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RESEARCH ARTICLE

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

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 Pim-2 is required for its antiapoptotic function. 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. This study was conducted on 90 patients with de novo AML. Using real time polymerase chain reaction RT-PCR, 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. There was an overexpression of both PIM-2 and NF-κB in patients before induction. 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 in patients with AML and with induction failure. 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.

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive disease caused by the transformation of hematopoietic progenitor cells due to the acquisition of genetic alterations (Patel *et al*., 2012). 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 contribute to the design of appropriate treatment for individual patients (Döhner *et al*., 2010). 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, consists of three serine/threonine kinase isoforms: PIM1, PIM2 and PIM3 (Nawijn *et al*., 2011). PIM2 is highly expressed in progenitor cells of the B-cell lineage and critically involved in signaling pathways regulating B-cell homeostasis (Woodland *et al*., 2008). Moreover, PIM2 has been reported being over-expressed and associated with progression of several malignancies that originate from the B-cell lineage such as chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), or myeloma (Huttmann *et al*., 2006). Nuclear factor kappa B (NF-κB) is a key regulator of the cell survival and differentiation. Its activation can lead to downstream protein expression that can promote tumor growth, antiapoptosis, invasiveness and chemotherapeutic resistance, which eventually allows tumor cells avoid cell death (Aggarwal *et al*., 2011). PIM2 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 PIM2 is required for its antiapoptotic function. There is evidence that leukemic transformation of some lymphoid cells expressing PIM-2 transgene is dependent on NF-κB activation (Hammerman *et al*., 2004). Similar observation on the PIM-2 dependence on NF-κB activity has been found in human hepatocellular carcinoma cells as well (Ren *et al*., 2010). 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.

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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 patients with hematological diseases to whom bone marrow examination is one of the required investigations 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, immunophenotypic which was established using Becton Dickinson, FACSCalibur flowcytometer equipped with Cell Quest software (Becton Dickinson, San Diego, CA, USA), (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) and cytogenetic evaluation. 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 (45 mg/m^2/day) for 3 days and cytarabine (100 mg/m^2/day as a continuous 24 h 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 (3 g/m^2 every 12 h on days 1, 3 and 5; total, 18 g/m^2). Patients were followed up once every 3 months with clinical examination and complete blood counts. BMA was done if there was any doubt of a relapse on clinical examination or peripheral smear. 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 230 nm 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 variability 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. Kaplan–Meier test was used for survival analysis. Significance of the obtained results was judged at the 5% level.

RESULTS

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

Using an RT-PCR approach, we detected the expression levels of PIM-2 and NF-κB in patients with AML at diagnosis. As shown in figures (1) and (2), the median expression levels of both PIM-2 and NF-κB were significantly higher in cases than in controls (P<0.001). On day 28 after induction chemotherapy, only 39 (43.3%) patients achieved complete remission, while 51 (56.7%) cases failed to respond to induction therapy. In relation to the clinical outcome, the median expression levels of both PIM-2 and NF-κB were significantly lower in AML patients who achieved complete remission than those with induction failure (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. Therefore, the older the patients, the higher the expression levels of PIM2 and NF-κB. Meanwhile, no correlation was found between their expression and gender. 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 and RBC count in patients with AML. However, a significant positive correlation was only found between PIM2 expression and blast percentage (Rs=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 (Rs=0.766, p<0.001) as well as in the cohort who failed to achieve complete remission (Rs=0.893, p<0.001) as presented in Table (2).

Table 1. PIM2, NFKB expression in AML patients in relation to the clinical outcome

	Outcome		p
	Complete remission (n = 39)	Failure to achieve remission (n = 51)	
PIM2 expression	0.90 (0.40 – 377.85)	190.0 (0.89 – 620.0)	<0.001*
NFKB 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 $p \leq 0.05$

Table 2. Correlation between PIM2 and NFKB expression and the clinical outcome in each group

		PIM2 expression		
		Cases (n=90)	Induction failure (n=51)	Complete remission (n=39)
NKPF expression	r_s	0.766*	0.893*	0.206
	p	<0.001*	<0.001*	0.207

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

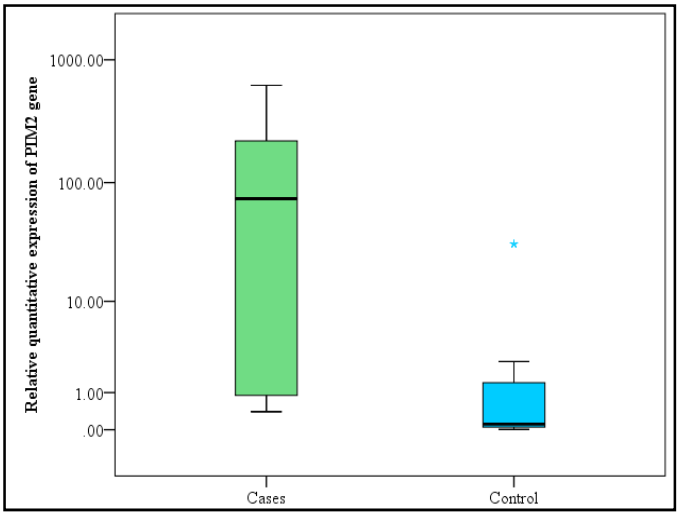

Figure 1. Comparison between patients with AML and controls regarding the relative quantitative expression of PIM2 gene

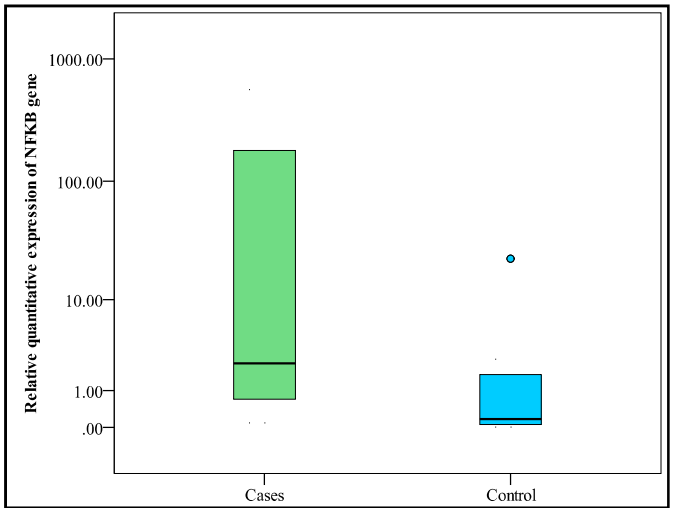

Figure 2. Comparison between patients with AML and controls regarding the relative quantitative expression of NFKB gene

DISCUSSION

PIM kinases are frequently over expressed in human cancer and especially in haematological malignancies where they support malignant cell proliferation and survival (Alvarado *et al*., 2012). In humans, both *PIM1* and *PIM2* mRNAs are highly expressed in CLL, DLBCL and mantle cell lymphoma (MCL), whereas *PIM2* is also overexpressed in follicular lymphoma, MALT lymphoma, nodal marginal zone lymphoma and multiple myeloma (Gomez-Abad *et al*., 2011). It was revealed that PIM2 is ubiquitously expressed with highest levels in brain and lymphoid cells, and potently synergizes in c-MYC induced lymphomagenesis (Allen *et al*., 1997). In the present study, PIM-2 expression level was significantly higher in patients with AML than in controls. On hematopoietic cells transformed by FLT3-ITD (FMS-like tyrosine kinase 3-internal tandem duplication) and BCR/ABL mutations, frequently expressed in AML, Green *et al* demonstrated that the suppression of PIM-1 and PIM-2 expression had led to a significant decrease in cell survival (Green *et al*., 2015). Tamburini *et al* observed significant levels of PIM2 in primary blasts from acute myeloid leukemia patients but were barely detectable in normal CD34+ hematopoietic progenitors (Tamburini *et al*., 2009). 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, it was reported that in AML, NF-κB has been detected in 40% of cases and its aberrant activity enable 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 important target for the intervention of AML (Zhou *et al*., 2015).

As regards follow up of the patients after induction chemotherapy, early assessment of response to therapy represents an in vivo assessment of chemo sensitivity and may be an essential tool to delineate the prognosis in these patients (Kern *et al*., 2003). Kern *et al* considered that failure of achieving blast clearance from the bone marrow aspirates after 1 or 2 weeks of induction chemotherapy a poor prognosis (Kern *et al*., 2003). Moreover, Gajjar *et al* assumed that the persistence of circulating blasts after 1 week of multi-agent chemotherapy indicated a poor prognosis (Gajjar *et al*., 1995). Meanwhile, Liso *et al*. evaluated the response to induction chemotherapy by assessing the degree of residual leukemic infiltration in the bone marrow after 14 days of chemotherapy (Liso *et al*., 2000). Due to bone marrow aplasia on day 14 and poor general condition of the patients, therefore in the current study, the evaluation of response to induction chemotherapy was carried out on day 28**.** 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. These results are in accordance with Upadhyay *et al.* who demonstrated that transgenic mice over-expressing PIM2 are predisposed to T cell lymphomas, whereas PIM2 acts synergistically with c-Myc to accelerate development of B-cell tumors (Upadhyay *et al*., 2013). Likewise, 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 (Zhang *et al*., 2004). In addition, perineural invasion, a major mechanism that leads to the spread of prostate cancer cells, has been found to be associated with elevated PIM2 expression (Ayala *et al*., 2004). Moreover, Rubenstein *et al*. observed higher levels of PIM2 mRNA in recurrent CNS lymphomas refractory to rituximab (Rubenstein *et al*., 2013). 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. 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 and increases resistance to chemotherapy. 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 (Kapelko-Slowik *et al*., 2016).

In 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.

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Declaration of Interest

The authors declare that they have no conflict of interest.

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