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Increased expression of *PIM-2* and *NF-κB* genes in patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) is associated with complete remission rate and overall survival

Zwiększona ekspresja genów *PIM-2* i *NF-κB* u chorych z ostrą białaczką szpikową i limfoblastyczną wykazuje związek z remisją całkowitą i przeżyciem

Katarzyna Kapelko-Słowik[1, A, B, D, E, F, G], Donata Urbaniak-Kujda[1, B, D, E], Dariusz Wołowiec[1, E, F], Bożena Jaźwiec[1, B, F], Jarosław Dybko[1, C], Jacek Jakubaszko[2, B], Mirosław Słowik[3, B, E], Kazimierz Kuliczkowski[1, A, G]

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

[1] Department of Hematology, Neoplastic Blood Disorders and Bone Marrow Transplantation Wroclaw Medical University, Wroclaw, Poland
[2] Department of Cardiosurgery, Wroclaw Medical University, Wroclaw, Poland
[3] Department of Ophthalmology, Wroclaw Medical University, Wroclaw, Poland

Summary

Introduction: *PIM-2* is a proto-oncogene that encodes for a serine/threonine kinase that interacts with various signaling molecules. *PIM-2* is highly expressed in neoplastic tissues and in leukemic and lymphoma cell lines, which is consistent with its role during oncogenic transformation. The nuclear factor kappa B (NF-κB) pathway appears to be deregulated in a variety of tumors, with sustained activity of NF-κB leading to apoptotic resistance in tumor cells. The aim of this study was to investigate whether expression of *PIM-2* and *NF*-κB is altered in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

Patients and methods: One hundred forty-three patients were included: 91 with AML and 52 with ALL, aged 18-84 (median 46.7). Eighty-three patients (51 AML and 32 ALL) reached complete remission (CR). Bone marrow samples were collected at the time of diagnosis. Control samples were obtained from 24 healthy donors. We analyzed *PIM-2* and *NF*-κB expression by RQ-PCR analysis.

Results: Expression of both *PIM-2* and *NF*-κB genes in all leukemic patients and both subgroups AML and ALL was significantly higher than in controls. AML patients who reached CR expressed *PIM-2* and *NF*-κB at significantly lower levels than patients with primary resistance to chemotherapy who did not reach CR (NCR). Survival analysis revealed that in AML patients, higher expression of *PIM-2* was related to significantly shorter patients' overall survival (OS).

Conclusion: Our data indicate that increased expression of *PIM-2* and *NF*-κB genes may be involved in pathogenesis of AML and ALL. Moreover, high *PIM-2* expression could be associated with CR rate and OS in AML patients.

Key words: **acute myeloid leukemia • acute lymphoblastic leukemia • *PIM-2* • *NF-κB***

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Author's address: Katarzyna Kapelko- Słowik, M.D., Ph.D, Wroclaw Medical University, Department of Hematology, 4 Pasteur St., 50-367 Wroclaw, Poland; e-mail: kks9999@wp.pl

Introduction

Inhibition of apoptosis is one of the most important phenomena inducing accumulation of neoplastic cells in leukemia patients. Despite extensive research, intracellular events leading to prolongation of cell life and resistance to pro-apoptotic factors are still not clearly defined. In recent years, the search for such events led to focusing on an anti-apoptotic factor, *PIM-2* (Proviral integration of Moloney virus-2). PIM-2, along with PIM-1 and PIM-3, belongs to a serine/threonine kinase family encoded by proto-oncogenes *PIM-2 PIM-1 and PIM-3* [2,7,26]. *PIM-2* gene expression is regulated at both the mRNA and protein levels by numerous cytokines (especially IL-3) involved in maturation of hematopoietic cells [13], and as such, kinase PIM-2 plays an important role in growth, differentiation, and survival of these cells. Its action is synergistic with another independent pro-survival pathway, PI3K/AKT/m-TOR. Murine model analyses led to the conclusion that incapacity of one of these pathways may be, at least partially, compensated by activities of the other [16].

The elevated expression of *PIM-2* was confirmed in human primary solid tumor cell lines (G361, A-549, SW-480) as well as hematological cell lines (HL-60, K-562, RAIJ) [4,24]. Alterations in *PIM-2* gene expression regulation were also shown in cells derived from prostate cancer and in some lymphatic system neoplasms [10,11,27]. Nuclear factor kappa B (NF-κB) is a key regulator of cell survival and differentiation [21]. In the inactive state NF-κB proteins occur as homodimeric or heterodimeric complexes in the cytoplasm bound to IκB proteins. After stimulation IκB is phosphorylated, ubiquitinated and degraded, which allows translocation of NF-κB to the nucleus and transcription of NF-κB targeted genes including many genes associated with cell survival: *XIAP*, and cellular inhibitors of apoptosis such as *FLIP*, *A1*, *BCL-2*, and *BCL-*X_L. The NF-κB pathway appears to be deregulated in a variety of tumors, with sustained activity of NF-κB leading to apoptotic resistance in tumor cells [20,22]. There is evidence that leukemic transformation of the FL5.12 lymphoid cells expressing Pim-2 transgene is dependent on NF-κB activation [17]. Similar observation on the PIM-2 dependence on NF-κB activity has been found in human hepatocellular carcinoma cells as well [28]. So far, limited data regarding *PIM-2* and *NF*-κB gene expression in acute leukemias are available. Significant levels of *PIM-2* mRNA were seen in primary blasts from patients with acute myeloid leukemia [25].

The aim in our study was to assess *PIM-2* and *NF*-κB expression in bone marrow samples collected from AML and ALL patients and to determine the correlation with clinical data and the outcome of induction treatment. Our promising preliminary data indicate increased levels of *PIM-2* mRNA as well as a relationship between *PIM-2* expression and CR rate in patients with AML compared with normal controls [19].

Patients, Cell lines and Methods

One hundred forty-three patients were included: 91 with AML and 52 with ALL, aged 18-84 (median 46.7). Eighty-three patients (51 AML and 32 ALL) reached complete remission (CR). Bone marrow samples were collected at the time of diagnosis. Leukemic bone marrow blasts accounted for more than 80% of the total cellularity, especially after Ficoll separation. Control samples were obtained from 24 healthy donors.

Clinical characteristics of patients and controls included in this study are given in Table 1. All of the patients underwent induction remission treatment according to the PALG (Polish Acute Leukemia Group) program for AML and ALL [14,18]. Patients were included in the study from January 1999 to June 2010, and they were observed during the period of 1 to 340 months (mean: 18 months). Complete remission was diagnosed according to standard criteria [9]. The control group consisted of 24 hematologically healthy bone marrow donors matching age and sex of the patients.

Human leukemic cell lines K-562, HL-60 and SD-1 were used as positive controls regarding expression of the examined genes.

Real-time PCR (RT-PCR)

Bone marrow was obtained from patients at diagnosis. Bone marrow mononuclear cells (BMNCs) were separated by centrifugation on Gradisol L (AquaMedica, Poland).

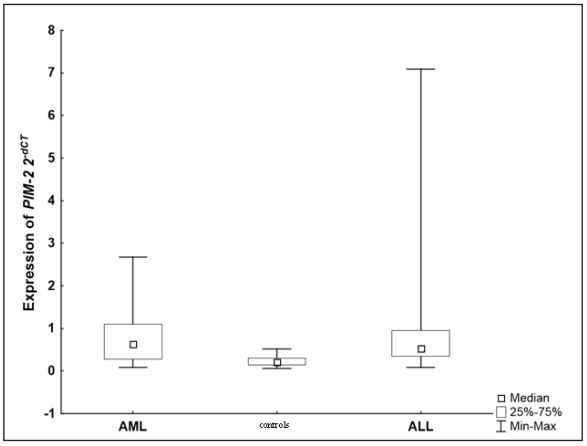

Fig. 1. Comparison of PIM-2 gene expression between AML and ALL patients and control group

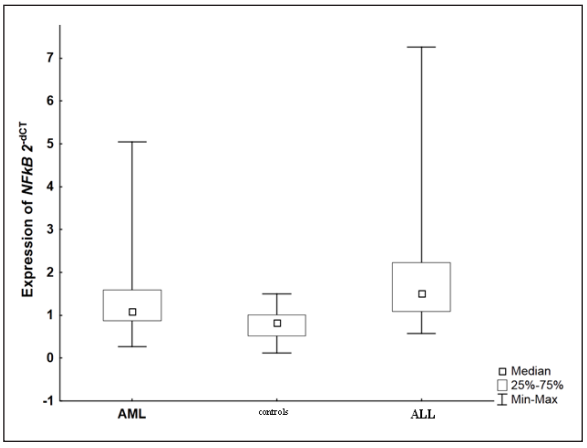

Fig. 2. Comparison of NF-κB gene expression between AML and ALL patients and control group

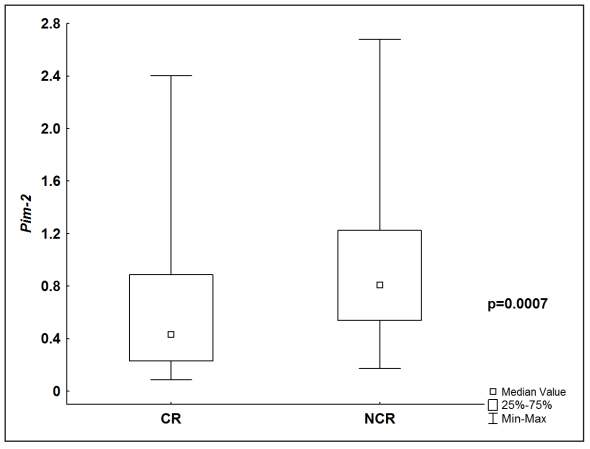

Fig. 3. PIM-2 gene expression among AML patients stratified according to treatment response (CR – complete remission, NCR – not complete remission)

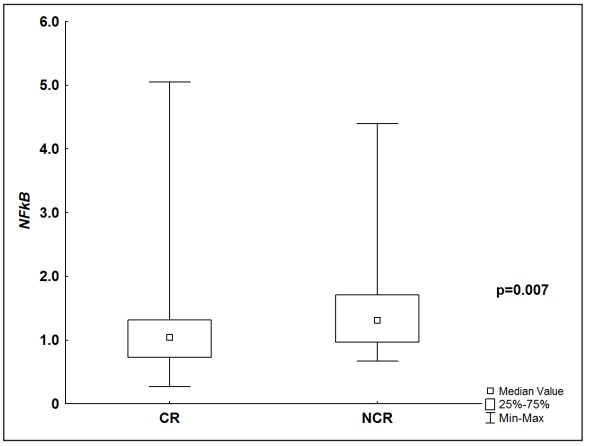

Fig. 4. NF-κB gene expression among AML patients stratified according to treatment response (CR – complete remission, NCR – not complete remission)

Total RNA was isolated from 5-10x10^6 BMNC using TriReagent® Solution (Ambient/Applied Biosystems) according to the manufacturer's protocol. DNA was removed from isolated RNA samples by DNase treatment using DNA-*free*™ reagent (Ambient/Applied Biosystems). Two micrograms of RNA were reverse transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according the manufacturer's protocol. Expression of *PIM-2* and *NF*-κβ genes was assessed on a 7500 Real Time PCR System (Applied Biosystems) with TaqMan real-time reverse transcription–polymerase chain reaction (RT-PCR) assay using inventoried TaqMan Gene Expression Assays Hs00179139_m1 and Hs00231653_m1 from Applied Biosystems. The beta-glucuronidase gene (*GUS*) was used as an internal control (TaqMan Gene Expression Assay Hs99999908_m1) [5].

The relative gene expression was calculated as the difference between the C_t values of *PIM-2* and *NF*-κ*B*, and *GUS* control (ΔC_t) and expressed as $2^{-\Delta Ct}$ for statistical analysis.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U test for independent samples. The correlation between quantitative variables was tested with Spearman's rank correlation test. Survival analysis was performed with Kaplan-Meier test.

Results

Expression of *PIM-2* and *NF*-κB genes

In leukemic patients either considered as a whole group (AML+ALL) or stratified into AML and ALL subgroups, the median expression of both *PIM-2* and *NF*-κB genes was significantly higher than in controls (Fig. 1, Fig. 2 and Table 1, Table 2). Only in AML patients who obtained CR was the median expression of *PIM-2* and *NF*-κB significantly

Table 1. Clinical data of patients and controls (AML-acute myeloid leukemia, ALL – acute lymphoblastic leukemia, F – females, M – males, CR – complete remission, NCR – no complete remission, HR – high risk group, IR – intermediate risk group, SR – standard risk group, Hb – concentration of hemoglobin, WBC – white blood cells, PLT – platelets, g/dL – gram/deciliter, G/L – Giga/liter, SD – standard deviation, M0 – minimally differentiated acute myeloid leukemia, M1 – acute myeloid leukemia without maturation, M2 – acute myeloid leukemia with maturation, M4 – acute myelomonocytic leukemia, M5a – acute monoblastic leukemia, M5b – acute monocytic leukemia, M6 – erythroleukemia)

	AML+ALL Patients	AML patients					ALL patients			Control group
		M0	M1	M2	M4	M5a+M5b	M6	B-cell	T-cell	
n	143	7	18	19	28	6+10	3	43	9	24
		91					52			
Age (median)	46.7	48.7					43			47.3
Sex F/M	61/82	41/50					20/32			11/13
CR/NCR	83/60	51/40					32/20			-
HR/IR/SR	69/55/19	25/55/11					44/0/8			-
Hb g/dL (mean±SD)	9.67±1.98	9.13±1.89					9.47±1.86			15.4±2.87
WBC G/L (mean±SD)	45.33±56.7	48.02±66.26					46.5±45.9			5.56±1.77
PLT G/L (mean±SD)	73.41±71.65	70.05±78.9					81.69±82.79			249.1±87.3
Blasts in peripheral blood, G/L (mean±SD)	48.9±37.5	39.6±57.7					62.2±85.2			0
% Blasts in myelogram (mean±SD)	86.78±4.31	87.68±5.69					85.8±7.43			0

lower than in patients who did not respond to induction treatment (Fig. 3, Fig. 4 and Table 2). The relationship of both *PIM-2* and *NF*-κB mRNA levels with response to induction therapy was not seen in the ALL group. No significant differences were observed between AML and ALL subgroups (Table 2) and between particular subgroups stratified according to the FAB classification in AML and ALL patients (data not shown).

For AML patients, a positive correlation between *PIM-2* and patient age (R=0.23, p=0.02) was observed. There was no correlation between *PIM-2* or *NF*-κB expression and absolute leukemic cell count in peripheral blood, hemoglobin concentration, platelet count or presence of chromosomal aberrations.

PIM-2 and *NF*-κB expression was additionally assessed in K-562, HL-60, and SD-1 leukemic cell lines, and was found

Table 2. Median expression of PIM-2 and NF-κB genes in patients and controls (AML – acute myeloid leukemia, ALL – acute lymphoblastic leukemia, CR – complete remission, NCR – no complete remission)

	Control group (1)	AML+ALL (2)	AML (3)	ALL (4)	AML-CR (5)	AML-NCR (6)	ALL-CR (7)	ALL-NCR (8)	p
NF-κB **(Median, min-max)**	0.83 (0.12-1.5)	1.3 (0.17-7.26)	1.22 (0.27-5.05)	1,51 (0.58-7.26)	1,04 (0.27-5.05)	1.32 (0.67-4.39)	1.51 (0.58-7.26)	1.46 (0.73-2.65)	2vs1=0.000001 3vs1=0.00001 4vs1=0.000000 5vs6=0.04 7vs8=N.S.
PIM-2 **(Median, min-max)**	0.21 (0.06-0.52)	0.66 (0.09-7.09)	0.77 (0.09-2.68)	0.53 (0.09-7.09)	0.43 (0.09-2.41)	0.81 (0.21-2.68)	0.59 (0.09-1.67)	0.51 (0.09-7.09)	2vs1=0.0000001 3vs1=0.0000001 4vs1=0.0000001 5vs 6=0.007 7vs8= N.S.

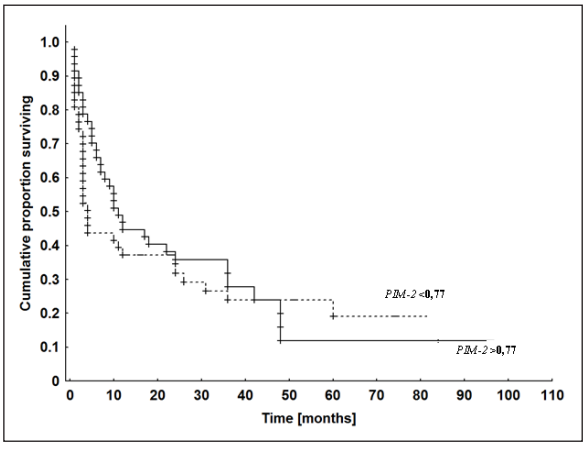

Fig. 5. Cumulative overall survival among AML patients stratified by PIM-2 gene expression. Patients were stratified as above or below the median value of PIM-2 gene expression (0.77)

to be significantly higher than in the control group, and was comparable to values obtained in leukemic patients (data not shown).

Survival analysis

In univariate analysis, a difference in overall survival between the AML patients with expression of *PIM-2* below and above the median value (estimated at 0.77) was observed (p=0.0377) (Fig. 5). Such a difference was not observed in AML patients with respect to *NF*-κB expression.

Discussion

The analyses of cell lines and some studies carried out on lymphoma cells indicated that increased *PIM-2* expression may be involved in the pathogenesis of hematological malignancies. In fact, *PIM-2* expression was found to be increased at both the mRNA and protein levels in chronic lymphocytic leukemia, follicular lymphoma, and diffuse large B-cell lymphoma compared to normal cells [10]. Recent data revealed that PIM-2 kinase inhibitor was able to induce apoptosis in CLL and myeloma cells, thus suggesting its anti-apoptotic function [3,8]. Therefore recently, interest in cancer research has focused on PIM-2 serine/threonine kinase, whose expression is regulated by hematopoietic cytokines, such as IL-3. Growth factor-induced increase in PIM-2 expression suppresses apoptosis and promotes cell survival in hematologic malignancies and solid tumors [6,13]. These events are consequences of PIM-2-mediated phosphorylation of the factors involved in apoptosis signaling, thus conferring apoptotic resistance in the neoplastic cells [23,31]. Yet, the influence of PIM-2 activity on cellular proliferation is still controversial.

In addition, a strong interrelation of *PIM-2* and *NF*-κB pathways in both leukemo- and tumorigenesis has been demonstrated. A key role of *NF*-κB in the *PIM-2* pathway has been reported by Hammerman et al. in a mouse model of lymphoma and by Ren et al. in human hepatocellular carcinoma [17, 28]. PIM-2 kinase activates apoptosis inhibitor 5 (API-5), which is a downstream factor for NF-κB. Moreover, apoptosis triggered by high PIM-2 expression could be reversed by NF-κB repressor [28]. So far, there are only a few publications regarding the role of these two factors in the development of acute leukemias in humans. A recent report by Adam et al. on hematopoietic cells transformed by FLT3-ITD (FMS-like tyrosine kinase 3-internal tandem duplication) and BCR/ABL mutations, which are frequently expressed in AML and ALL, demonstrated that the suppression of *PIM-1* and *PIM-2* expression led to a significant decrease in cell survival and immortality [1]. Mizuki et al. observed that *PIM-2* mRNA was significantly induced in the AML samples [25]. Moreover, Tamburini et al. found that PIM-2 kinase was constitutively expressed in AML blasts,

but was barely detectable in normal $CD34^+$ hematopoietic progenitors [30]. Therefore, we performed comparative analysis of *PIM-2* and *NF*-κB gene expression in bone marrow of AML and ALL patients and in normal hematopoietic cells. We found that levels of *PIM-2* and *NF*-κB transcripts were significantly and similarly higher in bone marrow cells of acute leukemia patients as well as in HL-60, K562, and SD-1 leukemic cell lines compared to normal cells. The current study is in accordance with the previous report conducted on pre-B–derived murine cell line FL5.12, indicating that lymphoid cells transfected with Pim-2 kinase demonstrated longer survival [17]. High *PIM-2* expression (both at the mRNA and at the protein level) was also demonstrated by Gong et al. in human hepatocellular cancer cells (HepG2). After *PIM-2* knock-down, the cancer cells lost survival ability in IL-3 starvation medium [15]. On the other hand, Dai JM et al. observed that antisense oligonucleotides against *PIM-2* induce a significant decrease in the proliferating fraction of the DU-145 human prostate cancer cell line, at least in part, due to the inhibition of cell cycle progression in G1 phase [12]; no signs of apoptosis of the tumor cells were also seen in this report. In contrast, Zhang et al. observed an increase of the apoptosis rate after silencing of *PIM-2* gene expression by siRNA (small-interfering RNAs) in the human colon cancer cell line SW-480, which proved its anti-apoptotic action [32]. Also, recent data revealed that PIM-2 kinase inhibitor was able to induce apoptosis in CLL and myeloma cells [3,8].

An important observation of our study was that *PIM-2* and *NF*-κB gene expression was found to be lower in patients with AML who reached CR in comparison to the AML group, in which induction treatment was ineffective. Our finding of a *PIM-2* relationship with the clinical outcome is in line with a recent report of Rubenstein et al., who observed higher levels of *PIM2* mRNA in recurrent CNS lymphomas refractory to rituximab [29]. Based on the fact that *PIM-2* and *NF*-κB promote cell survival in leukemic hematopoiesis, our observation points to the possibility that their high expression decreases blast cell sensitivity to apoptosis, including cell death induced by cytotoxic drugs. In addition, our results showed that the lower *PIM-2* expression in AML blasts corresponded with patients' overall survival, suggesting its possible prognostic significance.

Conclusion

In the current study, we found that expression of *PIM-2* and *NF*-κB genes was significantly increased in patients with AML and ALL, confirming their important role in the pathogenesis of acute leukemias. The high expression of the *PIM-2* gene was associated with a lower complete remission rate and worse overall survival. Although the latter relationship was revealed in univariate analysis in patients with AML only, it may suggest relevance of *PIM-2* expression as a possible prognostic factor in patients with acute leukemias. Nevertheless, it is possible that the capacity of *PIM-2* and *NF-κB* to protect blast cells against cytostatic drug-driven eradication requires cooperation with other anti-apoptotic intracellular factors, and needs further studies.

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