(^+\) counts, and one case with both features). On the contrary, 19 patients (70.4%) of group II had either a low iMFI index (7 patients) or a reduced number of CD20\(^+\)CD5\(^+\) cells (7 patients), or both signs (5 cases). The differences between the groups were significant (\( p = 0.000076 \)). Patients with the \( \text{NOTCH1} \) and \( \text{SF3B1} \) gene mutations did not differ in the number of cases with low CD20 antigen expression (\( p = 0.945 \)).

The number of CD23\(^+\) cells was also reduced in patients of group II, especially among patients

Table: Expression of some antigens in CLL cells depending on the mutational status of the \( \text{NOTCH1} \) and \( \text{SF3B1} \) genes

<table>
<thead>
<tr>
<th>Group of patients</th>
<th>Percentage of cells and relative index of mean fluorescence intensity of cells (iMFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-cells, %</td>
</tr>
<tr>
<td>I</td>
<td>79.88 ± 1.72</td>
</tr>
<tr>
<td>II</td>
<td>78.44 ± 2.34</td>
</tr>
<tr>
<td>( p )</td>
<td>0.254</td>
</tr>
<tr>
<td>Only ( \text{NOTCH1} ) mutations</td>
<td>76.98 ± 2.81</td>
</tr>
<tr>
<td>Only ( \text{SF3B1} ) mutations</td>
<td>79.15 ± 3.65</td>
</tr>
<tr>
<td>( p )</td>
<td>0.504</td>
</tr>
</tbody>
</table>
with the SF3B1 gene mutations (Table); however, we could not determine the cut-off by which these groups of patients would probably differ. Using the ROC analysis and the parameter of low CD20 antigen expression, we could predict the presence of NOTCH1 and SF3B1 gene mutations in 73.3 ± 0.06 % patients (area under the curve is 0.733, 95% CI within 0.615—0.852; \( p = 0.001 \)). The risk of the NOTCH1 and SF3B1 gene mutations in cases with low CD20 antigen expression was 6.96 (95% CI = 2.53—19.18; \( p = 0.0001 \)), only the NOTCH1 gene mutations — 7.33 (95% CI = 2.41—22.32; \( p = 0.0001 \)), only the SF3B1 gene mutations — 7.32 (95% CI = 1.28—36.06; \( p = 0.023 \)). As mentioned above, patients with the NOTCH1 and SF3B1 gene mutations were diagnosed in a more advanced Binet — Rai stage. The revealed regularities were statistically significant for patients in whom the diagnosis was established in all Binet — Rai stages except A0—A1 (Fig. 2).

Thus, our data confirmed the reduced CD20 antigen expression in CLL patients with the NOTCH1 and SF3B1 mutations [9, 10]. The obtained data are consistent with those obtained earlier regarding the mutational status of the TP53, POT1, and NOTCH1 genes in CLL after low-dose irradiation [18]. In addition, an approach was proposed to identify high-risk CLL patients for the prediction of unfavorable NOTCH1 and SF3B1 gene mutations. This high-risk group included previously untreated CLL patients at advanced Binet — Rai stages (BII, CIII, CIV) with a reduced number of double-positive CD20+CD5+ cells in the peripheral blood (according to our data, below 50% among all B-cells) and/or a low iMFI of CD20+ cells (according to our data, below 3.0).

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Immunophenotype of leukemic cells in chronic lymphocytic leukemia patients with NOTCH1 and SF3B1 gene mutations


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ІМУНОФЕНОТИП ЛЕЙКЕМІЧНИХ КЛІТИН У ХВОРИХ НА ХРОНІЧНУ ЛІМФОЦИТАРНУ ЛЕЙКЕМІЮ З МУТАЦІЯМИ ГЕНІВ NOTCH1 ТА SF3B1

Стан питання. Визначення типового імунофенотипу клітин при хронічній лімфоцитарній лейкемії (ХЛЛ) є основним завданням для постановки діагнозу, однак експресія окремих антигенів варіює, що має прогностичне значення. Є дані щодо зниження експресії антигену CD20 за наявності мутацій генів NOTCH1 і SF3B1.

Мета роботи: визначити групи ризику хворих на ХЛЛ щодо наявності несприятливих мутацій генів NOTCH1 і SF3B1 на підставі імунофенотипування клітин.

Методи. У групі 86 раніш нелікованих хворих на ХЛЛ проведено імунофенотипування методом протокової цитометрії та визначення мутацій генів NOTCH1, SF3B1 і TP53 методом полімеразної ланцюгової реакції з наступним секвенуванням.

Результати. Імунофенотип лейкемічних клітин у всіх хворих відповідав критеріям постановки діагнозу ХЛЛ. Мутації гена NOTCH1 виявлені у 21 хворого (24,4%), мутації гена SF3B1 — у 7 хворих (8,1%). Мутації гена TP53 були відсутні. Для хворих з мутаціями генів NOTCH1 і SF3B1 були характерними зниження кількості CD20+CD5+ клітин та тенденція до зниження іMFI CD20+ клітин. Базуючись на показниках іMFI (вище та/або нижче 3,0) та кількості CD20+CD5+ клітин серед B-лімфоцитів (вище та/або нижче за 50%), ми виділили випадки ХЛЛ з низьким та відносно високим рівнем експресії CD20 антигену. Використовуючи ROC аналіз та параметр низької експресії CD20 антигену, ми змогли передбачити наявність мутацій генів NOTCH1 і SF3B1 у 73,3 ± 0,06% хворих (p = 0,001). Ризик наявності мутацій генів NOTCH1 і SF3B1 у випадках з низьким рівнем експресії антигену CD20 становив 6,96 (95% CI = 2,53—19,18; p = 0,0001). Закономірності були значущими для хворих на різних стадіях ХЛЛ, крім стадії A0—Al.

Висновок. Отримані дані підтвердили зниження експресії антигену CD20 у хворих на ХЛЛ з мутаціями генів NOTCH1 і SF3B1. Крім того, запропоновано підхід для визначення хворих з високим ризиком щодо передбачення наявності прогностично неприйнятливих мутацій генів NOTCH1 і SF3B1. Ця група включає раніш нелікованих хворих на розгорнutyх стадіях ХЛЛ (BiI, BiII, BiIV за Binet — Rai) зі зниженою кількістю CD20+CD5+ клітин в периферичній крові та/або низьким рівнем іMFI CD20+ клітин.

Ключові слова: хронічна лімфоцитарна лейкемія, мутації генів NOTCH1 і SF3B1, CD20+CD5+ клітини.