https://doi.org/10.15407/exp-oncology.2023.04.457

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SOLUBLE CD150 ISOFORM LEVEL IN PLASMA OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

Background. SLAMF1/CD150 is an active player in B cell signaling networks in chronic lymphocytic leukemia (CLL). CD150-mediated signaling initiates through a homophilic CD150 binding, which spans the adjacent cells, or the interaction with the soluble CD150 isoform (sCD150). The expression of sCD150 isoform at the mRNA and protein levels *ex vivo* was confirmed. However, it is unclear whether sCD150 isoform present in the blood plasma of CLL patients is a factor in the constitutive activation of $CD150^+$ cells. The **aim** of this study was to develop an ELISA assay for the specific sCD150 evaluation and assess the sCD150 levels in the blood plasma of CLL patients with different CD150 expression on B cells. **Materials and Methods.** Blood plasma samples and peripheral blood mononuclear cells from 40 previously untreated CLL patients were analyzed. An ELISA method, *ex vivo* drug sensitivity assay, and a cell viability assay were used. **Results.** The sCD150 isoform was found in all studied plasma samples of CLL patients at different levels regardless of the cell surface CD150 expression status of B cells and sCD150 mRNA expression. CLL cases with low levels of the cell surface CD150 expression in B cells are characterized by high levels of sCD150 in blood plasma in contrast to the CLL cases with high cell surface CD150 expression on B cells. The elevated levels of sCD150 in blood plasma are associated with a better sensitivity of malignant B cells to cyclophosphamide and bendamustine. **Conclusions.** The sCD150 isoform is actively secreted by CLL B cells with its accumulation in blood plasma, which may be regarded as an additional factor in the CLL clinicopathologic variability.

Keywords: soluble CD150, isoform, plasma, chronic lymphocytic leukemia.

The SLAMF1/CD150 (Signaling Lymphocytic Activation Molecule Family Member 1) receptor expression profile is complex and multifaceted in chronic lymphocytic leukemia (CLL) B cells, which include the variable levels of the cell surface expression, differentially expressed CD150 isoforms, and mutually exclusive intracellular localization [1]. A high level of the cell surface CD150 expression is strikingly associated with the favorable outcome of CLL patients [2], while

Citation: Gordiienko I, Scherbina V, Shlapatska L. Soluble CD150 isoform level in plasma of chronic lymphocytic leukemia patients. *Exp Oncol*. 2023; 45(4): 457-462. https://doi.org/10.15407/exp-oncology.2023.04.457

the role of different CD150 isoforms in CLL pathogenesis is illusive. Since CD150 receptor functions as a self-ligand and is activated after homotypic head-to-head contact between two CD150 molecules, the soluble isoform of the CD150 receptors (sCD150) may be involved in the regulation of the tumor tissue microenvironment [3, 4]. The sCD150 isoform lacks 30 amino acids that encompass the entire 22 amino acid transmembrane region and as a result cannot be integrated into plasma membrane [5]. Our previous studies showed sCD150 expression at the mRNA and protein levels in CLL B cells and in CLL B cell culture supernatants independent of CD150 cell surface expression [1]. However, it is unclear whether sCD150 isoform is present in the blood plasma of CLL patients, and its role in CLL pathogenesis is unknown. Here, we have developed a sCD150-specific enzyme-linked immunosorbent sandwich assay (ELISA) and evaluated the sCD150 levels in the plasma of CLL patients with different CD150 expressions on B cells. Also, we tried to find out whether any association exists between the sCD150 plasma levels and anticancer drug sensitivity of CLL B cells.

Materials and Methods

Patients. Blood samples from 40 previously untreated patients with verified CLL diagnosis were obtained from the Department of Oncohematology of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine (IEPOR NASU, Kyiv, Ukraine) according to the Institutional Review Board and Research Ethics Committees of IEPOR NASU. Written informed consent from each patient was obtained, and all experimental procedures were performed in accordance with the Declaration of Helsinki.

Cell lines. B-lymphoblastoid cell line MP-1 and pre-B acute lymphoblastic leukemia cell line REH obtained from the Bank of Cell Lines from Human and Animal Tissues of the IEPOR NAS of Ukraine were used in the work.

sCD150 specific ELISA. The sCD150 content in the plasma of CLL patients was determined by ELISA. Nunc-Immuno 96-MicroWell plates (Sigma, USA) were coated with 100 μl of mouse anti-human CD150 mAb (IEPOR, NASU) in a concentration of 10 μg/ml diluted in a carbonate-bicarbonate buffer (pH 9.0) overnight at +4 °C. After the sorption, the solution was decanted from each well followed by blocking nonspecific binding sites with 150 μl of PBS solution containing 1% BSA, 4% sucrose, and 0.01% merthiolate for 1 h at 37 °C. Next, 50 μl of patients' sera samples were introduced into the wells simultaneously with 50 μl of rabbit anti-CD150 polyvalent antibody at a concentration of 5 μg/ml (Sino Biological, China) diluted in sample dilution solution (PBS containing 0.05% Tween-20, 12.5% dry milk, 2 M urea). The incubation was carried out for 1 h at 37 °C. The plates were washed 4 times with a washing solution (PBS containing 0.015% Tween-20). 100 μl of goat polyclonal antibody against rabbit immunoglobulins labeled with horseradish peroxidase (Santa Cruz Biotechnology, USA) were added to each well. The conjugate of antibodies with peroxidase was diluted in a dilution solution according to the manufacturer's instructions. Incubation was carried out for 1 h at 37 °C. The plates were washed 6 times with a washing solution. To visualize the reaction, 100 μl of chromogen 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, USA) solution was used. The reaction was stopped after 30 min of incubation by adding 50 μl of 7.66% orthophosphoric acid to the wells. The reaction was recorded using a spectrophotometer in the two-wave mode of 450/620 nm.

Flow cytometry. Peripheral blood mononuclear cells (PBMC) from CLL patients were immunophenotyped with CLL specific markers (CD5, CD23, CD19, CD20, CD22, and CD38) and CD150 using a standard indirect immu-

nofluorescent method as described earlier [1]. The results were analyzed according to the ratio of geometrical means of the mean fluorescent index (GeoMean MFI) in the samples labeled with specific mAb and samples incubated with isotype control mAb. GeoMean MFI ratio < 1.2 r.u. was considered negative and GeoMean MFI ratio ≥ 1.2 r.u. was considered positive as to the cell surface expression.

Ex vivo analysis of drug cytotoxicity was performed as described previously [6].

Statistical analysis. The statistical significance of the differences between groups was evaluated by a nonparametric Mann — Whitney U-test using Prism Software Version 4.0. The significance of the differences between the examined groups was assessed as $p < 0.05$.

Results and Discussion

The ELISA approach was optimized for the specific sCD150 evaluation in human blood plasma. To confirm that the anti-sCD150 developed ELISA system is specific for determining sCD150, the positive and negative controls were used. The lysates of the B-lymphoblastoid cell line MP-1, which expresses CD150 at a high level, were used as a positive control, while REH (pre-B acute lymphoblastic leukemia cell line) served as a negative control due to the lack of the CD150 expression at both protein and mRNA levels. It was established that the content of total CD150 in MP-1 cell lysate was 2.239 ± 0.035 r.u., while in REH lysate — 0.095 ± 0.003 r.u. The CD150 levels differed between positive and negative controls by more than 20 times, which validates the accuracy of our anti-sCD150 developed ELISA system.

Before the evaluation of sCD150 isoform levels in CLL blood plasma, the cell surface CD150 expression level as GeoMean MFI ratio of cells labeled by mAb against CD150 to cells incubated with MOPC antibody isotype control as well as a profile of the CD150 isoforms mRNA expression

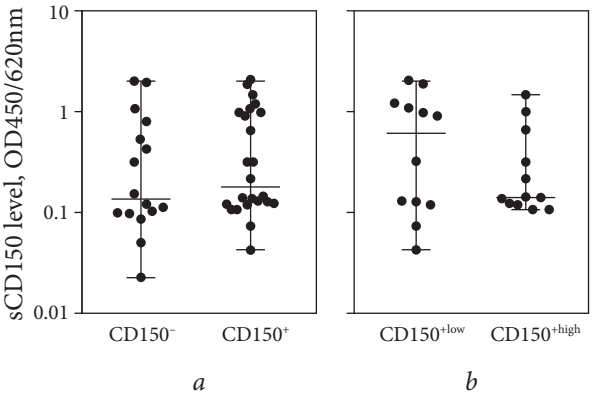

Fig. 1. The levels of the soluble CD150 isoform in plasma of CLL patients. (*a*) Levels of sCD150 in plasma of CLL patients depending on the cell surface CD150 expression status of B cells. (*b*) Levels of sCD150 in plasma of CLL patients with CD150 positive B cells grouped according to Me of CD150 GeoMean MFI, where $CD150^{+\,low}$ corresponds to the level of expression lower than Me (1.605), $CD150^{+\,high}$ being greater or equal to Me

in CLL B cells were determined. We revealed that the plasma sCD150 levels ranged within 0.043—1.908 r.u. in the CLL cases with CD150 positive B cells (24 patients, with GeoMean MFI CD150 > 1.2) and within 0.043—2.069 r.u. in the 16 CLL cases negative by CD150 expression on the plasma membrane of B cells (Fig. 1, *a*). The median value (Me) of sCD150 level in the blood plasma of CLL patients with the cell surface $CD150^+$ status of B cells was 0.182 r.u., while in CLL patients with the cell surface $CD150^-$ status — 0.138 r.u. (Fig. 1, *a*). We found no correlation between the sCD150 levels in the blood plasma of CLL patients and the cell surface CD150 expression in B-lymphocytes of these patients ($r = -0.04$, $p = 0.8$).

In addition, all analyzed CLL cases with $CD150^+$ B cells were grouped according to the Me of CD150 GeoMean MFI, which was 1.605. Surprisingly, a low sCD150 level (Me = 0.143 r.u.) was found in the blood plasma of CLL cases with $CD150^{+\,high}$ B cells to CLL cases with low CD150 expression on B cells (Me = 0.623 r.u.) ($p = 0.1$) (Fig. 1, *b*). In the group of patients with

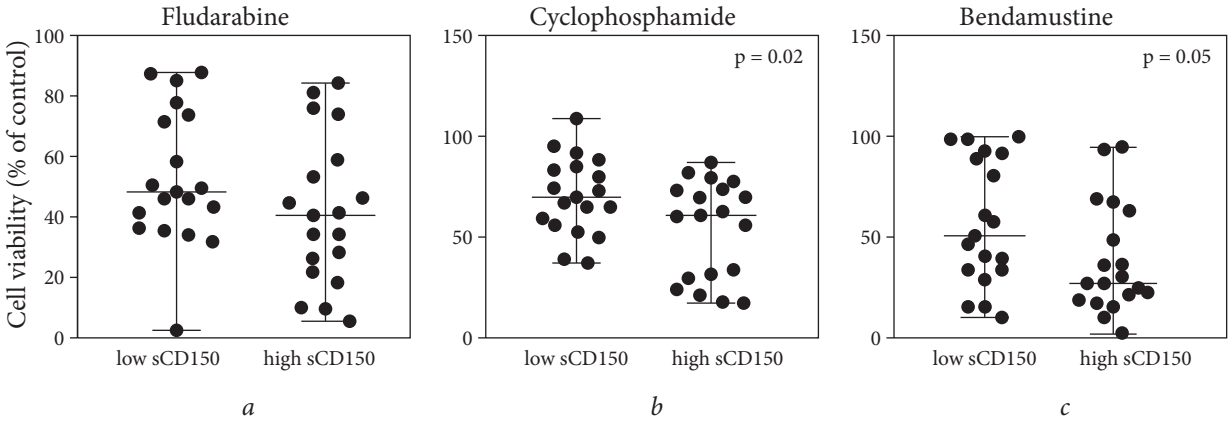

Fig. 2. Sensitivity of CLL B cells to (*a*) fludarabine, (*b*) cyclophosphamide, (*c*) bendamustine depending on the level of sCD150 in plasma of CLL patients. CLL cases were divided into two groups according to the sCD150 Me in blood plasma, where low sCD150 level was defined as less than Me value (0.143 r.u.) and high sCD150 level — greater than or equal to Me value

$CD150^{+\ high}$ B cells, a correlation of medium strength (r = 0.5, p = 0.06) was found between the content of sCD150 in plasma and GeoMean MFI CD150, while among patients with a low level of cell surface CD150 expression, no such correlation was found (r = 0.1, p = 0.6).

We also found no correlation between the sCD150 levels in the blood plasma of CLL patients and the levels of sCD150 mRNA expression in B cells when all CLL cases were analyzed (r = –0.2, p = 0.5). There was no correlation between the sCD150 level in blood plasma and sCD150 mRNA expression in B cells of the groups of patients differing in the cell surface CD150 expression status (r = –0.4, p = 0.3 and r = –0.1, p = 0.7 for CD150-negative and positive B cells, respectively).

Previously, we have shown that CLL B cells differed in their sensitivity to anticancer drugs *ex vivo* depending on the CD150 cell surface expression status and CD150 isoform mRNA profile [6]. Here, we have tested a hypothesis that the sCD150 level in the blood plasma of CLL patients may also be linked with B cell sensitivity to chemotherapy, possibly due to the modulation of the CD150-mediated signaling pathways in $CD150^+$ B cells. All studied cases of CLL were divided into two groups depending on the sCD150 Me in blood plasma, which was 0.148 r.u.

We revealed that B cell viability upon exposure to fludarabine was slightly lower in CLL cases with high sCD150 blood plasma levels (≥ 0.148 r.u.) than in CLL cases with low sCD150 blood plasma levels (< 0.148 r.u.) (p = 0.1) (Fig. 2, *a*). The viability of CLL B cells after the cyclophosphamide treatment was significantly lower (Me = 60.77 %) in CLL cases with high sCD150 plasma levels compared to CLL cases with low plasma levels of sCD150 (Me = 72.94 %) (p = 0.02) (Fig. 2, *b*). CLL B cell sensitivity to bendamustine was also higher in CLL cases with high sCD150 plasma levels compared to CLL cases with low plasma levels of sCD150 (p = 0.05) (Fig. 2, *c*).

Previously, the expression of sCD150 has been detected at the mRNA level in activated normal B and T cells, mature dendritic cells, primary cases of classical Hodgkin lymphoma, Hodgkin lymphoma cell lines, and B-lymphoblastoid cell lines. Also, sCD150 was found in the blood serum of healthy individuals and patients with rheumatoid arthritis [3, 5, 7—9].

This study confirmed that CLL B cells express the sCD150 isoform not only at the mRNA level but also as a protein and secret sCD150 into the

extracellular space, which allows us to determine sCD150 in patients' blood plasma. It is still unknown whether there is any association between the sCD150 isoform plasma level and CLL clinicopathological features, which requires further studies. We hypothesized that the sCD150 isoform may serve as a natural ligand for the transmembrane form of the CD150 receptor in CLL B cells or other $CD150^+$ immune cells. In earlier studies, it was confirmed that the recombinant sCD150 induces B cells proliferation only at high concentrations around 20 μg/ml, which are far from physiological ones [3]. However, the self-association affinity constant of the SLAMF1 extracellular domain has been re-examined recently, and the homotypic mechanism of the CD150 activation has been confirmed and supported by the published cell-based studies using the native soluble and transmembrane form of SLAMF1 [4].

In our study, the sCD150 level in the plasma of CLL patients did not correlate with the cell surface CD150 expression and mRNA expression in CLL B cells. The sCD150-mediated activation of CD150 signaling pathways may result in different biological outcomes in CLL cases with different CD150 cell surface expression statuses in B cells. $CD150^+$ B cells could be the main target of sCD150 in CLL cases positive by the CD150 cell surface expression, while $CD150^+$ immune cells including T cells, macrophages, and dendritic cells — in CLL cases with a negative CD150 surface expression status of B cells. Moreover, the elevated level of sCD150 in the blood plasma of CLL patients may act as a factor of the constitutive CD150 activation with the following transmembrane receptor internalization, which results in the negative CD150 cell surface phenotype. The absence of correlation between the plasma level of sCD150 and mRNA expression in CLL B cells can be explained by the fact that the sCD150 protein has a longer half-life in the blood than mRNA due to the accumulation and stabilization with other transport proteins. The association between the sCD150 plasma level and sensitivity to chemotherapy revealed in this study can partially confirm our previous results where the ligation of CD150 receptors with anti-CD150 mAb led to the increased sensitivity to bendamustine [6].

Taken together, sCD150 was found in CLL patients' plasma regardless of sCD150 mRNA expression and CD150 cell surface expression status in B cells. CLL cases with low levels of CD150 expression on B cells are characterized by a high level of sCD150 in blood plasma in contrast to CLL cases with high CD150 expression on B cells. The elevated level of sCD150 in blood plasma is associated with better sensitivity of malignant B cells to cyclophosphamide and bendsamustine. Further study is needed to clarify the sCD150 isoform role in CLL pathogenesis.

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Submitted: September 17, 2023

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РІВЕНЬ РОЗЧИННОЇ ІЗОФОРМИ CD150 У ПЛАЗМІ ХВОРИХ НА ХРОНІЧНИЙ ЛІМФОЛЕЙКОЗ

Стан проблеми. SLAMF1/CD150 — активний учасник сигнальних мереж В-клітин при хронічному лімфолейкозі (ХЛЛ). Передача сигналів, опосередкована CD150, ініціюється шляхом гомофільного зв'язування CD150, локалізованого на сусідніх клітинах, або ж взаємодією рецептора з розчинною ізоформою CD150 (sCD150). Експресію sCD150 ізоформи підтверджено на рівні мРНК і білка *ex vivo*. Однак незрозуміло, чи є ізоформа sCD150 у плазмі крові пацієнтів із ХЛЛ фактором конститутивної активації $CD150^+$ клітин. **Метою** цього дослідження була розробка ELISA метода для специфічної оцінки sCD150 та визначення рівня sCD150 у плазмі крові хворих на ХЛЛ. **Матеріали та методи.** Робота виконана на мононуклеарах периферичної крові та зразках плазми крові 40 хворих на ХЛЛ, яким попередньо не проводили лікування. У роботі використовували метод ELISA, аналіз чутливості клітин до хіміопрепаратів *ex vivo* та аналіз життєздатності клітин. **Результати.** sCD150 ізоформа була виявлена в усіх досліджених зразках плазми хворих на ХЛЛ на різному рівні незалежно від статусу експресії CD150 на плазматичній мембрані В-клітин та рівня експресії мРНК sCD150. Випадки ХЛЛ із низьким рівнем експресії CD150 на В-клітинах характеризуються високим рівнем sCD150 у плазмі крові, на відміну від випадків ХЛЛ із високою експресією CD150 на В-клітинах. Вищий рівень sCD150 у плазмі крові асоціюється з кращою чутливістю злоякісно трансформованих В-лімфоцитів до циклофосфаміду та бендамустину. **Висновки.** Ізоформа sCD150 активно секретується В-клітинами ХЛЛ із накопиченням у плазмі крові і може розглядатися як додатковий фактор клініко-патологічної варіабельності ХЛЛ.

Ключові слова: розчинна ізоформа CD150, плазма, хронічний лімфолейкоз.