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Expression and prognostic significance of the inhibitor of apoptosis protein (IAP) family and its antagonists in chronic lymphocytic leukaemia ☆

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ABSTRACT

Impaired apoptosis is still considered to be an important event in the development and progression of chronic lymphocytic leukaemia (CLL). However, mechanisms of this defect have not been fully elucidated. In this study, expression of inhibitor of apoptosis proteins, IAPs (cIAP1, cIAP2, XIAP and survivin), and their antagonists (Smac/DIABLO and HtrA2/Omi) was comprehensively analysed in 100 untreated CLL patients, using flow cytometry and Western blot techniques.

Expression of anti-apoptotic cIAP1 and cIAP2 in leukaemic cells was significantly higher than in non-tumour lymphocytes ($p = 0.000001$ and $p = 0.014$, respectively), whereas the IAP-antagonist, Smac/DIABLO, was decreased in CLL ($p = 0.010$). Higher expression of all analysed IAPs (cIAP1, $p = 0.002$; cIAP2, $p = 0.026$; XIAP, $p = 0.002$; survivin, $p = 0.00006$) and lower levels of Smac/DIABLO ($p = 0.006$) were found in patients with progressive disease, compared to those with stable CLL. High baseline expression of cIAP1 and survivin correlated with worse response to treatment. Co-expression of these proteins was associated with shorter overall survival of CLL patients ($p = 0.005$).

In conclusion, CLL cells show the apoptosis-resistant profile of IAPs/IAP-antagonist expression. Upregulation of IAPs is associated with a progressive course of the disease. Co-expression of cIAP1 and survivin seems to be an unfavourable prognostic factor in CLL patients. Further studies with longer follow up are warranted to confirm and expand these findings.

1. Introduction

Chronic lymphocytic leukaemia (CLL) is a heterogenous disease with a highly variable clinical course.[1] Although CLL is the most common leukaemia in western countries, its biology is still poorly understood. CLL is characterised by the relentless accumulation of monoclonal mature $CD5^+$ B cells in lymphoid organs, bone marrow, and peripheral blood. Virtually all circulating CLL lymphocytes are long-lived cells arrested in the G_0/early G_1 phase of the cell cycle.[2] The progressive expansion of lymphocytes has led to the notion that defective apoptosis rather than increased cell proliferation is

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the primary cause of CLL. The microenvironment likely plays a prominent role because the malignant cells progressively accumulate *in vivo*, whereas they rapidly undergo apoptosis when cultured *in vitro*.[3]

Apoptosis, defined as programmed cell death, is executed through the activity of caspases, cysteine proteases which are regulated by a number of pro- and anti-apoptotic proteins. Bcl-2 family proteins are major regulators of mitochondria-dependent apoptosis.[4] Several of them were found to be over-expressed in CLL[5] which is associated with resistance to apoptosis, poor response to chemotherapy (alkylating agents or purine analogues), and advanced stages of the disease.[6,7] Furthermore, increased Bcl-2/Bax rates have been correlated with refractory disease, its progression, and shorter survival.[8] Other important apoptosis-regulating proteins include the p53 protein family.[9] Mutations or deletions of the *p53* gene are known to be altered in a number of haematological malignancies, including progressive CLL.[10]

An essential checkpoint in apoptosis regulation is the control of caspase activation by the more recently discovered family of inhibitor of apoptosis proteins (IAPs). IAPs are the group of proteins which block both the mitochondrial-dependent and independent pathways of apoptosis. They also participate in the regulation of the cell cycle and intracellular signal transduction.[11,12] The family consists of eight proteins containing baculoviral IAP repeat (BIR) domains which allow them to bind to caspases, inhibiting these enzymes. They are: cIAP1 and cIAP2 (cellular inhibitors of apoptosis 1 and 2), XIAP (X-chromosome binding IAP), ILP-2 (IAP–like protein 2), livin, NAIP (neuronal apoptosis inhibitory protein), survivin and BRUCE (Apollon).[13–17] The majority of IAP activities are dependent on the BIR domain. Among the mammalian IAPs, c-IAP1, cIAP2, and XIAP have three BIRs in the N-terminal portion of the molecule and a RING finger at the C-terminus, NAIP contains three BIRs without RING, survivin and BRUCE each have just one BIR.[11–13] The BIR2 domain is capable of binding and inhibiting caspase-3 and -7, whereas BIR3 is an inhibitory segment for caspase-9. BIR1 does not display the inhibitory ability of caspase activation. BIR domains are also associated with the binding of cIAP1 and cIAP2 to TNF receptor associated factors (TRAF1 and TRAF2), the XIAP interaction with TAB1 protein and, as a consequence, with the activation of the nuclear factor κB (NF-κB) pathway. Additionally, the RING domain allows some proteins (XIAP, c-IAP1, c-IAP2 and livin) to act as E3 ubiquitin ligases. The E3 ubiquitin ligase activity of the IAPs is capable of promoting ubiquitination and proteasomal degradation of caspases, TRAF2 and several other partners. Furthermore, both cIAP1 and cIAP2 additionally contain the caspase recruitment domain (CARD) located between the BIR and RING domains. The functional significance of this domain for the anti-apoptotic function of IAPs is largely untested, but amino-terminal fragments of human c-IAP1 and c-IAP2 that retain only the BIR domains are sufficient to block apoptosis, implying that the CARD domain is not absolutely required. Other domains of potential interest in the IAP family include a functionally intact ubiquitin-conjugating (UBC) domain in BRUCE. This protein could conceivably provide a functional connection between apoptosis proteins and the ubiquitin proteasome pathway for protein degradation. The NAIP protein contains a P-loop consensus sequence similar to some ATP/GTP-binding proteins.[12–17] The structure of cIAP1, cIAP2 and XIAP allows them to inhibit caspase-3, -7 and -9 and ubiquitinate caspase-3, -7 and the IAP-antagonist, Smac/DIABLO. Additionally, they indirectly inhibit apoptosis through NF-κB activation. Most probably, survivin directly inhibits caspase-9 through the BIR domain and, indirectly, caspase-3 through p21Cip/WAF1. The other potential mechanism of survivin anti-apoptotic activity is the inhibition of apoptosis-inducing factor (AIF) released from the mitochondria (reviewed in 14). More recent reports brought a new insight into the activity of IAPs, including action through the external pathway of caspase activation.

The function of IAPs is negatively regulated by several proteins. Currently, three proteins are known to bind to different IAPs and inhibit their activity: Smac/DIABLO (second mitochondrial derived activator of caspase/direct IAP binding protein with low pI), HtrA2/Omi (high temperature requirement A) and XAF1 (XIAP-associated factor 1).[18–20] HtrA2/Omi and Smac/DIABLO are localised within the mitochondria and are released into the cytosol upon apoptotic stimuli. Cytosolic HtrA2/Omi results in displacement of XIAP from caspases and loss of their suppressive effect on caspase activity. Moreover, HtrA2/Omi was shown to be able to degrade XIAP protein. It is suggested that HtrA2/Omi may also be responsible for the proteolytic degradation of XIAP, cIAP1 and cIAP2. Smac/DIABLO is also an important molecule that regulates the function of IAPs. It can bind to the BIR domain of IAPs (XIAP, cIAP1, cIAP2, survivin, livin, and BRUCE), thereby interfering with either caspase-3/-7 or caspase-9 inhibition. XAF1 resides in the nucleus and can affect a relocation of the XIAP protein from the cytoplasm to the nucleus. It has been reported that XAF1 is mainly expressed in normal tissues but is missing or present at low levels in most cancer cell lines, which implies a tumour-suppressing function.[18–20]

There is growing evidence that IAP proteins can be involved in the pathogenesis of human malignancies.[21–23] Overexpression of IAPs was found in a wide variety of cancer cell lines and primary tumour biopsy samples, including primary acute leukaemias and B-cell lymphomas.[24–28]

Thus far, there are only a few clinical observations concerning IAP expression in CLL.[29–32] To date, there are no reports regarding the potential prognostic significance of these proteins and their antagonists in the disease. Therefore, the primary aim of this study was to perform a complex analysis of the IAP family members and their antagonists' content in leukaemic cells *in vivo*, in untreated CLL patients in comparison to healthy controls. Additionally, we aimed to assess possible differences in expression of these proteins between patients with stable and progressive CLL. Finally, we also tried to establish a potential prognostic significance of the IAP expression.

2. Materials and methods

2.1. *Patients*

The study group consisted of 100 previously untreated patients with CLL. The median age of patients was 69 years (range, 39–88 years). Twenty seven healthy donors, 12 women

and 15 men (median age 70 years, range 38–84 years), served as a control group. The study was approved by the Ethics Committee of the Medical University of Lodz, Poland, and all patients and healthy volunteers signed informed consent forms.

Diagnosis of CLL was established according to the National Cancer Institute–Sponsored Working Group (NCI-WG) recommended criteria.[33] The clinical stage of the disease was determined according to the modified Rai classification.[34] Characteristics of patients and the control group are summarised in Table 1.

Two groups of patients were evaluated: with stable disease (SD) (N = 52) and progressive disease (PD) (N = 48), as identified at the blood sample collection. PD was defined in all patients with stage III and IV according to the Rai classification, as well as in those patients with stages 0–II who fulfilled at least one of the following criteria: progressive lymphocytosis (doubling time shorter than 2 months), massive splenomegaly or bulky lymphadenopathy, recurrent disease-related infections, weight loss of more than 10% over a 6-month period, or fever of 38 °C or higher related to disease.

Response to the therapy according to either CC (cladribine, 2-CdA, and cyclophosphamide, CY) or FC (fludarabine, FA, and CY) regimens was evaluated in 50 patients (48 PD and two SD patients who progressed during the follow-up). Treatment effects (complete response, CR; partial response, PR; no response, NR) were monitored by physical examination, blood count evaluation, bone marrow aspiration, or biopsy, according to guidelines for response developed by the NCI-WG. Patients meeting the criteria of CR with the exception of presence of lymphoid nodules in bone marrow biopsy (nodular partial response, nPR) were analysed as having PR. Overall survival (OS) was measured from the time of diagnosis to death or last contact. The median follow-up time of the study was 42 months (range 15–205 months).

2.2. *Ex vivo studies*

2.2.1. *Cell isolation*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood samples by centrifugation in Histopaque-1077 (Sigma Diagnostic, St Louis, MO, USA) density gradients. A 1:1 (v/v) mixture of blood and Hanks' Balanced Salt Solution, HBSS (Biomed, Lublin, Poland), was layered on top of the Histopaque media in centrifuge tubes and centrifuged for 30 min at 200×g. The interphase region containing PBMC was collected and then washed twice, in HBSS and RPMI 1640 medium. Next, the cells were counted and divided into several portions. Some of them were re-suspended in phosphate buffered saline (PBS; Sigma Aldrich Chemie Gmbh, Steinheim, Germany) for the immunophenotypic study. Others were transferred to Eppendorf tubes and frozen at –20 °C (material for Western blot studies). The remaining cells were fixed in 1% formaldehyde (15 min/0 °C) and in 70% ethanol (30 min/0 °C), then stored at –20 °C – for subsequent protein expression studies by flow cytometry and immunohistochemistry.

2.2.2. *Cell immunophenotyping*

Immunophenotyping of leukaemic cells was performed routinely in the whole peripheral blood (the "lysed – not washed" method). A routine panel of MoAbs (CD3, CD5, CD10, CD11c, CD19, CD23, FMC7, Igκ and Igλ), fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE) or cyanine-5 (Cy5) conjugated (all BD Pharmingen, San Diego, CA, USA) was evaluated using flow cytometry. The diagnosis was confirmed based on detecting the leukaemic CD5+/CD19+/CD23+ clone. The rate of CLL cell population ranged from 55% to 97% of peripheral blood white cells (mean 80.5%).

2.2.3. *Assessment of ZAP-70 protein and CD38 expression*

Additionally, MoAbs against ZAP-70 protein (1E7.2clone Alexa fluor 488; Caltag Laboratories, Burlingame, CA, USA) and CD38 antigen (DAKO, Glostrup, Denmark) were evaluated to assess expression of CD38 or ZAP-70 as prognostic markers in CLL. ZAP-70 protein expression was evaluated on PBMC, after fixation and permeabilisation. The method was based on its detection in both leukaemic B-CLL cells and in healthy T or NK cells.[35] They were identified by following line-specific markers: CD19 (PECy5.5 conjugated), CD5 (conjugated with allophycocyanin, APC), CD3, CD56, and CD16 (all PE conjugated), combined in a four-colour analysis: ZAP-70 1E7.2 alexa 488/CD3 + CD16 + CD56 PE/CD19 PECy5.5/CD5 APC. Isotype control with IgG1 alexa 488 antibody was also performed (IgG1 alexa488/CD3 + CD16 + CD56 PE/CD19 PECy5.5/CD5 APC). Then, T and NK cells were gated on CD3 + CD56 + CD16+ expression. B-CLL cells were gated on CD19 + CD5+ expression. Then, the percentage of ZAP-70 positive B cells accord-

Table 1 – Characteristics of the examined group.

Characteristics	N = / Median (Min–Max)	
No. of patients/healthy controls	N = 100	N = 27
Sex	Female – 50; Male – 50	Female – 12; Male – 15
Age (years)	69.0 (39–88)	70.0 (38–84)
Rai stage	0 – 48; I – 13; II – 18; III – 9; IV – 11	NA
Lymphocytosis (G/l)	16.9 (6.3–368.8)	16.9 (4.3–10.5)
Haemoglobin (g/dl)	14 (3.8–17)	13.8 (10.9–15.8)
Platelet count (G/l)	200 (26–374)	243 (156–401)
LDH (U/l)	190 (80–447)	131 (65–191)
B2-microglobulin (ng/ml)	2666 (1230–12,683)	1405 (990–2683)
ZAP-70+ patients	N = 37	NA
CD38+ patients	N = 31	NA

NA – not applicable.

ing to a threshold set on ZAP-70 positive T cells was determined.

Expression of CD38 was measured according to the standard immunophenotyping method. According to currently accepted thresholds, more than 30% of CD38-positive and more than 20% of ZAP-70-positive B-CLL cells were respectively treated as positive cases.

2.2.4. *Assessment of apoptosis by Annexin V assay*

The rate of apoptosis was determined by Annexin V assay. In brief, after *ex vivo* isolation cells were washed twice with cold PBS and then re-suspended in 100 μl of binding buffer, containing 2 μl of FITC conjugated Annexin V and 10 μg/ml of propidium iodide (PI) (Becton-Dickinson, San Jose, CA, USA). Next, the samples were incubated for 15 min, at room temperature, in the dark. The fluorescence was measured immediately after staining by flow cytometry. The apoptotic index was calculated as a percentage of Annexin V – positive cells.

2.2.5. *Expression of IAPs/IAP antagonist proteins*

For assessment of the cellular expression of *IAPs/IAP antagonist proteins* by flow cytometry, cells were fixed with 1% methanol free paraformaldehyde and permeabilised with 0.1% polysorbate 20 (Tween-20) in PBS (Amersham Biosciences Inc., Piscataway, NJ, USA). Directly before staining, cells were washed in PBS and then incubated with the following primary Abs, commercially available at the time of the study start: anti-cIAP1 (polyclonal rabbit Ab), anti-cIAP2 (polyclonal goat Ab), anti-XIAP (polyclonal goat Ab), anti-survivin (polyclonal rabbit Ab), anti-Smac/DIABLO (polyclonal rabbit Ab), and anti-HtrA2/Omi (polyclonal rabbit Ab). All these Abs (R&D Systems, Minneapolis, MN, USA) were diluted in PBS containing 1% bovine serum albumin (BSA) for concentrations of 1:100. Samples were incubated for 90 min, room temperature (RT), and then washed in PBS and centrifuged (5 min, 1100/min). Next, secondary swine anti-rabbit or goat anti-mouse, FITC-conjugated Abs were used at dilutions of 1:20 (60 min incubation, RT, in the dark). Afterwards, samples were washed in PBS, re-suspended in 400 μl PBS and subjected to flow cytometry analysis. An increase or decrease in protein expression, compared to the parallel control, was defined as upregulation or downregulation, respectively.

2.2.6. *Western blot analysis*

Immunoblotting was carried out using cell lysates from PBMC. Cell lysates were prepared by incubating the pellets on ice for 15 min in 1 ml of a lysis buffer containing 10 mM Tris-HCl, ph 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN3, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1% aprotinin 100uM leupeptin, and 100 uM tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (Sigma, Saint Louis, USA). After normalisation, total protein content (20 μg/lane) was determined using a Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by 12% gels in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Nonspecific binding sites were blocked for 1 h in 5% milk in TBS-T(100 mM Tris-HCl, 100 mM NaCl, 0,1% Tween-20), and were probed with the primary antibodies overnight. Immunoblottings were performed using appropriate MoAbs against IAPs/IAP antagonist proteins and β-actin (Sigma, Saint Louis, USA) which was used as a control. Finally, membranes were incubated for 1 h in the presence of the secondary reagent: horseradish peroxidase-antimouse IgG (1:3000; Promega, Madison WI) and horseradish peroxidase-antirabbit IgG (1:5000, Promega). All immunoblots were revealed by enhanced chemiluminescence using Super Signal West Pico Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Data on X-ray films were quantified by scanning-densitometry using the NIH image analysis system. To normalise for variation in antibody concentration or time of exposure, the protein signal from the respective patient was normalised against the protein signal of the control cell line. The following cell line lysates were used as a positive control for particular IAPs or IAP-antogonist proteins: for cIAP1 and cIAP2 – NALM-6 cells (precursor B-cell lymphoblastic lymphoma/acute lymphoblastic leukaemia cell line); for XIAP – Jurkat cells (T cell lymhoma line), for survivin – HL-60 cells (promyelocytic leukaemia cell line), for Smac/DIABLO – Jurkat cells, and for HTRA2/Omi – HL-60.

2.2.7. *Flow cytometry analysis*

All fluorescence measurements were performed by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA). An acquisition gate was established based on FSC and SSC that included mononuclear cells and excluded dead cells and debris. Then, cell fluorescence was measured using standard emission filters: FL1 (green, λ 515–545 nm), FL2 (orange, λ 564–606 nm), FL3 (red, $\lambda > 650$ nm) and FL4 (APC – L17F12, λ 800 ± 20 nm), where necessary. For each analysis, 10,000 events were acquired and analysed using CellQuestPro software (Becton Dickinson, San Jose, CA, USA). For assessment of examined IAPs/IAP-antagonist expression, the levels of mean fluorescence intensity (MFI) were assessed.

2.2.8. *Statistical analysis*

Statistical analysis was performed with the SPSS 14.0 program and Statistica 7.0 (Tulsa, OK, USA) software. Differences in the expression of IAPs and their antagonists between analysed groups were evaluated using the Mann–Whitney test. OS probability was estimated using the Kaplan–Meier method. Univariate analysis of potential prognostic factors influencing OS was performed using the log rank test. Multivariate analyses were also carried out to assess independency of prognostic factors using Cox's proportional hazards model. Differences between examined parameters were considered statistically significant when the p value was <0.5.

3. Results

Characteristics of the examined group are shown in Table 1. The median OS of patients, calculated from the diagnosis, was 37 months (range 10–175 months).

3.1. *Spontaneous apoptosis of CLL cells in vivo*

Apoptosis of CLL cells measured *in vivo* by the Annexin V assay was significantly higher than in healthy lymphocytes from the control group (median apoptotic index 3.9% versus

0.7%; $p = 0.0001$). Among CLL patients, the frequency of cell apoptosis did not differ significantly in patients with SD and PD; however, there was a trend toward lower apoptosis in the PD group (median apoptotic index 0.81% versus 0.65%; $p = 0.075$).

3.2. *Overexpression of cIAP1, cIAP2 and downregulation of Smac-DIABLO in CLL cells*

Expression of cIAP1 and cIAP2 was significantly higher in CLL cells than in healthy lymphocytes (Fig. 1A). Median MFI levels of those proteins were as follows: cIAP1 – 81.1 versus 8.2, respectively ($p = 0.000001$) and cIAP2 – 313.5 versus 146.8 ($p = 0.014$). Surprisingly, XIAP expression was significantly higher in control than in CLL cells (median MFI – 551.9 versus 272.1; $p = 0.004$). There were not any statistically significant differences in survivin expression between leukaemic and healthy cells.

With regard to IAP antagonists, decreased expression of Smac/DIABLO in CLL cells, in comparison to control lymphocytes, was found (median MFI level 147.9 versus 301.3; $p = 0.010$) (Fig. 1B). No differences were seen for HtrA2/Omi protein. These results were confirmed in the Western blot analysis (Fig. 2).

3.3. *High expression of IAPs in progressive and advanced CLL*

Significantly higher expression of all analysed IAPs was found in progressive CLL, compared to patients with SD (Fig. 3A). Median MFI levels in PD versus SD patients were as follows; cIAP1 – 131.1 versus 60.1, respectively ($p = 0.002$), cIAP2 – 434.3 versus 301.5 ($p = 0.026$), XIAP – 463.7 versus 225.4 ($p = 0.002$), and survivin – 119.6 versus 38.6 ($p = 0.00006$). The Western blot analysis was consistent with these results (Fig. 3B). Moreover, expression of IAP antagonist, Smac/DIABLO, was significantly lower in PD than SD patients (median MFI levels 95.4 versus 198.4; $p = 0.005$). Differences in HtrA2/Omi expression were not statistically significant.

Expression of IAPs was significantly higher in advanced clinical stages according to the Rai classification (stage 0 versus III + IV), including cIAP1 ($p = 0.044$), cIAP2 ($p = 0.021$), XIAP ($p = 0.003$) and survivin ($p = 0.005$) (Fig. 4). There were not any statistically significant differences in IAP antagonist expression between patients with less or more advanced disease (data not shown).

Expression of IAPs in CLL cells was elevated in patients with higher (>15.0 G/L) lymphocyte counts. This included cIAP1 ($p = 0.0005$), cIAP2 ($p = 0.038$), XIAP ($p = 0.003$) and survivin ($p = 0.000005$). Expression of XIAP correlated with beta-2-microglobulin concentration ($p = 0.01$). No correlation was found between the levels of analysed proteins and other factors such as age, gender, LDH level and expression of ZAP-70 and CD38 antigen.

3.4. *Prognostic significance of IAP expression*

Among all examined proteins, low baseline expression of cIAP1 and survivin correlated with a better response to treatment ($p = 0.023$ and $p = 0.012$, respectively; Table 2). Additionally, proportions of cIAP1/ HtrA2/Omi and cIAP1/ Smac/DIABLO expression in responders (CR + PR; $N = 41$) were significantly higher than in NR patients ($N = 9$; ratios 0.5 versus 2.2, $p = 0.003$, and 0.4 versus 1.1, $p = 0.001$, respectively).

Low expression of all examined IAPs (below median MFIs) correlated positively with longer OS (log rank test = 0.033; Fig. 5A). Importantly, high (above median MFIs) concurrent expression of two IAPs, cIAP1 and surviving, was associated with shorter OS (log rank test = 0.005) in the study group (Fig. 5B). Moreover, co-expression of cIAP1 and survivin appeared to be an independent negative prognostic factor ($p = 0.010$) in our study group when entered into a model con-

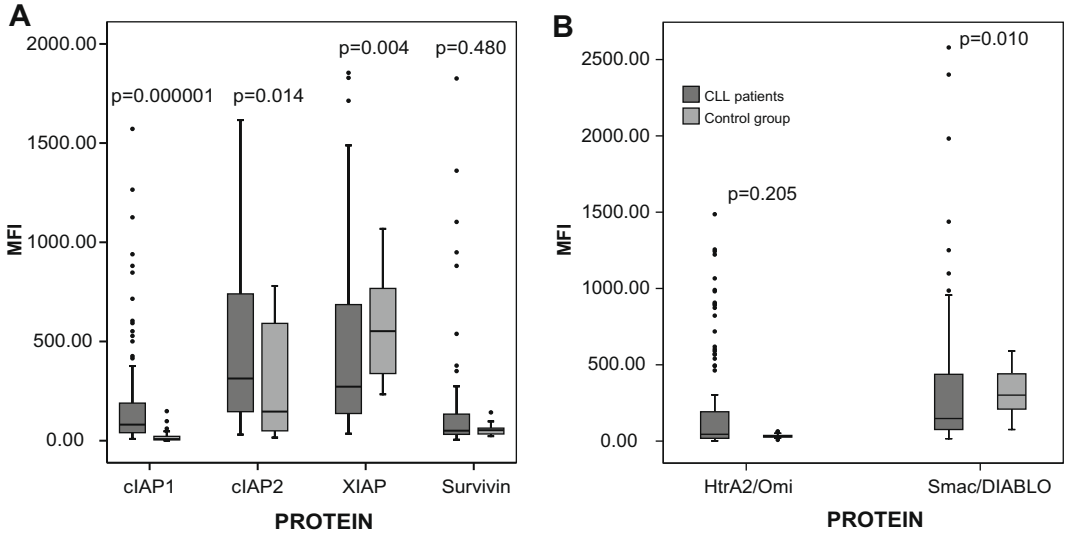

Fig. 1 – Expression of IAPs/IAP-antagonists in CLL cells in comparison to healthy lymphocytes. Protein expression was measured by mean fluorescence intensity (MFI) level. Medians, minimal and maximal values are presented. The individual dots are outliers (data which are numerically distant from the rest of results). (A) Increased expression of cIAP1 and cIAP2; (B) Decreased expression of IAP-antagonist, Smac/DIABLO.

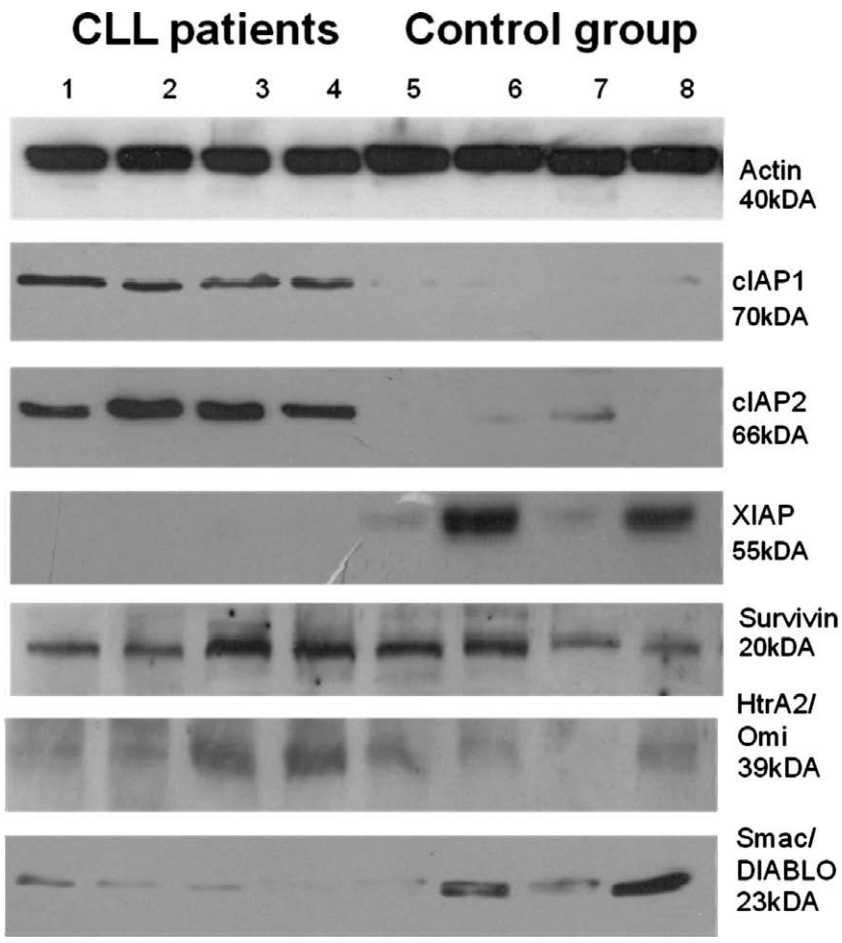

Fig. 2 – Expression of IAP proteins and their antagonists in CLL cells in comparison to healthy lymphocytes analysed by Western blot. To demonstrate significant differences in IAPs/IAP-antagonist expression found between the whole CLL group and the healthy subjects by flow cytometry analysis, the figure shows the most representative samples obtained from four CLL patients and four controls. MFI - mean fluorescence intensity. The bars represent medians, minimal and maximal values. The individual dots represent outliers.

taining variables of potential predictive value in CLL, such as: clinical stage, patient age, or levels of LDH and beta-2-microglobulin, as well as ZAP-70 and CD38 expression (Table 3).

4. Discussion

This is the first comprehensive analysis of the IAP family proteins and their antagonists content in CLL cells. The data we have presented demonstrates a significantly increased expression of anti-apoptotic cIAP1 and cIAP2 proteins and down-regulation of IAP antagonist, Smac/DIABLO, in the group of untreated CLL patients in comparison to healthy donors. Moreover, we found that concurrent high expression of two IAPs in a tumour cell was associated with a shorter OS.

In spite of the fact that recent knowledge may suggest an important role for IAPs and their antagonists in the pathophysiology of CLL, until recently there were only a few reports available concerning their expression in CLL cells. Our results are consistent with the data of Akyurek et al.[36] who showed high expression of cIAP1 and cIAP2 in CLL samples. However, the authors focused mostly on the comparative analysis of examined proteins in the different types of non-Hodgkin lymphoma, and the number of analysed CLL samples was very low (only nine patients). Significantly higher expression of cIAP2 mRNA was also confirmed by Nakagawa et al.[30] in bone marrow samples obtained from the CLL patients compared to the control group (21 versus 13 samples, respectively). In contrast with our data they did not observe differences in cIAP1 expression in the examined groups. On the other hand, Munzert et al.[37] reported a tendency toward higher levels of cIAP1 mRNA in CLL compared to healthy cells, whereas they did not find any differences in cIAP2 expression.

Thus, those reports are to some extent controversial. It is difficult, however, to compare results of existing single reports, which were obtained on a small number of samples, using various methods to determine the expression patterns. In our study, expression of IAPs and their inhibitors was investigated in individual cells of large cell populations by flow cytometry, and the data were in accordance with comparative studies by Western blot. To date, our group of patients studied on IAPs/IAP-antagonist expression is the most representative among available reports on CLL.

Survivin, another IAP family member, was found to be overexpressed in other haematological malignancies, includ-

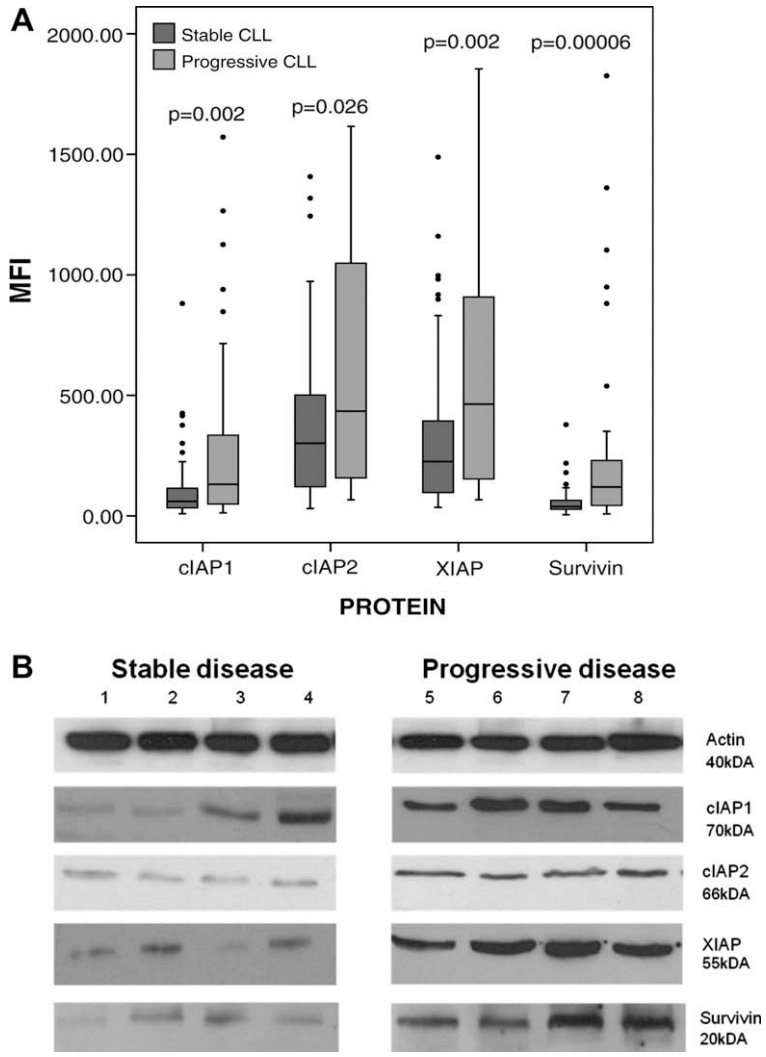

Fig. 3 – Expression of IAP proteins and their antagonists in CLL cells in regard to course of disease. (A) Significantly higher expression of analysed IAPs in progressive CLL, compared to patients with stable disease; the flow cytometry analysis. Protein expression was calculated by the level of mean fluorescence intensity (MFI). The bars represent medians, minimal and maximal values. The individual dots are outliers. (B) Examples of expression of analysed IAPs in eight representative patients with stable (1–4) and progressive (5–8) disease, underlying statistical differences found by flow cytometry. The samples were examined by Western blot assay.

ing *de novo* acute myeloid leukaemia cells[25,38,39] and some malignant lymphomas.[27,28,40] Nakagawa et al.[30] also indicated a significantly higher expression of survivin mRNA in CLL. In contrast to this report, we did not detect differences in its expression between CLL cells and lymphocytes obtained from healthy donors. Similar findings were reported by Munzert et al.[37] and, in part, by the group of Granziero et al.[41] who revealed the expression of survivin in only 5 of 28 examined CLL samples.

High expression of XIAP protein was found in many solid tumours[22,23,42] and also in some haematological malignancies.[21,24,26] Our study showed that XIAP protein expression in CLL cells was distinctly lower than in healthy lymphocytes. Similarly, Akyurek et al.[36] did not detect expression of XIAP in CLL samples. This was in contrast to Granziero et al.[41] who showed high expression of XIAP mRNA among all analysed CLL samples. Another XIAP expression pattern was reported by Munzert et al.[37] and Nakagawa et al.[30] who observed no differences in XIAP expression between the group of CLL patients and healthy donors.

In this study, we also assessed the expression of IAP antagonists, Smac/DIABLO and HtrA2/Omi, in CLL. Both flow cytometry assay and western blot analysis showed significantly lower expression of Smac/DIABLO in CLL cells compared to the normal lymphocytes, which correlated with the low frequency of *in vivo* spontaneous apoptosis of CLL cells.

Expression of HtrA2/Omi was similar to healthy controls. Until recently, there were only a few reports concerning the analysis of IAPs-antagonist expression in CLL. Schliep et al.[29] showed expression of Smac/DIABLO in CLL cells in all examined patients, whereas other authors reported a lack of Smac/DIABLO in all analysed samples.[43]

Comparing patients with different clinical courses of CLL we found a significantly higher expression of all analysed

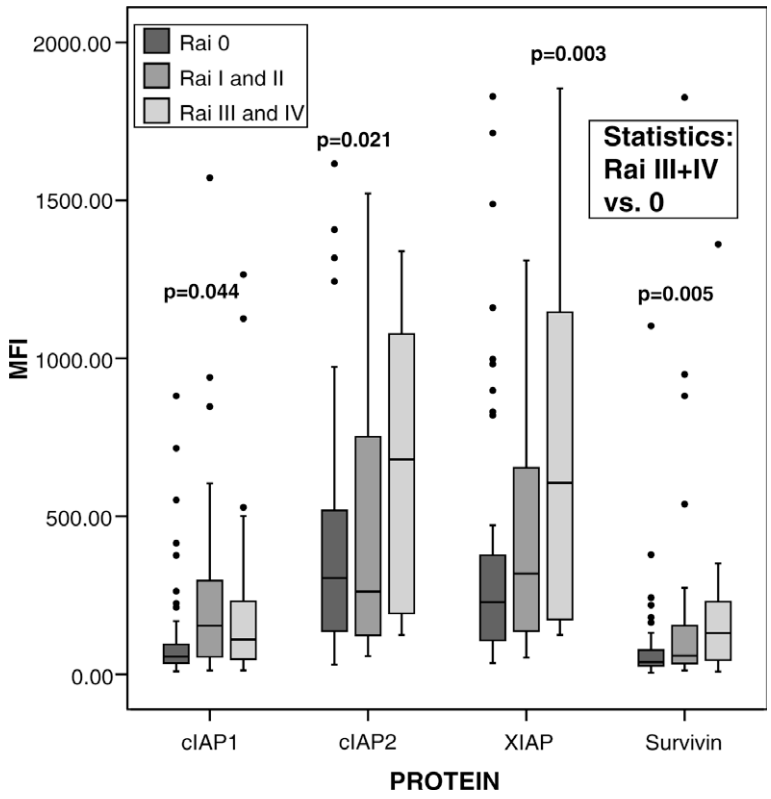

Fig. 4 – Increased expression of IAPs in advanced clinical stages according to the Rai classification. Protein expression was compared between groups of CLL patients with stage 0 versus I + II versus III + IV according to Rai. The bars represent medians, minimal and maximal values. The individual dots are outliers.

Table 2 – Pretreatment expression of IAPs and IAP-antagonists in patients who responded to treatment and non responders.

Proteins	Responders ($N = 41$) (Median MFI)	Non responders ($N = 9$) (Median MFI)	Statistics, p
cIAP1	77.9	592.5	0.023
cIAP1	156.9	334.4	0.077
XIAP	276.3	166.7	0.245
survivin	47.2	274.1	0.011
HtrA2/Omi	39.9	59.6	0.224
Smac/DIABLO	147.7	169.8	0.170

MFI – mean fluorescence intensity.

IAPs in PD. These results were consistent with our assessment of the spontaneous apoptosis *in vivo*, inhibited in PD patients. Thus, this indicates a role of inhibited proclivity of CLL cells to undergo spontaneous apoptosis in the disease progression. Two other studies showed comparable results with regard to survivin. Namely, expression of survivin mRNA was found by Granziero et al.[41] in patients with progressive CLL, whereas CLL cells in SD did not express this protein. Munzert et al.[37] also reported the lack of survivin in CLL samples obtained from non-progressive patients. However, due to the small number of examined groups, further studies to confirm these results seem warranted. Although in our study expression of anti-apoptotic survivin protein in CLL cells did not significantly differ from healthy lymphocytes, its higher expression in PD (versus SD) patients correlated with the anti-apoptotic profile of their CLL cells, which may result in progressive accumulation of leukaemic clone.

Regarding IAP antagonists, we revealed significantly lower expression of Smac/DIABLO protein in PD patients. There are no published data to compare this finding.

Regarding other clinical or laboratory parameters, we found a positive correlation between all examined proteins' expression and clinical stage according to Rai and higher lymphocytosis (>15 G/l), but only XIAP correlated with beta-2-microglobulin concentration. However, our study did not reveal any correlation between the levels of the analysed proteins and prognostic factors such as age, gender, LDH level and expression of ZAP-70 and CD38 antigen.

Interestingly, our study showed that simultaneous expression of two IAPs, cIAP1 and survivin, was associated with shorter OS. Co-expression of both IAP proteins occurred to be one of four independent risk factors in a multivariate analysis of our study group. Moreover, a high baseline expression of two IAPs, cIAP1 and survivin, correlated with a better response to the treatment. However, the ratio of IAPs and IAP antagonists also occurred to be associated with outcome. Namely, the ratios of cIAP1/ HtrA2/Omi and cIAP1/ Smac/DIABLO in responders were significantly higher than in patients with resistant disease. So far, little is known about the correlation between IAPs/IAP antagonists and the disease-related parameters. Consistent with our observations are two reports

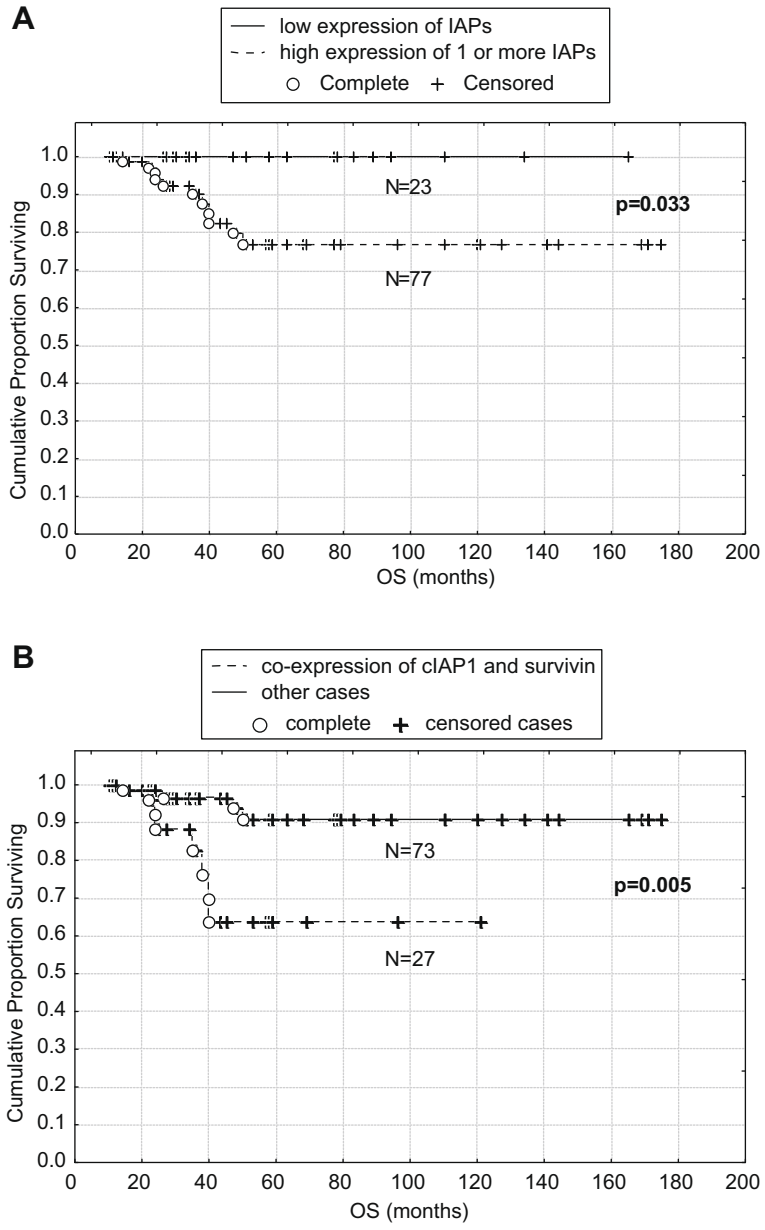

Fig. 5 – IAP expression and overall survival (OS) of CLL patients. (A) Kaplan–Meyer survival curves showing differences in OS between patients with low expression of IAPs and high expression of one or more IAPs. Low expression of all examined IAPs was the factor associated with longer OS (log-rank test – $p = 0.033$). (B) Kaplan–Meyer survival curves showing differences in OS between patients with co-expression of cIAP1 and survivin and other CLL patients. Co-expression of two IAPs (cIAP1 and survivin) was associated with shorter OS (log-rank test – $p = 0.005$).

Table 3 – Independent prognostic factors for overall survival (OS) in the study group. Multivariate analysis for predictors of recurrence based on the Cox proportional hazards regression model.

Prognostic factors for OS	HR	*p*
Co-expression of cIAP1 and survivin	1.7	0.012
Advanced clinical stage (Rai III + IV)	2.5	0.002
Age >75 y	2.1	0.008
ß$_2$microglobulin $\geqslant 2 \times N$	1.6	0.010

HR – hazard ratio.

which indicated that expression of IAP proteins in CLL did not depend on well known prognostic factors.[30,32] Concerning other haematological malignancies, Tamm et al.[24] revealed a statistically significant correlation between high expression of XIAP and survivin and shorter OS in AML patients. These results are in line with those reported by Adida et al.[28] and Schlette et al.[27] who observed shorter OS of non-Hodgkin's lymphoma patients with considerably higher expression of survivin in tumour cells. However, because of the small number of trials, particularly in CLL, further more comprehensive studies are necessary.

In conclusion, this study showed that CLL cells are characterised by overexpression of cIAP1 and cIAP2 and downregulation of Smac/DIABLO. The anti-apoptotic pattern of these apoptosis-regulating protein expressions may be a contributing factor in the pathogenesis of CLL. Elevated expression of all IAP proteins in PD may be responsible for the inhibited proclivity of CLL cells to undergo spontaneous apoptosis and may promote disease progression. Importantly, co-expression of cIAP1 and survivin proteins in tumour cells seems to be an unfavourable prognostic factor in CLL. Thus, all these data suggest that IAPs/IAP-antagonist systems play an important role in the pathophysiology of CLL.

Of course, the prognostic role of examined proteins should be interpreted with caution. The survival time analyses may be hampered by too short an observation time for the natural course of CLL. The number of patients being analysed in our centre is actually being extended. Thus, further studies, are warranted to confirm the prognostic usefulness of the present findings and to relate them within the context of other prognostic biomarkers of CLL.

Conflict of interest statement

None declared.

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