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ORIGINAL ARTICLE

Expression of Proviral Integration of Moloney Virus-2 (PIM2) Gene in Patients with Acute Myeloid Leukemia and its Clinical Significance

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ABSTRACT

Background: Despite extensive research in leukemia, intracellular events leading to prolongation of cell cycle 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). The aim of the present study was to assess the expression of PIM2 gene in patients with acute myeloid leukemia (AML) through quantitative real time polymerase chain reaction (QRT-PCR) and to correlate the results with clinical and laboratory findings of the patients as well as their response to the treatment.
Methods: 80 patients with AML and control group were enrolled in the present study. QRT-PCR was used to study PIM2 gene expression.
Results: The mean expression level of PIM2 gene was significantly higher in AML patients (3.5941±7.7736) compared with the control group (0.5303±0.4014) (P=0.034). Its expression level was not different in terms of achieving remission. A positive correlation was observed between PIM2 gene expression and total leucocytic count (R=0.059, P=0.719), while there was a negative correlation between the gene expression and platelet count (R=-0.118, P=0.470). No significant correlation was found between PIM2 gene and patients' response to treatment as (P=0.883) although it's level was higher in patients who did not achieve complete remission (6.1±11.9) than patients who achieved complete remission (3.1±5.3).
Conclusion: The present study showed higher PIM2 expression level in AML patients than normal population. Also, its level was higher in patients who did not achieve complete remission.

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Introduction

Despite high remission rates after induction therapy in Acute myeloid leukemia (AML), the overall long-term survival of patients is not satisfactory (1). Although many patients respond well to chemotherapy, acquired or intrinsic resistance is common. This has a fatal outcome due to relapse from the residual disease. It has been proposed that this phenomenon is associated with the resistance of leukemic cells to drug-induced apoptosis (2).

Inhibition of apoptosis is one of the most important phenomena inducing accumulation of neoplastic cells in patients with leukemia. 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, belong to a serine/threonine kinase family encoded by proto-oncogenes PIM-2, PIM-1 and PIM-3 genes (3). PIM-2 gene expression is regulated at both the mRNA and protein levels by numerous cytokines. IL-3 is one of them that is involved in maturation of hematopoietic cells (4). PIM-2 plays an important role in growth, differentiation, and survival of these cells (5).

High expression of PIM-2 gene 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) (6). Alterations in regulation of PIM-2 gene expression were also shown in cells derived from prostate cancer and in some lymphatic system neoplasms (7).

Several molecular mechanisms of drug resistance have been identified, including dysregulation of BCL-2 family members, increased PI3K/AKT/mTOR pathway activity, FLT3 activation and P-glycoprotein-mediated drug efflux ([2]). In all of these processes, post-transcriptional modification based on protein phosphorylation is mediated, at least in part, by a single family of PIM proteins, representing a group of small serine/threonine kinases (8). The PIM family is made up of three proteins: PIM1, PIM2, and PIM3 and is encoded by corresponding oncogenes. They phosphorylate key signaling molecules such as BAD, 4E-BP1, CHK1, 130 kDa FLT3, MYC, and possibly P-glycoprotein (9). This versatility of targets translates into a potentially significant clinical role played by PIM kinases in several hematological malignancies as well as in solid cancers (10). In previous studies, a significant relationship was found between PIM2 gene expression and complete remission (CR) rate in patients with AML (5).

The aim of this study was to assess the level of expression of PIM2 gene in patients with AML and control group. In an attempt to reveal the association if any, between its level and clinical and laboratory findings of the patients with AML, as well as their response to the treatment.

Materials and Methods

The current study was conducted on 80 de novo patients with AML and 80 age and sex matched healthy individuals as the control group. Both groups were recruited from the National Cancer Institute (NCI) and Beni-Suef University Hospital. Peripheral blood and bone marrow samples were obtained at the time of diagnosis following theirinformed consent as approved by the local Research Ethics Board. Diagnosis of AML was made by hematopathologists not involved in the direct care of the patients. AML was categorized according to the French-American-British (FAB) classification considering morphology, cytochemical studies (including myeloperoxidase, nonspecific esterase and dual esterase reactions) and immunophenotyping.

Prior to induction chemotherapy, patients were subjected to thorough history taking and clinical examination. CT scan of the chest, abdomen and pelvis was performed to assess any specific pathology and echocardiography to assess the cardiac function of the patients.

Detection and quantification of the Proviral Integration of Moloney virus-2 (PIM2) gene expression was performed by qRT-PCR through following steps:

1-RNA extraction: 5 ml of peripheral blood and bone marrow samples were withdrawn from every patient as well as the control group in sterile EDTA vaccutainers and centrifuged to obtain mononuclear cells. Samples were processed immediately using RNA isolation kit (QIAamp® RNA Blood Mini Kits Catalog No. 52304). RNA was used immediately or stored at -70^{0}c for further use.

2-Complementary DNA formation: cDNA was synthesized from RNA samples using high-Capacity cDNA Archive Kit. Applied Biosystems Part Number: 4322171.

3-Real-time PCR quantification was performed by TaqMan® Gene Expression Assay (TaqMan® MGB probes, FAM™ dye-labeled) Applied Biosystems. Part Number: 4331182, Assay ID: Hs00179139_m1. Gene Symbol: PIM2, Gene Name: proviral insertion site in Moloney murine leukemia virus 2. TaqMan® Universal PCR Master Mix, NO AmpErase® UNG Applied Biosystems (PN 4440043). TaqMan Endogenous Control Assays. Applied Biosystems Part Number: 4331182, Assay ID: Hs99999908_m1, Gene Symbol: GUSB, Gene Name: glucuronidase, beta,VIC® Dye Probe. QRT-PCR reactions and fluorescence measurements were made using the Applied Biosystem Step One ™ Instrument (USA).

Interpretation of results were carried out using the comparative C_T method for relative quantitation according to Arithmetic Formulas (11).

The PIM2 gene expression was determined by subtracting the cycle threshold of the reference gene from that of the target gene to get the normalized amount of the PIM2 gene, then comparing this value to the value of the calibrators.

Patients were treated according to the standard AML protocol of the National Cancer Institute (NCI)**.** All patients received the "7 and 3" protocols which consisted of a course of 12 mg/m² novantrone on day 1, 3 and 5 and Ara-C 100 mg/m² continuously every 12 hours from day 1 through 7. If the patient did not enter into remission, this protocol was repeated. If no or minimal response was achieved, patients were scheduled to receive high dose chemotherapy. Patients who entered into remission received 4 courses of high dose Ara-C as consolidation.

Data were analyzed using SPSS software, version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables (11). For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was performed using Kruskal-Wallis test (non-parametric ANOVA). Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. All tests were two-tailed. A $P<0.05$ was considered significant (12).

Results

This study was conducted on 80 patients with AML**.** The patients group included 50 (62.5%) men and 30 (37.5%) women aged 20-73 years. Eighty age and sex matched healthy unrelated subjects were included as the control group.

There was a statistically significant difference between patients with AML and the control group in mean WBC, Hb and Platelet count; (P=0.047, <0.001 and <0.001, respectively) (Table 1). The PIM2 Gene mean expression level was significantly higher in patients with AML than control group (P=0.034, Table 2). Hepatomegaly, splenomegaly, and lymph node enlargement was found in 26 (32.5%), 14 (17.5%), and 26 (32.5%) of the patients, respectively. There was no association between the mean PIM2 gene expression in the patients and the presence of hepatomegaly, splenomegaly, or lymphadenopathy (Table 3).

Although, the mean expression level of PIM2 gene was lower in patients who achieved complete remission (3.1±5.3) than those who did not (6.1±11.9), the was not significant (P=0.883, Table 3).

A positive correlation was observed between PIM2 gene expression and total leucocyte count (R=0.059, P=0.719), while there was a negative correlation between the gene expression and platelet count (R=-0.118, P=0.470) but it was not significant (Table 4).

There was no significant association between level of expression of PIM-2 gene and age of the patients, total leucocyte counts, hemoglobin level, platelets count or bone marrow blast percentage. There was negative correlation between level of PIM2 gene expression and age, platelet count and bone marrow blast, but didn't reach statistical significance (Table 4).

Table 1: Laboratory hematological parametrs in AML patients and Control group

CBC parameters	AML group	Control group	P value
WBC Count (×10^9/L):			
Mean±SD	65.0691.04±	7.381.80±	0.047
Range	1.29-450.00	4.20-10.30	
Median	28.90	7.65	
Hemoglobin (gm/dl):			
Mean±SD	7.92.2±	12.90.7±	<0.001
Range	4.3-13.8	11.9-14.0	
Median	7.1	12.8	
Platelets (×10^9/L)			
Mean±SD	62.064.4±	284.573.2±	<0.001
Range	2-271	189-410	
Median	34.5	274	

Table 2: Expression of PIM2 gene in AML patients and Control group

	AML group N=80	Control group N=80	P value
Mean±SD	3.5941±7.7736	0.5303±0.4014	0.034
Range	0 .0081-34.4899	0.007-1.130	
Median	0.9158	0.5180	

Table 3: PIM2 Gene expression in the patients with AML

Patients characteristics	Number	Mean expression of PIM2±SD	P value
Patient (No)	80		
Sex (Female/male)	30/50	5.08.26 4.01± ,0.95±	0.562
Hepatomegaly Present/absent	26/54	1.949.25±4.39 ,2.57±	0.441
Splenomegaly Present/absent	14/66	2.318.43±3.87 ,3.44±	0.781
Lymph node enlargement Present/absent	26/54	1.889.24±4.42 ,2.61±	0.732
Treatment outcome Remission/Not remission	30/50	3.111.9±6.1 ,5.3±	0.883

Table 4: Association between level of PIM2 gene expression with age and laboratory data in the patients with AML

Parameter	r-value	P value
Age	-0.129	0.427
Total WBC count	0.059	0.719
Hemoglobin	0.174	0.284
Platelets	-0.118	0.470
BM blasts	-0.003	0.985

Discussion

Cytogenetic abnormalities have been considered to be the most crucial independent prognostic factors in AML. Gene mutations also constitute the key events in AML pathogenesis (13). Further research in the role of the underlying genetic and epigenetic mechanisms of the malignant cells provide more information to deeply understand the mechanism of leukemogenesis in AML, as well as offering important prognostic criteria and highlighting potential therapeutic targets (14).

The PIM-2 kinase belongs to a family of serine/threonine kinases first identified as preferential proviral insertion sites in Moloney Murine Leukemia Virus (MoMuLV) induced T-cell lymphomas (6). PIM family proteins are highly conserved serine/threonine kinases that have been implicated in cancer progression and emergence of resistance to chemotherapy. Three PIM kinases (PIM-1, 2 and 3) have been identified, each with variant isoforms of the expressed protein due to alternate start sites. In humans, PIM-1, 2 and 3 genes are located on chromosome 6p21, Xp11.23 and 22q13, respectively (15). At the amino acid level, there is substantial homology between PIM-1 and PIM-2 (53%) (16) and PIM-3 (69%) (17). PIM kinases overlap in their function and compensate for each another. Regulators of transcription, translation, cell cycle, survival, and drug resistance are considered as their targets.

As regards to cancer biology, increased levels of PIM kinase proteins have been strongly implicated in cell survival and tumorigenesis. PIM kinases are overexpressed in both solid tumors such as colon, prostate cancer and hematologic malignancies including lymphomas, chronic lymphocytic leukemia and acute leukemias (18-22). Specifically in AML, up-regulation of PIM may be due to overexpression of HOXA9 and STAT activation (23, 24), which can act as transcription factors for PIM (25). These oncogenic kinases appear to play critical roles in leukemogenesis and resistance to chemoradiotherapy (26).

PIM2 gene was initially identified as an oncogene by screening the common integration sites of the Moloney murine leukemia virus in mutant mice lacking PIM1 expression (27). Its expression is induced by a variety of cytokines, hypoxia, Epstein–Barr virus infection or UVC radiation, which promotes cell survival and resistance to apoptosis (28).

Clinical studies revealed that the PIM-2 gene is commonly overexpressed in several types of hematological malignancies (29). In normal hematopoietic cells transformed with FLT3-ITD or BCR-ABL fusion genes, inhibition of PIM2 expression caused a significant decrease in cell survival (30).

We found that there was a statistically significant difference between AML patients and control group regarding PIM2 gene expression level as it was 3.5941±7.7736 in AML patients compared to 0.5303±0.4014 in control healthy subjects (P=0.034). In line with our results, Tamburini and colleagues reported a lack of PIM-2 expression in normal CD34 positive hematopoietic cells compared to 23 cases of AML where PIM-2 protein expression was detected (31). Mizuki and co-workers analyzed the expression of PIM-2 mRNA in bone marrow samples from 84 patients with newly diagnosed AML in comparison to healthy bone marrow. PIM-2 mRNA was significantly observed in the AML samples (32). Moreover, another study showed that the median PIM-2 gene expression in AML and ALL patients was significantly higher than healthy control group (5).

Green et al. showed that PIM kinases (particularly PIM-2) were overexpressed in FLT3-ITD positive AML patients who are refractory to FLT3 inhibitors, regardless of their FLT3-TKD mutational status. They found that in murine FLT3-ITD negative MPN and AML models, ectopic expression of PIM2 decreases efficacy of FLT3 inhibitors. They also noted that PIM kinase inhibition reduces AML cell viability and PIM-2 knockdown blocks disease propagation in FLT3-ITD positive AML xenografts (33).

In our study, a positive correlation was observed between PIM-2 gene expression and total leucocyte count (R=0.059, P=0.719), while there was a negative correlation between the gene expression and platelet count (R=-0.118, P=0.470) and patient age (R=-0.129, P=0.427). Kapelko and colleagues found a positive correlation between PIM-2 gene expression and patient age (R=0.23, P=0.02), but there was no correlation between PIM2 expression and absolute leukemic cell count in peripheral blood, hemoglobin concentration or platelet count (34).

Despite extensive studies, associations between AML outcome and PIM-2 expression have not yet been fully defined. Investigators in this area have presented evidence that high PIM-2 mRNA expression in leukemic cells of AML patients is associated with poor prognosis in contrast to patients with ALL (5). Survival analysis in their AML patients indicated that low PIM2 expression prolonged event free survival (EFS) and leukemia free survival (LFS) as they found that AML patients with high expression levels of the PIM2 gene (above the median) had significantly shorter EFS and LFS than patients with low expression of PIM-2 (P=0.0053 and 0.027); however, overall survival (OS) was not significantly different. Their results were validated by the analysis of data obtained from TCGA (The Cancer Genome Atlas) AML study, which confirmed that high PIM-2 gene expression was associated with shorter OS and EFS. Moreover, they showed that high PIM-2 expression was associated with adverse cytogenetics and molecular risks in AML patients (5). Garcia et al. demonstrated that PIM kinase inhibition has an antiproliferative effect primarily in hematologic cell lines with very limited activity in cell lines from solid tumors (35). Although the role of PIM kinase in some solid tumors has been extensively described in the literature, data suggest that PIM kinases play a more significant role in hematologic malignancies than in solid tumors. Their results strongly suggest that the use of potent and selective pan-PIM inhibitors, either as single agent or in combination with other agents, will be promising for the treatment of hematologic malignancies in general, and in multiple myeloma and AML in particular (29).

There were differences between the results and statistics

of our study results and other studies which may be due to ethnic differences or our small sample size. We recommend further studies with larger sample size to validate our results and to find any correlation between expression level of PIM2 gene and disease outcome and survival in AML patients.

Ethical approval: All procedures performed in the study involving human participants were in accordance with the ethical standards of the Research Ethics Committee, Faculty of Medicine, Beni-Suef University and with the 1964 Helsinki declaration.

Written Informed consent was obtained from all individual participants included in the study.

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Conflict of Interest: None declared.

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