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PROGNOSTIC VALUE OF BCL2-ASSOCIATED X PROTEIN (BAX) EXPRESSION IN ADULT EGYPTIAN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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AML, BAX gene, new molecular marker

Abstract

Searching for new molecular markers in acute myeloid leukemia (AML) that allow prediction of clinical outcome has recently directed towards genes involved in the regulation of apoptosis. **Aim:** To assess whether BAX gene expression per se is an independent prognostic parameters for outcome in Egyptian patients with AML. Our case control study was conducted on fifty four subjects, thirty four adult de novo AML patients and twenty volunteers as a control group. All participants were subjected to medical history, thorough clinical examination and laboratory measuring of routine CBC and comprehensive metabolic profile. Quantitative real-time PCR for BAX was done for all subjects. BAX gene expression was detected in AML patients and there was a significant correlation between the patients and control group (p value< 0.001). The complete remission rate after first induction chemotherapy was seen in 15 patients (71.4%) with high BAX expression and in 6 patients with low BAX expression, while only 4 out of 13 (30.7%) with poor clinical outcome expressed high BAX gene levels. This should deduce that BAX, as a promoter of apoptosis, could act as a prognostic indicator in our sample of AML patients.

Introduction

Acute myeloid leukemia (AML), also known as acute non lymphocytic leukemia, represents a heterogeneous group of clonal hematopoietic stem cell disorders in which both a block in differentiation and unchecked proliferation of hematopoietic stem cells (HSPCs) result in the accumulation of immature myeloid progenitors (myeloblasts) at the expense of normal hematopoietic precursors ***(Sekeres et al., 2014)***.

AML encompasses multiple specific and clearly defined subtypes that are highly heterogeneous in terms of their genetics, biology and clinical behavior, highlighting the requirement for specific therapies for each subtype. However, despite increased understanding of AML pathogenesis, the mainstay of treatment for the majority of AML subtypes has not significantly changed and continues to be based on standard cytotoxic chemotherapy, consisting of anthracyclines and cytarabine (***Vardiman et al., 2009***; ***Roboz, 2012***).

The complete remission rate currently is approximately 80%, but the majority of patients relapse. It is becoming clear that major determinants of outcome for the patient are the biology of the leukemia and the initial response to treatment. The mechanism associated with primary resistance to chemotherapy-induced cell death may be the failure to activate the apoptotic machinery. Therefore, the search for new prognostic markers which may reflect tumor prognosis has recently focused on genes involved in the regulation of apoptosis ***(Köhler et al., 2002)***.

The cytogenetics of AML patients and their remission status after the first course of treatment have been shown to be highly predictive prognostic indicators, and are used to categorize patients into good-, standard- and poor risk

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groups, to allow tailoring of treatment strategies. Understanding of the molecular pathways that participate in the biology of leukemia may lead to the design of new therapies which may improve patients' survival ***(Kumar, 2011)***. Apoptosis is a physiological process whereby unwanted cells in the body are selectively removed; it not only plays a key role in the development of malignancy, but also influences the treatment strategy of leukemia, since the main aim of chemotherapy is to induce catastrophic genomic damage and initiation of apoptosis. This mode of cell death is controlled by a variety of genes now identified as positive and negative regulators. Among them are "bcl-2", which suppresses apoptosis, and "BAX", which enhances it. BAX is a pro-apototic protein, also known as BCL2-like protein 4. BAX gene, located on 19q13.3-q13.4 ***(Apte et al., 1995)***.

De-regulation of apoptosis disturbs tissue homeostasis and has been implicated in leukemogenesis ***(Duke et al, 1996)***. Bcl-2 seems to inhibit apoptosis by the preservation of mitochondrial membrane integrity as its hydrophobic carboxyl-terminal domain is linked to the outer membrane ***(Wei et al., 2000; Kuwana et al., 2002)***.

BAX protein which is a proapoptotic family member of bcl-2 superfamily is a monomeric protein in the cytosol, which integrates into the mitochondria during apoptosis and subsequently oligomerizes, resulting to the release of apoptogenic factors like cytochrome C and the activation of the caspase cascade. Because bcl-2 function can be modulated by dimerization with family members, like BAX, in this study we hypothesize that the relative expression of this protein in primary leukemic cells might alter the prognostic impact, so the level of spontaneous apoptosis in untreated leukemic blasts would indicate their susceptibility to chemotherapy induced apoptosis, thus measuring BAX would provide valuable information on the intrinsic chemo sensitivity of AML, as reflected by the clinical response to treatment ***(Tzifi et al., 2012)***.

The present study aimed to assess to measure BAX gene expression, as an apoptosis related gene, and its clinical role in predicting clinical outcome in AML patients at diagnosis, and post treatment (after first course of induction chemotherapy).

Materials and methods

Subjects and methods:

Our case control study was conducted on fifty four subjects, thirty four adult de novo AML patients and twenty volunteers as a control group. Patients were recruited between June 2014 and January 2015 from Hematology Oncology unit at Ain Shams University Hospitals. This study was approved by the ethical and moral committee. Before inclusion, an informed written consent was obtained from each patient after full explanation of the study protocol. They were divided into two groups: Group 1: Included thirty four adult de novo AML patients included 20 (58.9%) males and 14 (41.1%) females. Their age ranged between 18 to 60 years (19 above 50 years and 15 below 50 years), with mean age 46.44 ±10.06 years. Group 2: Included twenty normal subjects (13 males and 7 females) as a control group aged 43.5 ± 10.01 years. They were age and sex matching (p= 0 .775).

All subjects were subjected to full medical history and thorough clinical examination. The diagnosis of acute leukemia was based on the French-American-British (FAB) morphological and immunocytochemical criteria. M3 patients were excluded from the study due to the different modality of treatment depicted in these patients. Risk group assignment of AML patients was based on karyotype and percentage blast in bone marrow as shown in table 1.

All patients received standard chemotherapy protocol for AML ("3 and 7," that consists of 3 days of a 30-minute infusion of an anthracycline (idarubicin or daunorubicin), combined with 100 mg/m2 of cytarabine (arabinosylcytosine; ara-C) as a 24-hour infusion daily for 7 days. Dosages have been as follows: (Idarubicin: 12 mg/m 2/d for 3 days) (Daunorubicin: 45-60 mg/m 2/d for 3 days) at diagnosis.

The patients' remission status following the first induction therapy was determined morphologically. Complete remission was defined as less than 5% blasts in a normocellular bone marrow, partial remission with blasts between 5% and 15%, and resistant disease with blasts greater than 15%. Good outcome are those patients who had complete

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remission after first induction chemotherapy, while poor outcome are those patients who had partial remission or failed induction chemotherapy.

Laboratory measurements

All subjects were subjected to a routine complete blood count (CBC), comprehensive metabolic profile (including basic liver, kidney and blood sugar tests), coagulation studies (including PT and PTT). Computed tomography and echocardiography were also done as a routine work up as a pre- treatment baseline for patients.

In addition to the base line CBC, patients had additional laboratory investigations including bone marrow samples, with examination of Leishman stained peripheral blood (PB) and bone marrow (BM) smears, immunophenotyping (IPT) of PB and/or BM samples. Quantitative real-time PCR using Light Cycler system for BAX (Roche diagnostics, GmbH, Germany) was done for all subjects (patients and controls).

Real-Time Polymerase Chain Reaction (RT-PCR) of BAX gene expression:

The Procedure of quantitative PCR determination involved RNA extraction, cDNA synthesis, and amplification. Real time quantitative PCR (qPCR) was performed as described by ***Savli et al., 2003***.

1. ***RNA isolation and complimentary (c) DNA synthesis***; RNA isolation was performed using isolated MagNA pure LC system and MagNA pure LC RNA isolation kit (Roche Applied Science, Mannheim, Germany). Isolated RNAs were reverse transcribed with first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany). First strand cDNA synthesis was performed as suggested by manufacturer. cDNAs were stored at -20o c until real time quantitative PCR was performed.

2. Real-time quantification PCR of BAX gene expression; cDNA was used as a template in a PCR amplification reaction on a Lightcycler system (Roche molecular biochemical, Mannheim, Germany). For the PCR, two reactions were used for each sample, one used to determine BAX level and the other used for determining β2-microglobulin (β2M). Each of them were 20 µL reaction volume used with 5 µL of cDNA and 15 µL reaction volume containing 4.0 µL of the enzyme mixture from Light Cycle Fast Start DNA Masterplus SYBR Green I kit (Roche molecular biochemical, Mannheim, Germany) and 0.5 µM of each primers. Primers were supplied by TIB MOL BIOL (Berlin, Gemany). BAX primers were forward TggAgCTgCAgAggATgATT; reverse CAgTTgAAgTTgCCgTCAgA; and for β2M (housekeeping gene) were forward gAgTATgCCTgCCgTgTg; reverse AATCCAAATgCggCATCT. PCR reaction was initiated with a 10 min denaturation at 95^0C and terminated with a 30s cooling step at 40^0C. The cycling protocol consisted of denaturation step at 95^0C for 10s, annealing at 60^0C for 15s, and extension at 72^0C for 20s, and repeated for 45 times. Fluorescence detection was performed at the end of each extension step. Expression of BAX ratios in the samples were obtained by comparing the BAX copy number of each sample against its β2M level. Copy numbers of BAX were normalized by β2M that were used as a reference gene. The results of the real-time PCR assay for each sample were reported as a specific copy numbers of transcripts per nanogram of RNA.

Statistical analysis:

Data analysis was performed using the SPSS program (version 21, IBM Corporation, USA). Data were expressed as mean ± standard deviation (SD) was used for quantitative data, whereas number and percent (%) were used for qualitative data. Independent-samples t test was used when comparing between two groups. Probability (p-value) less than 0.05 was considered significant and less than 0.01 was considered as highly significant.

Results

This study was conducted originally on 36 patients; 2 of them died during induction chemotherapy, so they were excluded from the study. Thus, this study was finally conducted on 34 de novo AML patients, their clinical and demographical characteristic are presented in table 2.

BAX gene expression was detected in all patients 34/34 (100%) and there was a significant correlation between the patients and control group (p value< 0.001, table 3, 4). It was of high expression (expression above median) in 15 patients (44.2%), and low expression (expression below or equal to median value) in 19 patients (55.8%); the cut off value was 1.5 (table 5).

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The mean total leukocyte count (TLC) of the patients was 81.169 ± 60,000, the count was less than 50,000/cc in 21 patients (61.8%) and total count was above 50,000/cc in 13 patients (38.2%). When correlating these levels with BAX expression, there was a significant positive correlation of increased TLC and blast percent (Blast %) to BAX expression. On the other hand no significant correlation of BAX was found in relation to age, gender, hemoglobin (Hb) level or platelets count (table 6).

In this study population, the complete remission rate after first induction chemotherapy (good clinical outcome) was seen in 15 patients (71.4%) with high BAX expression and in 6 patients with low BAX expression, while only 4 out of 13 (30.7%) with poor clinical outcome (partial remission or failed induction) expressed high BAX gene levels representing a positive significant correlation (P = 0.0337) between those with good clinical outcome and high levels of BAX gene expression, which emphasized that BAX gene expression had a significant positive correlation with patient remission after first induction (table 7).

Discussion

Most cytotoxic drugs affect the malignant cells by inducing apoptosis in their target cells; this suggests that the chemo sensitivity of a tumor may be the outcome of the balance between pro- and anti-apoptotic mechanisms functioning within the malignant cells. Measurement of certain apoptosis-related genes could guide risk-adapted therapy to aid treatment stratification and predicting outcome. Although this hypothesis has been supported by work with a variety of cell-line model systems, there is still considerable controversy as to the predictive value of measures of apoptotic proteins in the clinical settings.

Our objective was to assess the relationship between levels of BAX expression at diagnosis as a predictor of clinical outcome after first induction chemotherapy in Egyptian AML patients.

Pretreatment levels of BAX were found significantly high in our patient sample, indicating the value of the expression. Furthermore, when following patients' response to treatment, we found that those with higher levels of BAX gene expression had higher incidence of remission after first induction chemotherapy.

Our results was consistent with Del Poeta et al., 2003, who studied 255 de novo AML patients and found that BAX expression was higher in chemosensitive patients, and so, represent both a sensitive indicator of clinical outcome and potential target of novel proapoptotic molecules in order to circumvent chemoresistance. It was also, in agreement with Ong et al., 2000 who studied 56 newly diagnosed AML patients and declared that Multivariate analysis revealed BAX to be an independent predictor of survival. On the contrary, a study of 165 pts with newly diagnosed AML by Kornblau SM et al., 2000, reported that levels of BAX expression by immunoblotting did not correlate with response to induction chemotherapy or survival.

In our study, Bax was significantly more frequently expressed in de novo AML patients with better outcomes. This goes with Cingeetham et al., 2015, who studied 221 AML patients and 305 age- and sex-matched healthy controls.

According to our study, values of pretreatment TLC were directly correlated to Bax Gene expression levels, agreeing with findings of Kaparou et al., 2013, although no such correlation was found by Del Poeta et al., 2003.

Tables:

Table 1. Risk group assignment of AML patients based on karyotype and percentage blast in bone marrow.

Good risk	Any patient with favorable cytogenetics i.e. t(8;21), t(15;17), inv(16)
Standard risk	Any patient not in either good-risk or poor-risk groups, i.e. neither favorable nor adverse genetic abnormalities

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Poor risk	Patients with adverse genetic abnormalities, i.e. 25, 27, del(5q), abn(3q), complex karyotype ± and without favorable genetic abnormalities

Table 2. Demographic and laboratory data of AML patients.

Age	< 50y	19 (55.8 %)
	>50y:	15 (44.2 %)
Sex	Male	20 (58.9%)
	Female	14 (41.1 %)
TLC	<50,000/cc	21(61.8%)
	>50,000/cc	13(38.2%)
Hb	<9	22 (64.7%)
	>9:	12 (35.3%)
Platelets	<50,000 /cc	25 (73.5%)
	>50,000/cc	9 (26.5%)
Blasts in peripheral blood	<30%	18 (52.9%)
	>30 %	16 (47.1%)
CD 34	Positive	21 (61.8%)
	Negative	13 (38.2%)
Fab classification	M0: M1: M2 M4: M5	02 (5.8 %) 10 (29.4 %) 14 (41.2 %) 05 (14.7 %) 03 (8.9 %)
Risk category	Good	7
	Intermediate	16
	Poor	12
Cytogenetics	Favorable	12 [t(8;21) n5, inv(16) n1, t(15;17) n6]
	Intermediate	26 [trisomy 8 n4, abn (12) n1, abn Xq n1, del(9) n1, inv (9) n1, tetraploid n1, trisomy 21 n2, normal n15]
	Unfavorable	7 (all complex karyotype)
Achieved complete remission after first course status		27

Table 3. BAX gene expression at diagnosis.

	Patients	**Controls**	**t**	**P**
BAX at diagnosis	1.7647 ± 0.99144	0.8474 ± 0.47419	3.7	0.001

Table 4. BAX expression in AML patients and control subjects.

Group	**sample size(no)**	**Detectable expression (%)**	**P- value**
AML	34	34/34 (100%)	p<0.0001
Control	20	0/20 (0%)	

Table 5. BAX gene expression ratio in AML patients at diagnosis.

BAX gene expression ratio	AML patients (N=34)	P	Significance
Low BAX expression	19 (55.8 %)	<0.001	S
High BAX expression	15 (44.2 %)		

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Table 6. Relation of BAX gene expression to studied parameters.

Parameter	BAX gene expression	
	P-value	Significance
Age	>0.05	NS
Gender	>0.05	NS
Hb	>0.05	NS
TLC	0.015	S
Blast %	0.028	S
Platelets count	>0.05	NS

Table 7. BAX gene expression in comparison to clinical outcome after first induction chemotherapy.

Parameter		BAX expression		p	Significance
		Low	High		
Clinical outcome	Good (*complete remission after first induction chemotherapy)*	6	15	0.0337	S
	Poor (*partial remission or failed induction chemotherapy)*	9	4		

Conclusion

Our data provides a rationale to use BAX transcript levels as independent predictive factor to assess prognosis in AML patients at diagnosis. BAX gene expression was positively significantly correlated to de novo AML patients with better outcomes. However, longer-term follow-up is needed and larger number of AML patients treated with well-defined, uniform chemotherapy regimens need to be prospectively investigated before it can be accepted as a standard prognostic indicator.

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