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Association of *PIM-2* and *NF-κB* Over-Expression with Poor Clinical Outcome in Egyptian Patients with Acute Myeloid Leukemia

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ABSTRACT

Background: Acute myeloid leukemia (AML) is a highly fatal disease. Therefore, accurate predictors of clinical outcome can contribute to the design of appropriate treatment for individual patients. Proviral integration of Moloney virus-2 (*PIM2*) is a key mediator of hematopoietic cell growth and apoptotic resistance. Nuclear factor kappa B (*NF-κB*) activation by *PIM2* is required for its anti-apoptotic function.

Objectives: The aim of the present study was to assess *PIM-2* and *NF-κB* expression in adult patients with AML and to determine their correlation with the induction outcome.

Patients and Methods: This study was conducted on 90 patients with de novo AML and 36 age and sex matched patients were selected as a control group. Using real time polymerase chain reaction, *PIM2* and *NF-κB* expression was analyzed. Patients were followed up by bone marrow examination on day 28 after induction to assess the response to chemotherapy.

Results: There was an over-expression of both *PIM-2* and *NF-κB* in patients before induction compared to controls. After induction, those who failed to respond to induction therapy had higher expression levels than those who achieved complete remission. A significant positive correlation was evident between their expression levels in patients with AML and in patients who had induction failure. The high expression of *PIM-2* was associated with a lower complete remission rate and worse prognosis.

Conclusion: Over-expression of *PIM-2* and *NF-κB* in patients with AML is associated with resistance to induction therapy and low complete remission rate.

Key Words: *PIM-2 – NF-κB – AML.*

INTRODUCTION

Acute myeloid leukemia (AML) is a fatal disease caused by the transformation of hematopoietic progenitor cells due to a number of genetic alterations **[1]**. Although chemotherapy can induce complete remission in around 70% of cases, many patients relapse and die of their disease. Therefore, identification of accurate predictors of the clinical outcome can guide the design of appropriate treatment for individual patients **[2]**.

The proviral insertion in murine (PIM) lymphoma family proteins, whose gene locus was discovered as a proviral integration site for Moloney murine leukemia virus infection, includes three serine/threonine kinase isoforms: PIM1, PIM2 and PIM3 **[3]**.

The proto-oncogene *PIM2* is an essential mediator of hematopoietic cell growth and apoptotic resistance. *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 **[4]**, and as such, *PIM-2* kinase plays an important role in growth, differentiation, and survival of these cells. The elevated expression of *PIM-2* was confirmed in human primary solid tumor cell lines, hematological cell lines, cells derived from prostate cancer and in some lymphatic system neoplasms. *PIM2* has been implicated in the transformation of both T and B lymphocytes and is over expressed in human leukemia and lymphomas **[5]**.

Nuclear factor kappa B (*NF-κB*) is a key regulator of the cell survival and differentiation; its pathway appears to be deregulated in a variety of tumors, with sustained activity of *NF-κB* leading to resistance of apoptosis in tumor cells **[6]**. *PIM -2* expression maintains high levels of

NF-κB activity and *NF-κB*–dependent gene expression in the absence of growth factor stimulation, and *NF-κB* activation by *PIM -2* is required for its anti-apoptotic function. There is evidence that leukemic transformation of some lymphoid cells expressing *PIM-2* transgene is dependent on *NF-κB* activation **[7]**. Similar observation on the *PIM-2* dependence on *NF-κB* activity has been found in human hepatocellular carcinoma cells as well **[8]**. However, there is paucity of data regarding *PIM-2* and *NF-κB* gene expression in acute leukemias.

The aim of the present study was to assess *PIM-2* and *NF-κB* expression in adult patients with de novo AML and to determine their correlation with the outcome of induction treatment.

PATIENTS AND METHODS

This study was conducted on 90 patients with de novo AML. Patients, who met the diagnostic criteria for AML, were selected from the Hematology unit of Alexandria main University Hospital between January 2014 and December 2015. The patients were 42 males and 48 females with age range from 15 to 80 years (median age 41.5). Thirty six age and sex matched normal bone marrow transplantation (BMT) donors were selected as a control group. They were 9 males and 27 females with age range from 25 to 85 years (median age 48.5). The selection of these patients was based on the following criteria: Full history taking; thorough clinical examination; standard diagnostic methods, including cytomorphological, cytochemical, cytogenetic and immunophenotypic evaluation which was established using Miltenyi Biotec MACS Quant™ flowcytometry analyzer equipped with MACS Quantify software version 2.4. (positivity by flowcytometry was defined as an expression in at least 20% of cells in the gated population of interest, compared to internal negative control cells). Inclusion criteria for the study were newly diagnosed AML with different FAB subtypes and normal karyotyping by conventional cytogenetics on bone marrow aspirate (BMA) at the time of diagnosis. To establish cytogenetically normal (CN-AML), 20 or more metaphase cells from the samples had to be examined to assure normal karyotypes. Patients with therapy-related AML were excluded from the study.RNA isolation from bone marrow aspirates or peripheral blood and cDNA preparation followed by quantitative real time RT-PCR were done to assess expression of *PIM2* and *NF-κB* in both cases and controls.

Then patients received the standard '3 + 7' induction chemotherapy protocol: Doxorubicin (45mg/m^2/day) for 3 days and cytarabine (100mg/m^2/day as a continuous 24h intravenous infusion) for 7 days. BMA was done between 21 and 28 days after the initiation of chemotherapy to demonstrate the morphological remission. Consolidation is comprised of three to four courses of high-dose cytosine arabinoside (3g/m^2 every 12h on days 1, 3 and 5; total, 18g/m^2). Patients were followed up once every 3 months for one to two years with clinical examination and complete blood counts. BMA was done if there was any doubt of a relapse on clinical examination or peripheral smear. Complete remission (CR) was a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other BM elements, with neutrophil count of ≥1 x 10^9/L and a platelet count of ≥100 x 10^9/L.

The study was approved by the medical ethics committee and informed consents were obtained from all participants involved in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 **[5]**.

PIM2 and NF-κB expression:

Purification of total cellular RNA from human whole blood was done using the PureLink® RNA Mini Kit (Ambion by life technologies, USA). The concentration and purity of RNA were determined by measuring the absorbance at 260, 280 and 230nm using Nanodrop2000 spectrophotometer (Thermo Scientific, USA). A260:A230 ratio greater than 1.9 and A260:A280 ratio greater than 2.1 indicates highly pure RNA. Reverse transcription (cDNA synthesis) was done using high capacity cDNA reverse transcription kit (Applied Biosystems, USA) by Touchgene gradient thermal cycler (USA). Quantitative RT-PCR for *PIM2* and *NF-κB* expression was performed using TaqMan® Universal Master Mix II (Applied Biosystems, USA). Expression data were normalized to the geometric mean of the housekeeping gene beta glucuronidase (GUSB) to control the vari-ability in expression levels by RT-PCR, using real-time cycler Rotor gene Q®

(Qiagen, USA). Data analysis was done using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups for categorical variables were assessed using Chi-square test (χ^2). Student t-test was used to compare two groups for normally distributed quantitative variables. Mann-Whitney test was used to compare two groups for abnormally distributed quantitative variables. Paired *t*-test and Wilcoxon signed ranks test were assessed for comparison between different periods. Spearman coefficient was used to correlate between quantitative variables. Significance of the obtained results was judged at the 5% level.

RESULTS

PIM-2 and NF-κB expression in patients with AML:

Blood samples were taken from 90 leukemic patients before therapy. Demographic findings, initial peripheral blood counts and parameters showing tumor burden were done. Using an RT-PCR approach, we detected the expression levels of *PIM-2* and *NF-κB* in patients with AML at diagnosis. The median expression levels for the patients were 75.0 and 2.35 respectively, while the levels in controls were 0.11 and 0.17 respectively. The median expression levels of both *PIM-2* and *NF-κB* were significantly higher in cases than in controls ($p<0.001$).

Only 39/90 (43.3%) patients achieved complete remission, while 51/90 (56.7%) cases failed to respond to induction therapy without any induction deaths. In relation to the clinical outcome, the median expression levels of both *PIM-2* and *NF-κB* were significantly lower (0.90, 0.70 respectively) in AML patients who achieved complete remission than those with induction failure (190.0, 170.0 respectively, $p<0.001$) as shown in Table (1). Meanwhile, no significant differences were observed between the particular subtypes stratified according to the FAB classification in AML.

Correlation studies:

A significant positive correlation was found between *PIM2* and *NF-κB* expression and age in patients with AML (r_s=0.575 and 0.574) respectively. Therefore, the older the patients, the higher the expression levels of *PIM2* and *NF-κB*. Meanwhile, no association was found between their expression and gender (p=0.110, 0.129) respectively.

As regards the peripheral blood and bone marrow aspirate parameters, a significant negative correlation was evident between *PIM2* and *NF-κB* expression with both hemoglobin concentration (r_s =–0.428, –0.381, $p<0.05$) and RBC count in patients with AML (r_s=–0.506, –0.532, $p<0.05$) respectively. However, a significant positive correlation was found between blast percentage and only *PIM2* expression (r_s=0.472, p=0.008). On the contrary, no correlations were observed between *PIM2* and *NF-κB* expression and WBC count or platelet count.

A statistically significant positive correlation was evident between *PIM2* and *NF-κB* expression in patients with AML (r_s=0.766, $p<0.001$) as well as in the cohort who failed to achieve complete remission (r_s=0.893, $p<0.001$) as presented in Table (2).

Table (1): *PIM2*, *NFκB* expression in AML patients in relation to the clinical outcome.

Gene	Outcome		*p*
	Complete remission (n=39)	Induction failure (n=51)	
PIM2 expression	0.90 (0.40 – 377.85)	190.0 (0.89 – 620.0)	<0.001*
NFκB expression	0.70 (0.09 – 2.70)	170.0 (0.60 – 560.0)	<0.001*

Abnormally quantitative data expressed in Median (Min. – Max.) and was compared using Mann Whitney test.
*: Statistically significant at $p\leq0.05$.

Table (2): Correlation between *PIM2* and *NFκB* expression and the clinical outcome in acute myeloid leukemia according to clinical response.

	All Cases (n=90)	Induction failure (n=51)	Complete remission (n=39)
r	0.766*	0.893*	0.206
p	<0.001*	<0.001*	0.207

r: Spearman coefficient.
*: Statistically significant at $p\leq0.05$.

DISCUSSION

PIM kinases are frequently over-expressed in human cancer, especially in hematological malignancies, where they support malignant cell proliferation and survival **[9]**. In humans, increased levels of *PIM-2* were described in different hematological malignancies. In fact, *PIM-2* expression was found to be increased at both the mRNA and protein levels in several malignancies that originate from the B-cell lineage such as chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL) or myeloma **[5]**. It was revealed that *PIM-2* kinase inhibitor was able to induce apoptosis in CLL and myeloma cells, suggesting its anti-apoptotic function **[10]**.

In the present study, *PIM-2* expression level was significantly higher in patients with AML than in controls. Significant levels of *PIM2* were observed in primary blasts from AML patients but were barely detectable in normal CD34+ hematopoietic progenitors **[11]**. This agrees with the present results which revealed a significant positive correlation between *PIM2* expression and blast percentage.

In addition, the expression of *NF-κB* was found to be significantly higher in patients with AML than in controls. Similarly, constitutive *NF-κB* has been detected in 40% of AML cases and its aberrant activity enables leukemia cells to evade apoptosis and stimulate proliferation. These facts suggest that *NF-κB* signaling pathway plays a fundamental role in the development of AML and it represents an attractive target for the intervention in AML **[12]**.

As regards follow-up of the patients after induction chemotherapy, early assessment of response to therapy represents an in vivo assessment of chemosensitivity and may be an eminent tool to delineate the prognosis in these patients **[13]**. It has been considered that failure of achieving blast clearance from the bone marrow aspirates after 1 or 2 weeks of induction chemotherapy indicates a poor prognosis **[13]**. Moreover, another study has assumed that the persistence of circulating blasts after one week of multi-agent chemotherapy indicated a poor prognosis **[14]**. Meanwhile, the response to induction chemotherapy has been evaluated by assessing the degree of residual leukemic infiltration in the bone marrow after 14 days of chemotherapy **[15]**.

Due to bone marrow aplasia on day 14 and poor general condition of the patients, the evaluation of response to induction chemotherapy, in the current study, was carried out between 21 and 28 days. Those patients who failed to respond to induction therapy had higher expression levels of both *PIM-2* and *NF-κB* than those who achieved complete remission. 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. 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, the suppression of *PIM-1* and *PIM-2* expression had led to a significant decrease in cell survival **[16]**. These results are in accordance with another study which demonstrated high *PIM-2* expression (both at the mRNA and at the protein level) in human hepatocellular cancer cells (HepG2). After PIM-2 knock-down, the cancer cells lost survival ability in IL-3 starvation medium **[17]**. Likewise, an increase of the apoptosis rate was observed 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 **[18]**. In addition, it was 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 **[19]**. Moreover, another study has observed higher levels of *PIM2* mRNA in recurrent CNS lymphomas refractory to rituximab **[20]**. In the present study, a statistically significant positive correlation was evident between *PIM2* and *NF-κB* expression in patients with AML as well as in the cohort who failed to achieve complete remission. This could be explained by the strong interrelation of *PIM-2* and *NF-κB* pathways in both leukemo- and tumorigenesis. A key role of *NF-κB* in the *PIM-2* pathway has been reported in a mouse model of lymphoma and in human hepatocellular carcinoma **[7,8]**.

In our studied cohort, no mortality was detected. However, a recent study has shown that elevated *PIM2* gene expression was associated with poor survival of patients with acute myeloid leukemia suggesting another aspect of its possible prognostic significance **[21]**. In this aspect, our results showed that high expression of PIM-2 gene was associated with a lower complete remission rate and worse prognosis. This relationship may suggest relevance of *PIM-2* expression as a possible prognostic factor in patients with acute myeloid leukemia.

In conclusion, overexpression of *PIM-2* and *NF-κB* in patients with AML is associated with resistance to induction therapy and low complete remission rate.

The authors declare that they have no conflict of interest.

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