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RESEARCH ARTICLE

Significance of PAX (CDX2) gene expression in adult acute Myeloid leukemia.

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Abstract

Background, CDX2, a gene of the paraHox cluster, encodes a homeodomain transcription factor that plays numerous roles in embryonic development. Its abnormal ectopic expression in acute leukemia is associated with a pro-oncogenic function. We **aimed** in this study to investigate CDX2 gene expression and its clinical significance in adult patients with acute myeloid leukemia. **Methods**, bone marrow and peripheral blood samples were collected from 70 newly diagnosed adult patients, the CDX2 gene expression in patients and normal controls (50 apparently normal persons) was detected by RT-PCR. **The results**, CDX2 gene expression was positive in 54 out of 70 AML patients (77.2%) however, no expression of CDX2 was observed in all control group. We did not find any significant difference between the two patients groups as regards the clinical manifestation, most of the laboratory parameters and response to induction therapy. Patients with positive CDX2 expression had significantly shorter relapse free survival (RFS) and overall survival (OS) (P=0.018 and 0.024 respectively). **In conclusion**, in spite of CDX2 gene expression does not significantly influence the response to induction therapy,it has a negative impact on the disease progression and survival rate in adult AML patients.

INTRODUCTION

Acute myeloid leukemia (AML), the most common acute leukemia in adults, is characterized by variable degrees of myeloid differentiation, ability to self-renew and proliferate indefinitely. The proliferation, differentiation, and survival of hemopoietic cells are tightly regulated by cytokines (Zipori et al.,1990).One pivotal class of regulatory factors, is the highly conserved family of homeobox genes which has been identified as a major player not only in normal hematopoiesis but also in leukemogenesis. Homeobox genes are divided into 2 classes. Class I includes clustered genes (HOX) that comprises 39 members and the class II divergent homeobox genes, which are dispersed through the genome and include smaller families such as the MSX, Para-Hox (CDX), PAX, DLX, and Engrailed (EN) groups (Rawat et al.,2012).

CDX2, is a gene of the paraHox cluster, encodes a homeodomain transcription factors classically known as regulators of axial elongation and anterior-posterior patterning during early embryogenesis. Many studies have revealed an unsuspected role during hematopoietic development via activation of specific Hox genes (Davidson et al.,2003;Davidson and Zon,2006). Whereas CDX2 exerts a tumor suppressor function in the gut, its abnormal ectopic expression in acute leukemia is associated with a pro-oncogenic function (Renouf et al.,2012). It is not normally expressed in adult murine and human normal hematopoiesis (Savory et al.,2011).

The N-terminal part of CDX2 is essential for the activation of Hox genes, supporting the concept that activation of downstream Hox genes is a key mechanism of CDX2 induced transformation (Taylor et al.,1997). Thus, perturbation of CDX2 might be linked to critical alterations in down streamHox genes that are important regulators of

normal early hematopoietic development in the adult with a distinct expression profile in human early progenitor cells (Pineault et al.,2002).

Data from experimental models and gene expression profiling in patients with acute myeloid leukemia (AML) have identified CDX2 as a powerful oncogene when aberrantly expressed in adult hematopoietic progenitor cells (Chase et al.,1999). It was shown that CDX2 is among the most frequent aberrantly expressed proto-oncogenes in AML, its high expression is closely associated with HOX gene deregulation (Rawat et al., 2008).

As most of the currently available studies have focused on the effectors through which CDX2 contributes to AML development so, here we determine CDX2 expression in healthy persons and AML patients and try to explore whether it has an impact on the clinical course of the disease, response to therapy and patients survival.

Material and methods:

Seventy consecutive patients with de novo AML attending the Medical Oncology Department, Zagazig University Hospital were enrolled in this study. They were 42 males and 28 females with male to female ratio of 1.5/1. Age ranged from 24 to 58 years. Age and sex matched 50 healthy subjects were included as control group only to detect the expression of CDX2. Patients having acute promyelocytic leukemia were not enrolled in the study. The study protocol was approved by the Institutional Ethical Committee and informed consent was obtained from all subjects.

Patients with acute promyelocytic leukemia, impaired cardiac, renal, liver function, prior history of myelodysplasia, other antecedent hematologic malignancies, cytotoxic chemotherapy and radiation therapy were not included in the study. All patients were diagnosed according to FAB cooperative group criteria using standard methods including morphology, cytochemistry and immunophenotyping.

Treatment:

Patients received induction chemotherapy doxorubicine 25-30 mg/m2/d intravenously from day 1-3 and cytarabine 100 mg/m2/d as a continuous infusion day 1-7. Those who attained a CR after one or two courses of induction therapy were randomly assigned to four cycles of High dose cytarabine 2 gm/m2/12h over 3 hours on days 1, 3, and 5 as a post-induction therapy (Lowenberg et al., 2001). Those not responding to induction therapy received more intensified chemotherapy. Thereafter patients were followed up with CBC every 3 months for 2 years, then every 6 months up to 5 years, bone marrow (BM) examination only if the peripheral smear was abnormal or cytopenia developed.

All patients in the study were subjected to the following:

Full history taking, clinical examination, radiological studies and routine laboratory investigations including: complete blood count (CBC), liver and kidney function tests. BM aspiration and staining of films with Leishman and myeloperoxidase.Immunophenotyping by flowcytometry (FACScan, Becton Dickinson; San Diego, USA) for establishment of the diagnosis. Cytogenetic analysis was performed for all samples. The chromosomes were interpreted according to the International System of Human Cytogenetic Nomenclature. The presence of t(8;21) or inv/t (16) was considered as an ‘‘favorable-risk’’ abnormality. An ‘‘unfavorable-risk’’ abnormality was considered in patients with del/t 11q23 aberrations, -5/5q, -7/7q, t(6;9), or a complex karyotype (three or more numerical and/or structural abnormalities). Patients with a normal karyotype and patients with the remaining abnormalities were defined as in an ‘‘intermediate risk.

Determination of CDX2 gene expression:

Genomic RNA was extracted from whole blood using a commercial kit (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). RNA was reverse transcribed with reverse transcriptase enzyme by incubation for 30 min at 60^{o}C. Following a denaturation step, random hexamer primers was used for cDNA synthesis in a total volume of 20 ul [4 ul 5X Reaction Buffer, 1 ulRibo-LockTMRNase Inhibitor (20 u/ul), 2 ul 10 mMdNTP Mix, 2 ul M-MuLV Reverse Transcriptase (20 u/ul), 1 ul hexaprimer (100 uM, 0.2 ug/ul), 2 ug RNA]. Subsequently, the cDNA was heated to inactivate the reverse transcriptase enzyme and was stored at-20C until used for PCR amplification. The protocol of Eda et al.(2000) was followed. The following primer sequences were used: for CDX2 gene sense (5'-CGGCTGGAGCTGGAGAAGG-3') and antisense (5'-TCAGCCTGGAATTGCTCTGC-3') and for GAPDH (as an internal control) sense (5'-CCACCCATGGCAAATTCCATGGCA-3') antisense (5'-TCTAGACGGCAGGTCCACC-3').

Amplification of cDNA for CDX2 gene was done for 35 cycles of denaturation at 94^{o}C for 30s, annealing at 60C for 30s, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The size of the amplified product was read with the use of a DNA marker of different molecular weights (Ladder) (NoLimits 100 bpDNA Fragment, Fermentas Inc., Glen

Burnie, Maryland).The sample was considered positive when a band was observed at the specific molecular weight390 bp for CDX2 and 600 bp for GAPDH (Fig 1).

Disease outcome:

Patients were followed up for three years to detect clinical course of the disease. Complete remission was determined to be achieved, after one or two courses of induction chemotherapy, when the BM was normocellular, containing <5% blasts and when neutrophil in peripheral blood had recovered to $1.5X10^9$/L and platelets to 100 $X10^9$/L according to the criteria of the Cancer and Leukemia Group B (CALGB).

Relapse was diagnosed when the BM contained more than 5% leukemic blasts or when new leukemic infiltration occurred at any other extra-medullary site. Overall survival (OS): was measured from the protocol on-study date until the date of death regardless of cause, censoring for those alive at last follow-up. Relapse Free Survival (RFS): was estimated from the time of first CR to relapse or death in CR.

Statistics:

Analysis of data was performed with statistical package for social science computer program (SPSS Inc., version16.0, Chicago, IL, USA). Comparison of non parametrical quantitative variables between the studied groups was done using Mann–Whitney test. Categorical variables were expressed as frequency and percentage Fisher exact test was used for the comparison between groups. An effect was considered statistically significant at $P<0.05$. The Kaplan–Meier's method was used to construct survival curves and the results were compared using the log-rank test.

Results:

Our patients were divided into two groups according to CDX2 gene expression. The first group revealed negative CDX2 expression 16/70(22.8%) while, the second group had positive gene expression 54/70 (77.2%), the expression of CDX2 gene was negative in all individuals of control group.

No significant difference was observed between the two groups as regards the demographic, clinical and laboratory parameters except for the median of bone marrow and peripheral blood blasts percent which was significantly higher in patients with positive gene expression compared to those with negative gene expression ($p =0.017$ and 0.026 respectively) (Table 1).

As regards the response to therapy, there was no significant difference between the two groups ($p= 0.271$) while, a significant difference was found as regard disease outcome. Patients with positive CDX2 expression had poor outcome as 55.6% of them relapsed or remain NR and 27.8% remain in CR, whereas, only 25% of patients with negative CDX2 expression relapsed or remain NR and 62.6% remain in CR ($p = 0.045$) (Table2).

The patients were followed up for 36 months. Kaplan–Meier's curves were constructed to examine the clinical relevance of CDX2 expression on survival. Patients with positive CDX2 expression had significantly shorter RFS (median,17 months) than those with negative CDX2 expression (median, 25 months) ($P = 0.018$) (Fig2). Also, patients with positive CDX2 expression had a statistically significant shorter overall survival (median 21 months) than those with negative gene expression (median OS not reached) ($p=0.024$) (Fig3).

Discussion

CDX2 is one of the most frequent ectopically expressed proto-oncogenes in AML which is leukemogenic in experimental models (Chawengsaksophak et al.,1997). However, their role in adult malignant hematopoiesis as well as their functional relevance, how it affects the course of the disease and response to therapy in adult acute myeloid leukemia has not been well characterized thus far and is therefore the subject of this study.

Many observations indicate that there is a causal relationship between aberrant CDX2 expression and myeloid leukemogenesis for example, selective knockdown of CDX2 in a human AML cell lines inhibited proliferation and colony formation of AML cells in vitro. Also, murine hematopoietic progenitor cells transduced with CDX2 generated a fully penetrant AML in BM transplant recipients, demonstrating the leukemogenic potential of CDX2 in vivo (Rawat et al.,2004).

We have found that CDX2 gene is aberrantly expressed in (77.2%) of our AML patients. This was more than that previously published by Li Y.X and coworkers (Li et al.,2011), they recorded CDX2 gene expression in only 55% but in pediatric patients. While, it was shown to be expressed in (90%) of acute myeloid leukemic patients in a study conducted by Thoene et al.(2009). It is most likely that ethnic difference, small number of studied groups and differences in the sensitivities of the respective PCR assays account for the disparity between our findings and previous observations. On the other hand, CDX2 gene was not expressed among all normal control group which is in agreement with all other studies which revealed that, CDX2 was not expressed in healthy human (Arnaoaut et al.,2014).

The mechanism by which CDX2 expression is reactivated in leukemia is not well understood. No differences in CDX2 promoter methylation were found in CDX2-expressing leukemic cells from AML patients compared to CDX2-negative AML patients and monocytes/granulocytes from healthy individuals. Also no mutations in the coding region or gene amplification was detected (Schol et al.,2007; Yuasa et al.,2005).

Only a significant difference between CDX2 gene expression and percentage of blast cells in bone marrow and peripheral blood was found. While, no significant difference as regards different clinical and other laboratory findings, including FAB classification and cytogenetic risk categories, among our patients indicating that there is no significant impact of this gene expression on diagnosis. Scholl et al. (2007), found that CDX2 expression levels correlated with disease burden but the expression levels within the different AML subgroups differed slightly but not significantly.

As the most important prognostic parameter represents the early in vivo evaluation of therapy response and prediction of the clinical course of the disease, we further analyzed the impact of CDX2 expression on the prognosis through following up the patients. In spite of, we did not find any significant difference between the two groups as regards the response to induction therapy however, patients with positive gene expression had a significantly poor disease outcome compared to the other group. Li et al.(2011) also recorded that CDX2 positive expression is not related to the CR rate in acute leukemic patients.

During follow-up period, we found that patients with positive CDX2 expression had significantly shorter RFS and OS compared to those with negative CDX2 expression. This was in line with the previously addressed that, high expression of CDX2 was associated with a significantly inferior overall survival (Thoene et al., 2009).

CDX2 contribute to AML pathogenesis through upregulated expression of 50 located Hox genes known to be leukemogenic and itsability depends on the N-terminal transactivation domain. Dysregulated HOX gene expression in AML may be caused by chimeric oncoproteins involving MLL (Ernst et al.,2002) or rare chromosomal translocations directly affecting individual HOX genes (Borrow et al.,1996, Raza et al 1996). This hypothesis was supported by the finding that a deletion mutant of CDX2 missing the N-terminal transactivation domain did not exhibit any leukemogenic effect (Rawat et al.,2008; Rings et al.,2001).

Another essential roles for CDX2 in enhancement of self-renewal are direct targets of the Wnt pathway (Ikeya and Takada.,2001;, Lickert et al.,2001) and silenced of zinc-finger protein KLF4 transcription, an established tumor suppressor, through binding to the KLF4 upstream regulatory region and a decrease in histone 3 lysine 4 trimethylation (H3K4me3) at the KLF4 promoter mediated by the H3K4 demethylase KDM5B (Rowland and Peeper.,2006, Guan et al.,2010) so, it is tempting to speculate that CDX2 may be a component of shared different pathways of transformation converge to enhance self-renewal. So far it is unknown whether the stage of differentiation of the cell, which is initially hit by aberrant CDX2 gene expression, determines the phenotype of the leukemia or not.

We concluded that, aberrant expression of CDX2is a frequent and functionally relevant event in human adult AML. Its expression is significantly associated with poor disease outcome, short RFS and inferior overall survival so it may be useful as a risk stratification marker.Analysis of larger cohorts from different populations should further support the validity of this association also, it may serve as an interesting target for the development of new agents in the therapy of acute leukemia.

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Conflict of interest

There is no conflict of interest or funding support.

(Table 1) : Comparison of patients data in relation to CDX2 gene expression in 70 AML patients at diagnosis.

Character	-ve CDX2 n=16(22.8 %)	+ ve CDX2 n=54 (77.2%)	p-value
Age (years)			
Median (range)	37 (24-57)	39 (26-58)	0.474#
Sex			
Male 42	11(26%)	31(74%)	0.416$
Female 28	5(18%)	23(82%)	
Organomegaly			
Hepatomegaly 8	2 (25%)	6 (75%)	0.589$
Splenomegaly 27	7 (25.9%)	20 (74.1%)	0.631&
Lymphadenopathy 29	10 (34.4%)	19 (65.6%)	0.056&
Hb (g/dL)			
Median (range)	7.8 (4.1-10.6)	8.2 (5.2-11.3)	0.321#
Platelet (10^9/L)			
Median (range)	48 (25-88)	42 (30-112)	0.453#
TLC (10^9/L)			
Median (range)	63.5 (11-213)	71.0 (21-193)	0.291#
BM Blasts (%)			
Median (range)	54 (37-79)	68 (32-94)	**0.017#***
P B Blasts (%)			
Median (range)	32 (11-65)	46 (10-92)	**0.026#***
FAB Classification			
M0 5	2 (12.5%)	3 (5.6%)	
M1 10	2(12.5%)	8 (14.8%)	
M2 17	3 (18.7%)	14 (25.9%)	0.314$
M4 19	5 (31.3%)	14 (25%.9)	
M5 19	4 (25%)	15 (27.8%)	
Cytogenetic subgroups			
Good 18	4 (25%)	14 (25.9%)	0.729$
Intermediate 41	9 (56.3%)	32 (59.3%)	
Poor 11	3 (18.7%)	8 (14.8%)	

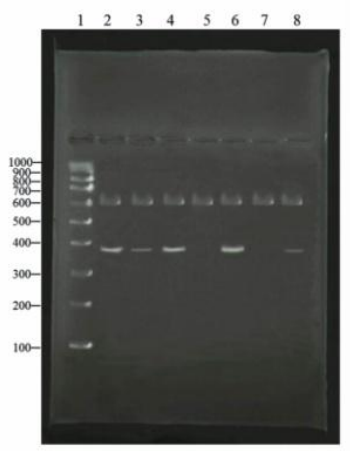
TLC, totalleukocytic count; Hb, hemoglobin; PLTs, platelets; PB, peripheral blood; BM, bone marrow ; SD, standard deviation.
*Level of significance <0.05; &Chi square$ Fisher's exact test; #Mann-Whitney U test

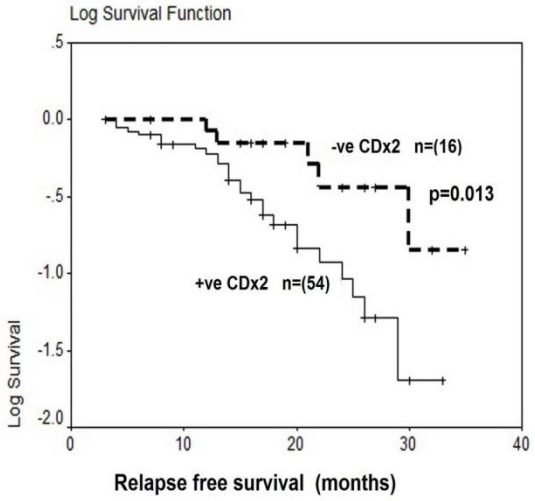
(Table 2) : CDX2 expression and its relevance for treatment response and disease outcome in patients.

Character	-veCDX2 n=16(22.8%)	+ ve CDX2 n=54 (77.2%)	p-value
Response to induction therapy			
CR (43)	12 (75%)	31(57.4%)	
NR (24)	3(18.8%)	21(38.9%)	0.271$
Died (3)	1 (6.2%)	2 (3.7%)	
Disease outcome during follow up (3 years)			
Remain in Remission (25)	10 (62.5%)	15(27.8%)	
Relapsed/ remain non Resp (34)	4 (25%)	30 (55.6%)	0.045$
Died (11)	2(12.5%)	9 (16.6%)	

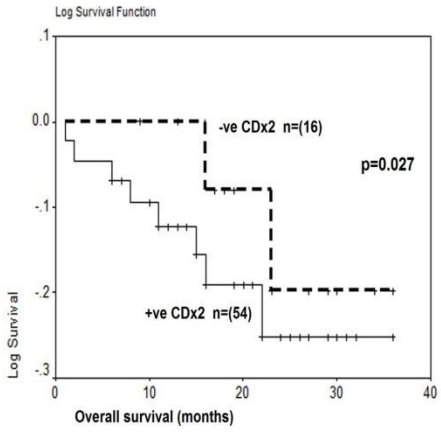
CR complete remission NR: no remission , $ Fisher's exact test

(Fig 1): CDX2 gene expression by RT-PCR of some AML patients. Lane 1 represents the molecular weight (MW) marker (NoLimits 100 bp DNA Fragment, Fermentas Inc., Glen Burnie, Maryland). Lanes 2, 3, 4, 6, 8 represent CDX2 expression (band at 390 bp) in AML positive patients. Lane 5, 7 represents AML patients with negative gene expression (no band at 390 bp).GAPDH expression at 600 bp.





(Fig 2): Kaplan-Meier estimates of Relapse free survival (RFS) of patients according to CDX2 expression.



(Fig 3): Kaplan-Meier estimates of overall survival (OS) of patients according to CDX2 expression.

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