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B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA-ASSOCIATED NUCLEAR ANTIGENS

Abstract: One- and two-dimensional polyacrylamide gel electrophoresis were used to compare the composition of nuclear polypeptides from normal and B-cell chronic lymphocytic leukemia mononuclear cells. Against two electrophoretically-specific nuclear proteins with molecular weight of 38/39 and 44/46 kDa from leukemic cells rabbit sera were obtained. As it was analyzed by Western blot technique the available antisera recognized the 38/39 kDa antigen in 53 of the 56 (94.6%), while the 44/46 kDa in 46 of the 49 (93.9%) of examined B-CLL nuclear fraction preparations, but not in normal ones. The pI values of described leukaemia-specific antigens were determined; p38/39 had pI in the range of pH 6.55–7.00 and p44/46 – in the range of pH 6.2–6.4.

Key words: B-CLL specific antigens, electrophoresis, immunoblotting

1. INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is a hematological cancer characterized by an accumulation in blood, bone marrow and lymph nodes, transformed clone of B lymphocytes (ROZMAN, MONSERRAT 1990; GALE et al. 1994). The etiology of this disease is still not fully understood. It was stated that the malignant cells appearing in peripheral blood of patients seems to have a low rate of proliferation and resistance to programmed cell death (ZWIEBEL, CHESON 1998). The remarkable clinical variability in the course of this leukemia was observed. These diversities could be a consequence of many cytogenetic abnormalities found in B-CLL cells. Although, a variety of genetic aberrations have been described, the deletion of chromosome 13 arm (13q14), as well as deletions of chromosome bands 11q22.3–23.1, and the trisomy of chromosome

12 are most common (HAMBLIN, OSCIER 1997; REED 1998). Interestingly, the deletions involving of chromosome bands 17p13 and 11q22.3–23.1 seems to correlate with higher B-CLL aggressiveness, shorter survival and resistance to therapy (JULIUSSON, MERUP 1990; DÖHNER et al. 1999, 2000). These genetic alterations occurred in neoplastic B-lymphocytes could lead to changes in the level of protein expression or the appearance of new polypeptides on cell surface as well as among cellular compartments of transformed B-cells.

Our previous investigations (CHRUŚCIEL et al. 1996, 1999; KILIAŃSKA et al. 1998, 2001) revealed that within nuclei of mononuclear cells isolated from peripheral blood samples of B-CLL patients took place specific expression of non-histone proteins with a mol. mass of 38/39 and 44/46 kDa described as p38/39 and p44/46. Similar nuclear proteins were also detected in mononuclear cells obtained from the blood of patients with acute lymphoblastoid leukemia (ALL), but not from the blood of patients with T-cell chronic lymphocytic leukemia (T-CLL) or healthy donors (CHRUŚCIEL et al. 1999).

In present report our special interest is focused on electrophoretic and immunological investigation of nuclear proteins from normal and leukemic mononuclear cells. Using the rabbit polyclonal antisera raised against 38/39 and 44/46 kDa polypeptides we verified the appearance of these nuclear components among large number of B-CLL mononuclear cell preparations as well as normal ones.

2. MATERIALS AND METHODS

2.1. Patients and mononuclear cells isolation

Peripheral blood samples were obtained from 63 B-CLL patients aged from 43 to 83 years. Diagnosis was made in all cases according to standard clinical, cytological and immunological criteria. Clinical staging was performed according to the Rai classification (RAI et al. 1975). Number of patients, i.e., 7, 12, 11, 10, 21 were stage 0, I, II, III and IV, respectively. Additionally, 2 patients were without classification data. All patients had documented lymphocytosis with an absolute leukocyte count greater than $15\,000/\mu\text{l}$ and had more than 30% of lymphocytes in the bone marrow. None of the patients were treated before or during the study. Peripheral blood mononuclear cell suspensions were obtained by density gradient cell separation using Ficoll-Paque and centrifugation ($400\times g$, 18°C , 30 min) according to BÖYUM method (BÖYUM 1968). The study was approved by the Local Ethics Committee of Medical University of Lodz (no. RNN 970/98) and the patients had signed the informed consent form.

Blood samples of healthy donors were used as controls. Mononuclear cells were counted by hemocytometer and subjected to isolation of nuclear fraction.

2.2. Morphological and cytochemical studies

Smears of peripheral blood and aspirates of bone marrow were stained with May-Grünwald-Giemsa, for peroxidase, α -naphthyl butyrate esterase and with periodic-acid Schiff (PAS) solution by standard methods.

2.3. Immunophenotypic analysis

A panel of monoclonal antibodies was used for immunologic typing including CD2, CD3, CD5, CD7, CD9, CD10, CD20, CD23, TdT, FMC7 (Dakopatts, Denmark), conjugated fluorescein isothiocyanate or phycoerythrin fluorochromes. Surface antigens of the mononuclear cells on all the peripheral blood and bone marrow were analyzed by two colours flow cytometer Coulter Epics (Coulter, Hialeah). A minimum of 10 000 cells were analyzed per sample and data were collected in list mode and examined with the XL2 and Immuno 4 program (Coulter).

2.4. Preparation of nuclear fraction

Nuclei were isolated from leukemic and normal mononuclear cells by the sucrose method (BLOBEL, POTTER 1966) using 0.5% Triton X-100 to remove membrane ghost and 1 mM phenylmethyl sulfonyl fluoride (PMSF) to inhibit protease activity.

2.5. One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Nuclear protein preparations isolated from 63 individual patients' blood samples were mixed with 0.9 vol of solubilizing buffer [10% glycerol, 4% sodium dodecyl sulfate (SDS), 25 μ g pyronine Y, 125 mM Tris-HCl, pH 6.8, 1 mM PMSF], with 0.1 vol of 2-mercaptoethanol, and heated in boiling water for 5 min. The polyacrylamide gel electrophoresis was performed as described by LAEMMLI (1970) at 15 mA slab until the pyronine Y marker reached the end of the 3.0% stacking gel and then at 30 mA/slab in the 11.2% resolving gel until the marker dye reached the bottom of gel. About 20 μ g of proteins were loaded per slot. The proteins were stained with silver nitrate (WRAY et

al. 1981). The approximate relative molecular mass (M_r) was determined by comparing the mobilities of proteins to those of known molecular mass standards (Sigma Chemical Co.).

2.6. Two-dimensional polyacrylamide gel electrophoresis (2-DE)

Two-dimensional electrophoresis was carried out according to the method of O'FARRELL (1975) as modified by CUPO et al. (1990). The first-dimensional equilibration isoelectrofocusing gel containing a 2.0% total carrier ampholyte mixture (composed of pH range 4.0–6.5, 6.5–9.0 and 3.0–10.0). The final acrylamide concentration was 4.0% with a cross-linking of 2.5% N,N'-methylenebisacrylamide. The detergent present in the gel was 0.025 M 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonate (CHAPS). Nuclear protein preparations obtained from 17 individual patients' blood samples were solubilized in 8.0 M urea, 2.0% carrier ampholytes, 0.065 M dithiotreitol, 0.5% Nonidet P-40, 0.065 M CHAPS and the samples of about 200 μ g were overlaid on the gels. The gels were electrophoresed for 14 hrs at 300 V and for 2 hrs at 400 V. The electrolyte solutions were: 0.02 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). The pH of the "blank gels" was measured at 0.5 cm intervals in 0.01 M KCl using a Beckman pH-meter. After isoelectric focusing, the gels were equilibrated for 30 min in 10.0% glycerol, 5.0% 2-mercaptoethanol, 2.3% SDS, and 0.065 M Tris-HCl at pH 6.8. Electrophoresis in the second dimension was carried out on 11.2% polyacrylamide gel according to LAEMMLI (1970). Proteins were visualized by silver staining (WRAY et al. 1981).

2.7. Antisera production

New Zealand white rabbits were immunized with B-CLL electrophoretically-specific non-histone protein bands visualized after SDS-PAGE by silver staining method (WRAY et al. 1981) as was described previously (CHYTIL 1978; CHRUSCIEL et al. 1996, 1999; KILIAŃSKA et al. 1998). The gel slices were equilibrated in 50 mM Tris-HCl buffer (pH 8.0) for 30 min and after cutting into 1 mm pieces emulsified in a sterile mortar with an equal volume of 0.9% NaCl and complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the following schedule. Approximately 500 μ l (containing 250–500 μ g) of p38/39 or p44/46 proteins were injected at multiple sites intradermally on day zero. The rabbits were boosted at weekly intervals for four weeks. The rabbits were bled 1 week after the final injection and the sera were assayed for antibodies by immunoblotting technique. The rabbits were bled prior to the antigens injection, and the sera were used for control studies. No cross reactivity was observed in the presence of preimmune rabbit serum.

2.8. Immunoblotting assay

Electrophoretically separated proteins by SDS-polyacrylamide gel electrophoresis or 2-DE were blotted to Immobilon P according to TOWBIN and colleagues (TOWBIN et al. 1979). For immunodetection of antigens immobilized on Immobilon P the alkaline phosphatase technique was used. The filters were incubated for 1 h at room temperature in 3.0% nonfat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) to saturate the non-specific protein binding sites. Immobilon filters were incubated with primary antisera (at 1:200 dilution) in TBS in a cold room overnight. After washing several times in TBS containing 0.05% Tween 20 (TBST), the filters were incubated with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma Chemical Co.) at 1:6000 dilution in TBS for 2 hrs at room temperature. After washing with TBST, specific antigen-antibody reactions were visualized by incubation with substrate solution (0.33 mg/ml of nitro blue tetrazolium, 0.17 mg/ml of 5-bromo-3-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM MgCl₂), prepared according to LEARY et al. (1983).

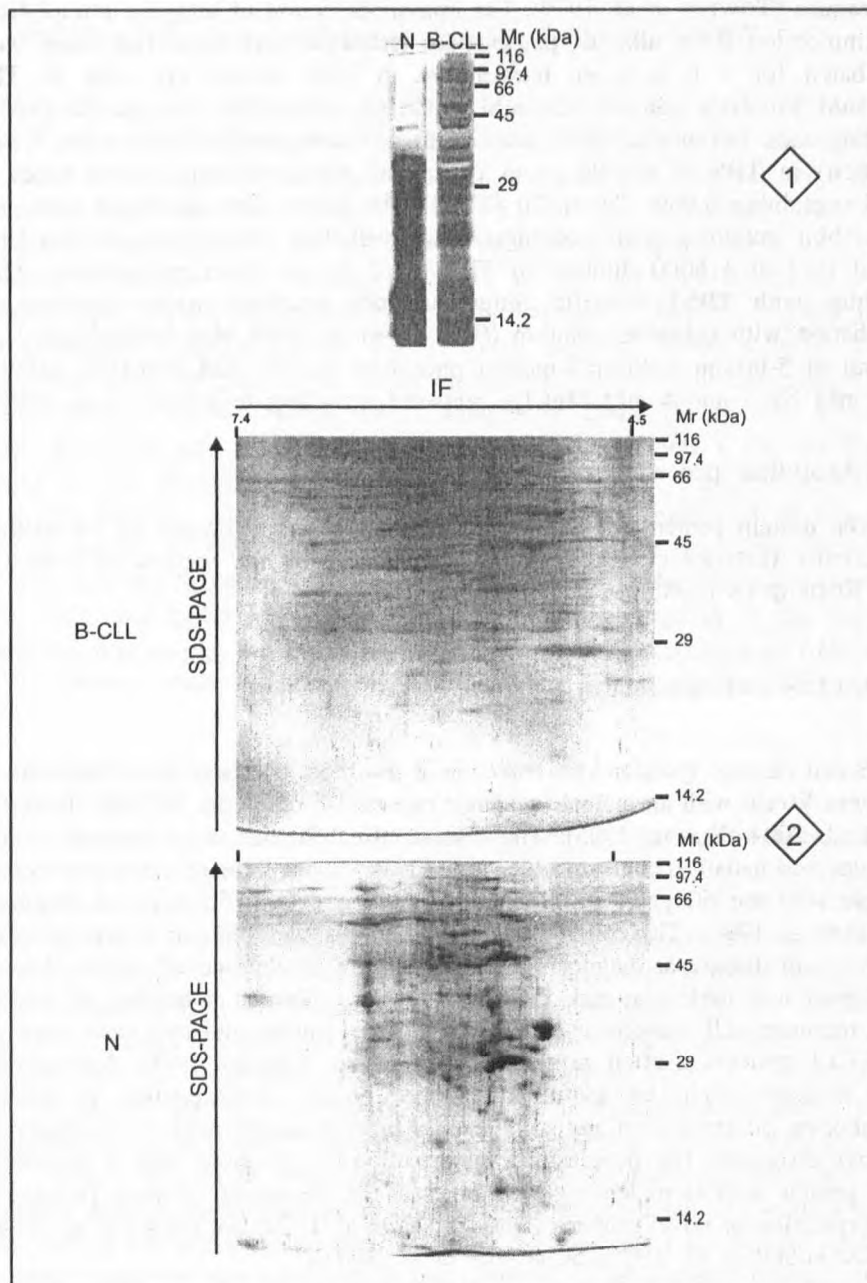
2.9. Analytical procedures

The protein content for SDS-PAGE technique was estimated by the method of LOWRY (LOWRY et al. 1951), while for 2-DE by the method of RAMAGLI and RODRIQUES (1985).

3. RESULTS AND DISCUSSION

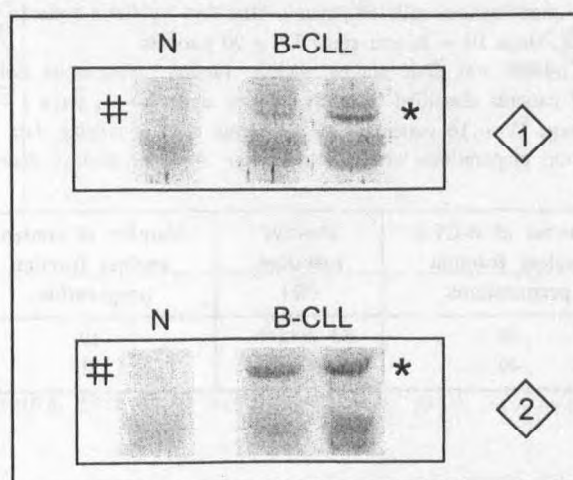
B-cell chronic lymphocytic leukemia is the most common adult leukemia in Western World with an annual incidence rate of 3-5 cases per 100 000 (RADAELLI et al. 2004; ROBAK 2005). The disease affects males more frequently than females and usually occurs in older individuals; about 90% of cases are seen in people who are 50 years or older, with median age of 65 years at diagnosis (Diehl et al. 1999). The course of B-CLL is highly variable and is dependent on the stage of disease at diagnosis and the presence or absence of certain clinical, biological and molecular risk factors. However, although a number of routine hematopoietic cell markers is known, the clinical course and treatment response of B-CLL patients is often unpredictable (ZWIEBEL, CHESON 1998). A search for new markers might be useful in a more precise determination of control lymphocyte differentiation and sufficient for precise classification of lymphoproliferative disorders. The neoplastic transformation of lymphoid cells is associated with genetic alterations leading to changes in the expression of some proteins or the expression of novel proteins (DEL-GIGLIO et al. 1992; SAUNDERS et al. 1994a, b; HANAUSEK et al. 1996; SCHROERS et al. 2005).

Fig. 1: (1) – SDS-polyacrylamide gel (11.2% resolving gel) electrophoresis of nuclear proteins (about 20 μ g) from normal (N) and leukemic (B-CLL) mononuclear cells followed by silver staining; (2)– 2-DE of nuclear proteins (~200 μ g) from normal (N) and leukemic (B-CLL) mononuclear cells followed by silver staining.



Our previous investigations revealed some differences in electrophoretic pattern of cellular compartment proteins, especially isolated from nuclei of leukemic (CLL and ALL) cells and those from normal cells of peripheral blood (CHRUŚCIEL *et al.* 1996, 1999; KILIAŃSKA *et al.* 1998). In present study we have concentrated on electrophoretic and immunological characterization of nuclear proteins originating from mononuclear cells from blood of B-CLL patients and healthy donors. The results obtained by us shown some differences in one- and two-dimensional gel electrophoretic patterns of nuclear proteins isolated from normal and B-CLL mononuclear cells (Fig. 1). Profound analysis of electrophoretic behavior of nuclear proteins obtained from control and leukemic cells confirmed many common components, however some differences were observed. Main diversities have seen in electrophoretic banding of nuclear proteins from normal and transformed cells, particularly in the region of gels with molecular mass of 38–46 and 62–105 kDa. Moreover, a few constituents characterized by molecular mass of 38/39 kDa and 44/46 kDa in slightly acidic/neutral area of gels as well as large complex of polypeptides with molecular weights/pI of 14–16/5.9–7.4; 35–36/6.2–7.4; 40–52/5.0–5.6; 64–69/5.1–5.7 and 95–105/5.2–5.5 showed altered expression in nuclei of transformed cells during disease development (ROGALIŃSKA *et al.* 2001).

Fig. 2: Immunoblot analysis of nuclear proteins from normal (N) and leukemic (B-CLL) mononuclear cells, by alkaline phosphatase method in the presence of antisera raised against electrophoretically-specific polypeptides with Mr of 38/39 kDa (1) and 44/46 kDa (2) separated from B-CLL nuclear fraction. The symbols indicate the presence (*) or lack (#) of the p38/39 and p44/46 antigens, respectively.



Using polyclonal antisera raised against two electrophoretically-specific nuclear proteins of leukemic cells with molecular mass of 38/39 and 44/46 kDa we confirmed by Western blot method the expression of both antigens with high frequency among studied leukemic cell preparations. The exemplary immunoblot analysis of nuclear proteins from B-CLL and control mononuclear cells obtained by alkaline phosphatase method in the presence of antisera raised against nuclear antigens (p38/39, p44/46) is illustrated on Fig. 2. In the study reported here we detected the antigen with molecular mass 38/39 kDa (p38/39) in 53 of the 56 (94.6%), and the 44/46 kDa (p44/46) – in 46 of the 49 (93.9%) of the examined B-CLL nuclear fraction preparations, but not in any of samples from healthy donors (Table 1). For further characterization of both leukemia-specific antigens we employed two-dimensional gel electrophoresis (2-DE) of leukemia nuclear proteins followed by Western blot analysis in the presence of available anti-p38/39 and anti-p44/46 sera (Fig. 3). As stated by this combined 2DE/Western blot techniques leukemia-specific antigen p38/39 indicated isoelectric point (pI) in the range of pH 6.55–7.00, while the p44/46 – in the range of pH 6.2–6.4. The data described here provide a basis for the supposition that the detected nuclear antigens – p38/39/pI 6.55–7.00 and p44/46/pI 6.2–6.4 whose expression seems to accompany the transformation of normal mononuclear cells into B-CLL cells may serve as potential biomarkers of this type of leukemia.

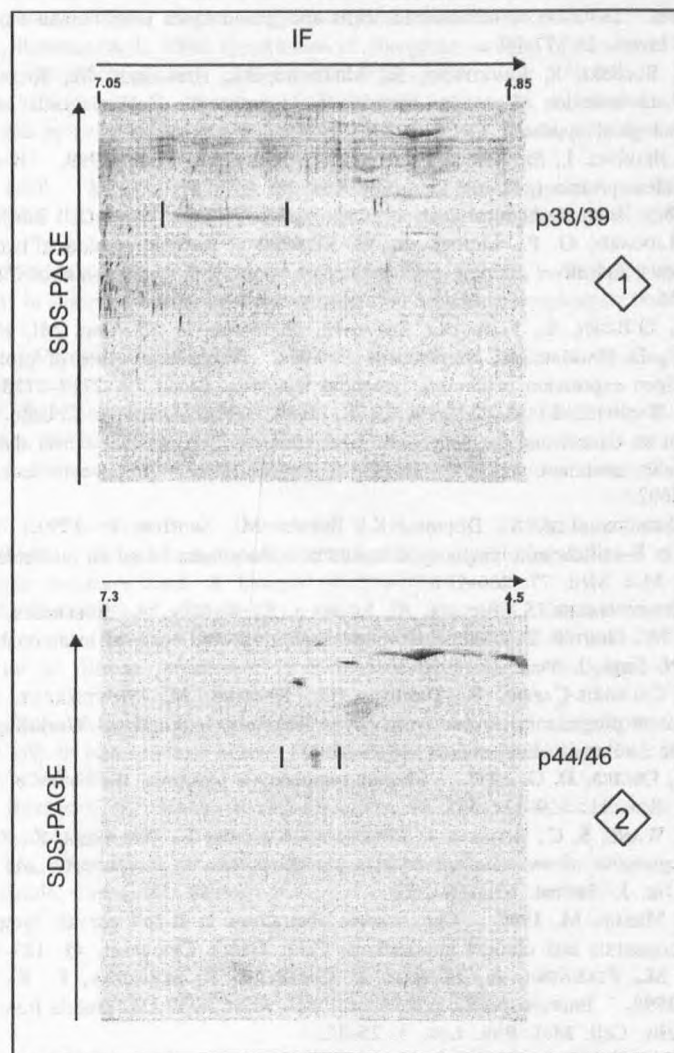
Table 1: Immunodetection of p38/39 and p44/46 antigens among control and B-CLL nuclear fraction preparations. Immunodetection of p38/39 was performed among nuclear fraction preparations isolated from B-CLL mononuclear cells of patients classified by Rai's system: stage 0 – 5, stage I – 12, stage II – 11, stage III – 8, and stage IV – 20 patients.

Immunodetection of p44/46 was done among nuclear fraction preparations isolated from B-CLL mononuclear cells of patients classified by Rai's system: stage 0 – 4, stage I – 11, stage II – 8, stage III – 8, and stage IV – 16 patients, and 2 patients without staging data.

Control nuclear fraction preparations were obtained from blood of healthy donors.

Antigen	Number of B-CLL nuclear fraction preparations	Positive reactions (%)	Number of control nuclear fraction preparations	Positive reactions (%)
p38/39	56	53 (94.6%)	19	0
p44/46	49	46 (93.9%)	11	0

Fig. 3. Immunoblot analysis of nuclear proteins from B-CLL mononuclear cells resolved by 2-DE technique, by alkaline phosphatase method in the presence of antisera raised against electrophoretically-specific polypeptide p38/39 (1) and p44/46 (2).



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