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RESEARCH ARTICLE

RUNX3 gene expression confers an independent Overall survival advantage in non-M3 adult acute myeloid leukemia patients in Egypt

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ABSTRACT

Background: Acute Myeloid Leukemia (AML) is a heterogeneous hematologic malignancy. It is a disease of the elderly with median age 67 years and it affects men more than women. It is infrequent fatal disease, although it has the largest number of annual deaths among leukemias. **Aim of the work**: Evaluation of RUNX1,2,3 expression along with the hedgehog ligands Indian hedgehog(IHH) and Sonic hedgehog (SHH)and correlated their expressions with various clinicopathological parameters, to determine one or more of these genes as a the prognostic biomarker of Acute Myeloid leukemia. **Materials and Methods**: The present study includes 46 Pretreatment bone marrow samples from newly diagnosed AML patients and five normal samples as control group, The expression level of RUNX family and Hedgehog genes were evaluated by qPCR and prognostic impact of studied genes were analyzed in acute myeloid leukemia (AML). **Resultes**: AML patients had significantly increased expression of RUNX1 & RUNX2 and significantly decreased expression of the Hedgehog ligands (IHH&SHH) compared to normal bone marrow samples.Spearman Correlation Coefficient test showed a positive correlation of RUNX3 gene expression with platelet count, but a significant negative correlation with total leucocytic count and peripheral blood blasts. This reduced circulating tumor burden induced by RUNX3 overexpression, was not significantly reflected upon complete remission induction. But was manifested by a significantly improved overall survival (P=0.015) on univariate analysis. Multivariate analysis showed that higher expression of RUNX3 & increased hemoglobin level were the only independent factors which affect OS significantly. This significant effect was not achieved by any of the other four studied genes. Ectopic co-expression of the hedgehog ligands: Indian hedgehog (IHH) & Sonic hedgehog (SHH) in AML was observed. No significant correlation between RUNX3 expression & either IHH or SHH was observed. The link between HH pathway & RUNXs family members was provided by a significant positive correlation between RUNX2 & IHH. **Conclusion:** RUNX3 overexpression is a good prognostic factor in Egyptian adult non-M3 AML.

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INTRODUCTION

Acute Myeloid Leukemia (AML) is an aggressive heterogeneous neoplastic disorder characterized by uncontrolled proliferation and inhibition of differentiation of undeveloped myeloid precursor cells in bone marrow. Which distinguished by its aggressive impact leading to impaired hematopoiesis and bone marrow failure[1]. It has broad variability in the pathogenesis, clinical course, and response to therapy[2]. AML accounts for ~80% of all acute leukemia cases in adults, and the incidence increase with age[3].

Based on the data of the National Cancer Registry Program (NCRP) of Egypt, population of Egypt is anticipated to increase around 160% of the 2013 population size and followed by increasing number of cancer patient cases from approximately 115,000 in 2013 to more than 331,000 in 2050, about a 3-fold increase. The

estimated increase of incident myeloid leukemia cases from 1125 patients in 2013 to reach 2412 patients in 2050 almost 2-fold increase[4]. Despite progress in diagnosis and treatment, over last decades all subtypes of AML were treated uniformly, it's usually a combination of chemotherapy drug: cytarabine (AraC) with an anthracycline, for example, doxorubicin for 7+3 days or idarubicin for 5+2 days. Both anthracycline admitted in dose reduced regimens and consolidation therapy. The adverse features in elderly AML patients, fragilities and comorbidities frequently present, make therapeutic management a real challenge and further studies[1].

The Runt-related transcription factors (RUNXs) which is part of metazoan genes family. This genes family is known to be involved in different essential developmental processes. They are heterodimers consisting of a and b subunits. In humans, the subunit consists of three proteins RUNX1,2,3 that contain a conserved domain, named Runt domain. It is responsible for DNA binding and involved in the heterodimerization process with core-binding factor B which represents b subunit. The b subunit includes a single protein, enhances the DNA-binding activity of subunit and protects it from degradation. RUNX proteins are known to be involved in different signalling pathways, cellular processes and its contribution to hematopoiesis. They act as transcriptional activators or repressors and can be proto-oncogenes or tumor suppressors. Because of lineage-specific expression, each RUNX gene has been studied in isolation without reference to the other two genes in the family. However, there is a growing realization that RUNX genes function in a complementary manner and must be studied concurrently, also increased dosage of RUNX genes was documented at the third mechanism of oncogenesis in AML[6]. Therefore, we studied the expression level of RUNX1,2,3 together in AML adult patients[5,7]. Hedgehog (HH) signaling pathway is considered as one of the major developmental pathways which interact with RUNX family. (HH) is a developmental signaling pathway in which human HH ligands are: sonic hedgehog(SHH), Indian hedgehog(IHH) and desert hedgehog (DHH).

On binding with Patched (Ptch) receptor, it relieves inhibition of Smoothened (Smo) receptor, allowing Gli family of transcription factors to translocate to nucleus and activate HH target genes[7-8]. Hypothesis suggests That cancer and leukemia stem cell (LSC) act as a source of leukemic cell renewal. Different pathways and factors have been founded in stem cell self-renewal and resistance to chemotherapy, include Wnt, transforming growth factor b (TGFb), Notch, and Hedgehog (HH) signaling pathways which are key player in stem-cell biology, malignant transformation. We investigated the expression level and prognostic impact of RUNX1,2,3 beside IHH and SHH in acute myeloid leukemia (AML)[9].

MATERIAL AND METHODS

Forty-six newly diagnosed adults with *de novo* AML patients, 29 (63.0%) males and 17(37.0%) females with a mean age of 33.1 years (SD 10.2) ranging from 18 to 57 years, were evaluated for expression of RUNX1,2,3, IHH and SHH by qRT-PCR. All of these patients were treated at National Cancer Institute (NCI), Cairo University (CU), diagnosed between June 2015 and November 2016. Patients were diagnosed and classified according to standard morphological and immunophenotyping (IPT) criteria. All of the clinical features and IPT of AML patients are listed in Tables 1 and 2. Written informed consents were obtained from the patients or their legal guardians, and this study was approved by the ethical committee of NCI, CU, Egypt. Five normal donors were also enrolled for normalization. The normal bone marrow (BM) samples were obtained from five healthy volunteers with normal blood picture for evaluation of studied genes expression. Bone marrow (BM) samples from both AML patients and healthy donors were collected on lysis buffer and frozen at −80°C until use. The expression of RUNX1,2,3, IHH and SHH was determined before treatment. Among 46 patients, 36 (78.3%) received standard induction chemotherapy 3+7 type regimen (doxorubicin 45 mg/m2 per day on days 1-3 and cytarabine 100 mg/m2 per day on days 1 to 7). Twenty-five out of 46 patients (54.3%) reached complete remission (CR). Remaining 21 patients (45.7%) died before treatment was initiated.

RNA isolation and qRT-PCR

The total cellular RNA from BM aspirate samples was purified using GeneJet Whole Blood RNA Purification Mini Kit (Thermo Fisher Scientific), the purity and the concentration of the purified RNA was evaluated using spectrophotometer nano-drop (Quawell, Q-500, Scribner, USA) and stored at –80˚C till further processing. RNA reversely transcribed to cDNA, which was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

Table 1. Clinicolaboratory data of studied group (n=46)

Variables		n	%
FLT3-ITD	46		
W/W		36	78.3
W/ITD		10	21.7
Molecular cytogenetics			
-ve		38	82.6
Inv (16)		6	13.0
t (8.21)		1	2.2
t(8,21),t(9,22)		1	2.2
FAB classification			
M1		2	4.3
M2		19	41.3
M4		19	41.3
M5a		3	6.5
M5b		3	6.5
Genetic risk			
Favorable		13	28.3
Intermediate		22	47.8
Adverse		11	23.9
Initial CBC			
TLC (*11^9/L)		Median	range
		60.6	2.2-440
=< 11		11	23.9
> 11		35	76.1
Hb (g/dL)		Mean	SD
		7.8	±1.8
Hb (g/dL)			
< 10		39	84.8
>= 10		7	15.2
PLT(*100/L)		Median	range
		29.0	6-205
PLT(*100^9/L)			
< 100		39	84.8
>= 100		7	15.2
Initial BM blast (%)		Median	range
		72.5	14-95
Initial BM blast			
< 50%		6	13.0
>= 50%		40	87.0
PB.blast		Median	range
		60	0-98
PB.blast			
< 50%		16	34.8
>= 50%		30	65.2

AML acute myeloid leukemia, FAB French–American–British classification of acute myeloid leukemia

Table 2. Immunophenotyping (IPT) of AML patients

IPT*	n	%
CD34	32	69.6
CD117	37	80.4
CD4	20	43.5
CD14	25	54.3
CD11c	24	52.2
HLA/DR	38	82.6

Total number (n=46)

The qRT-PCR was performed using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific) according to the manufacturer's instruction on DT lite Real-Time PCR System (v7.7, DNA technology, Moscow, Russia). The forward and reverse primers sequences for RUNX1, RUNX2, RUNX3, IHH, SHH and GAPDH (Table 3). The primers were designed using Primer-BLAST tool. CT values were obtained for all genes and normalized to GAPDH. Then, calculate the fold changes using $2^{-\Delta\Delta CT}$ method. All of the CT values were in the linear range of detection.

Statistical Analysis

Statistical analysis was done using IBM SPSS® Statistics version 22 (IBM® Corp., Armonk, NY, USA). Mean and standard deviation or median and range were used to represent numerical data. while frequency and percentage were used with qualitative data. In the examination of qualitative variables, Pearson's Chi-square test or Fisher's exact test were used, while in not normally distributed data, the comparison between the two groups was analyzed using a non-parametric t-test (Mann-Whitney test). Correlation between numerical variables was analyzed using the Spearman-rho test method. Kaplan-Meier method was used to perform survival analysis, and the log-rank test used to compare two survival curves. All tests were two-tailed with significant P value ≤0.05. Overall survival (OS) and Event-free survival (EFS) were calculated starting from the date of diagnosis to the date of death or last follow-up.In contrast, Disease-free survival (DFS) was calculated from the date of complete remission until the date of relapse or death or last follow-up. Multivariate analysis was done using the Cox-regression method for the factors affecting survival on univariate analysis. Hazard ratio (HR) with 95% confidence interval (CI) were used for risk estimation.

RESULTS

Expression pattern of RUNX1,2,3, IHH and SHH in AML patients

The profiling of RUNX1,2,3, IHH and SHH in bone marrow (BM) cells of the AML patients exhibited differential expression compared to normal controls as shown in Table 4. The fold changes of the studied genes were then analyzed to define lower and higher expression groups as shown in Table 5, median values of fold changes were used as a cut-off value for each gene.

Spearman's Correlation Coefficient test showed a correlation between RUNX1,2,3, IHH and SHH as shown in Figure 1. RUNX3 gene expression was positively correlated with RUNX1 gene expression (r=0.302, *P*=0.042) (Fig 1,A1). RUNX2 gene expression was positively correlated with IHH gene expression (r =0.307 *P*= 0.038)(Fig 1, B2), while not with SHH gene expression. IHH and SHH genes expression were strongly positive correlated (r=0.949, *P*-value<0.001) (Figure 1B1). There was no other significant correlation between genes expression found.

Table 3. The forward and reverse primer sequences of RUNX1,RUNX2, RUNX3,IHH and SHH

Gene	Primer sequence
RUNX1	
Primer forward	GAAGACATCGGCAGAAACTAGATGA
Primer reverse	TGGATCTGCCTTGTATCCTGCATCT
RUNX2	
Primer forward	AGGCGCATTTCAGATGATGACACTG
Primer reverse	GGACATACCGAGGGACATGCCT
RUNX3	
Primer forward	TCGCCTTCAAGGTGGTGGCATTGGG
Primer reverse	CAGTGATGGTCAGGGTGAAACTCTTCCCT
IHH	
Primer forward	GAACTCGCTGGCTATCTCGG
Primer reverse	CTCGGACTTGACGGAGCAAT
SHH	
Primer forward	TGCTGGTATGCTCGGGACTG
Primer reverse	TCGGGGTTGTAATTGGGGGT
GAPDH	Hs_GABDH_1_SG QuatiTect Primer Assay (QT00079247)/QIAGEN

Table 4. Comparison the level expression of RUNXs, IHH and SHH between AML group and control group

Genes	control N=5 Median (Range)	AML Patients N=46 Median(Range)	P Value
Runx1 2^-∆ct	0.001586 (0.000086-0.002405)	0.016186 (0.000001- 0.378929)	<0.001
Runx2 2^-∆ct	0.001822 (0.001289-0.004187)	0.007813 (0.000000- 0.659754)	0.002
Runx3 2^-∆ct	0.007813 (0.000523-0.020617)	0.012691 (0.000213- 1.071773)	0.195
IHH 2^-∆ct	0.058315 (0.012691- 0.143587)	0.00267 (0.000075- 3.249010)	0.018
SHH 2^-∆ct	0.041235 (0.005921- 0.088388)	0.001643 (0.000040 -2.143547)	0.02
Mann-Whitney Test			

Table 5. The frequency of high and low expression of the studied genes

Genes	Frequency
Runx1 =< 24.4348	23 (50) %
Runx1 > 24.4348	23 (50) %
Runx2 =< 3.7842	26(56.5) %
Runx2 > 3.7842	20 (43.5) %
Runx3 =< 3.0738	25 (54.3) %
Runx3 > 3.0738	21 (45.7) %
IHH =< 0.0504	23 (50) %
IHH > 0.0504	23 (50) %
SHH =< 0.0498	23 (50) %
SHH > 0.0498	23 (50) %

Table 6. descriptive data of the present cohort

parameters	Median/ mean* for age and HB (Range)
Age	33.1* (18.0 - 57.0)
TLC	60.6 (2.2 - 440.0)
Hb	7.8* (2.3 - 11.6)
PLT	29 (6.0 - 205.0)
PB blast %	60 (0.0 - 98.0) %
BM blast %	72.5 (14.0 - 95.0) %
Runx1.Fold.change	24.43 (0.002-572.05)
Runx2.Fold.change	3.78 (0.0002-319.6)
Runx3.Fold.change	3.07 (0.05-259.6)
IHH.Fold.change	0.05 (0.0014-61.4)
SHH.Fold.change	0.049 (0.0012-64.9)

Relation of expression pattern of RUNX1,2,3, IHH and SHH with each other with patient's clinicolaboratory data

In this study, the expression of RUNX1,2,3, IHH and SHH was compared with all clinical parameters of AML patients (Table 6). The data was analyzed using the median value for all clinical data except age and hemoglobin (Hb) where the mean was applied. Using Spearman's Correlation Coefficient test the present study showed that the expression of either IHH and SHH genes was increased with elder age (r=0.287, *P*=0.053 and r=0.317, *P*=0.032 respectively) (Figure 1C1,2). IHH and SHH are significantly negatively associated with CD4. 14/20 (70.0%) (*P*=0.017) of positive CD4 were low expressers, while 16/26 (61.5%) of negative CD4 were high expresser in SHH with border significant *P*-value=0.074. Moreover, IHH and SHH genes expression have no other significant impact (Table 7).

Spearman's Correlation Coefficient test showed positive correlation of RUNX3 gene expression with platelets count (PLT) (r=0.317, *P*=0.032) (Figure 1A2) while a significant negative correlation with peripheral blood blasts (PB blast) (r=-0.436, *P*=0.002) (Figure 1A4) and total leucocytic count (TLC) (R=-0.426, *P*=0.003) (Figure 1A3). RUNX3 gene expression had a significant influence on age (*P*=0.018), PLT (*P*=0.002), PB Blast (*P*<0.001), genetic risk (*P*=0.044), HLA/DR (P=0.055) according to Chi-Square test and TLC (*P*=0.005) according to Mann-Whitney test (Tables 8, 9).

Neither RUNX1 nor RUNX2 gene expression had significant impact on any of the studied clinicopathological data. However, lower expression of RUNX2 gene was significantly associated with hyper bone Marrow (BM) cellularity (*P*=0.056) and had inverse association with CD4 marker with *P*=0.011 (Tables 10, 11).

This study confirmed that the presence of HLA/DR on AML blast is significantly associated with favorable and intermediate-risk group with *P*=0.011. Also 11/13(84%) of $CD4^-$ were present in favorable cytogenetic group (*P*=0.018). CD4 was also strong positive associated with CD11c and CD14 with *P*<0.001. There was no other significant association between genes expression and clinical laboratory data found.

Effect of different gene expressions on response to chemotherapy in studied group

Among 46 Adult AML patients, 25 (54.3%) achieved complete remission, ten patients (21.7%) relapsed, 32 (69.6%) died. Hemoglobin was only parameter significantly affecting CR induction (*P*=0.011) (Table 12). The last follow up time was September 2019. The median follows up period was 12.93 months (range 0.03 to 51.18 months). The cumulative overall survival at 1 year (12 months) was 52.2%, at 2 years 24 months was 33.7%, and at the end of the study was 28.9%. The univariate analysis revealed the lower expression of RUNX1 gene and higher expression of RUNX2 gene indicated better OS with no significance (*P*=0.372, *P*=0.955 respectively). Both, event-free survival (EFS) (*P*=0.483/*P*=0.707 respectively) disease-free survival (DFS) (*P*=0.703/*P*=0.682 respectively) had no significant association.

This study reported that the lower expression of IHH and SHH genes were correlated with better OS outcome with the non-significant association and neither of them had any significant impact on EFS and DFS. AML patient's survival in present study with overexpression of RUNX3 gene was significantly higher than patients with lower expression of RUNX3 gene (Figure 2) (*P* value=0.015) with no significant impact on EFS and DFS.

Table 7. IHH and SHH associated with clinicopathological datz

factors		IHH		p value	Shh		p value
		=< 0.0504	**> 0.0504**		**=< 0.0504**	**> 0.0504**	
age	< 40 years	20 (58.8) %	14 (41.2) %	0.044	21 (61.8) %	13 (38.2) %	0.007
	>= 40 years	3 (25) %	9 (75) %		2 (16.7) %	10 (83.3) %	
CD4	-ve	9 (34.6) %	17 (65.4) %	0.017	10 (38.5) %	16 (61.5) %	0.074
	+ve	14 (70) %	6 (30) %		13 (65) %	7 (35) %	
TLC	=< 11	3 (27.3) %	8 (72.7) %	0.084	4(36.4) %	7 (63.6) %	0.3
	> 11	20 (57.1) %	15 (42.9) %		19(54.3) %	16 (45.7) %	
Chi-Square Tests							

Table 8. RUNX3 association with clinicopathological parameters (Chi-Square Tests)

Factors	median	RUNX3		P value
		=< 3.0738	> 3.0738	
age	< 40 years	22 (64.7) %	12 (35.3) %	0.018
	>= 40 years	3 (25) %	9 (75) %	
PLT	< 100	25 (64.1) %	14 (35.9) %	0.002
	>= 100	0 (0.0) %	7 (100.0) %	
PB.blast	< 50%	3 (18.8) %	13 (81.3) %	< 0.001
	>= 50%	22 (73.3) %	8 (26.7) %	
Genetic.risk	Favorable	4 (30.8) %	9 (69.2) %	0.044
	Intermediate/Adverse	21 (63.6) %	12 (36.4) %	
HLA/DR	-ve	7 (87.5) %	1 (12.5) %	0.055
	+ve	18 (47.4) %	20 (52.6) %	

Table 9. RUNX3 association with clinicopathological parameters (Mann-Whitney test)

Relation of numeric variables with Runx3			median(range)	p-value
TLC	Runx3	=< 3.0738	115 (2.6-440)	0.005
		> 3.0738	27 (2.2-132)	

Table 10. RUNX2 association with bone Marrow (BM) cellularity

RUNX2 AND BM CLLULARITY			
BM CLLULARITY	Runx2		p-value
	=<.3.7842	> 3.7842	
Normo, Hypo	3(30.0) %	7 (70.0) %	0.056
Hyper	23 (63.9) %	13 (36.1) %	
Chi-Square Tests			

Table 11.RUNX2 association with CD4

Relation of numeric variables with Runx2			median(range)	p-value
Runx2	CD4	Negative	4.2 (1.65- 319.57)	0.011
		Positive	2.1(0.0002- 8.6939)	
Mann-Whitney Test				

Table 12. Influence of clinicopathological parameters on CR

FACTERS		CR		P-Value
		YES	NO	
RUNX1	=< 24.4348	14(56.0%)	9(42.9%)	
	> 24.4348	11(44%)	12(57.1%)	0.375
RUNX2	=<.3.7842	14(56%)	12(57.1%)	
	> 3.7842	11(44%)	9(42.9%)	0.938
RUNX3	=< 3.0738	13(52%)	12(57.1%)	
	> 3.0738	12(48%)	9(42.9%)	0.727
IHH	=< 0.0504	14(56%)	9(42.9%)	
	> 0.0504	11 (44%)	12 (57.1%)	0.375
SHH	=< 0.0498	14(56%)	9(42.9%)	
	> 0.0498	11 (44%)	12 (57.1%)	0.375
Hb	< 10	18 (72)%	21 (100)%	0.011
	>= 10	7 (28)%	0 (0.00)%	
FT3-ITD	YES	5	5	ND
	NO	20	16	ND
INV(16)	YES	4	2	ND
	NO	19	19	ND
Chi-Square Tests				

Our study confirmed that the presence of HLA/DR indicated a better patient's OS (Figure 3) but with no significant (*P*=0.1). While it's worth mentioning that a higher Hb above 10 confers better significant complete remission CR induction (*P*=0 .011) and better OS (Figure 4) though non-significant (*P*=0.249). As there is significance on a univariate level, so the analysis is eligible for the multivariate level. Multivariate analysis was done including (RUNX3 gene expression, CD4, TLC, Hb, PB blast and HLA/DR) the significant effect on OS was only in high expression of RUNX3 gene (*P*=0.003, HR 3.3, 95% CI: 151-7.1) and high level of Hb (*P* =0.041, HR 3.1, 95% CI: 1.1-9.3).

DISCUSSION

RUNXs transcription factors dysregulation has been reported in many types of cancer [5,7,10-17] including AML. Three RUNX family members are master regulators, their functions linked to major developmental pathways which include hedgehog among others. They have an important role in carcinogenesis as either tumor suppressors or oncogenes[5,7,18]. Based on the median of the level of expression we showed an up-regulation of RUNX1 in 23/46 (50%), up-regulation of RUNX2 in 20/46 (44%), they were both significantly up-regulated (*P-value*<0.001, 0.002 respectively) when compared to control. up-regulation of RUNX3 was in 21/46 (46%) with no significance when compared to the control.

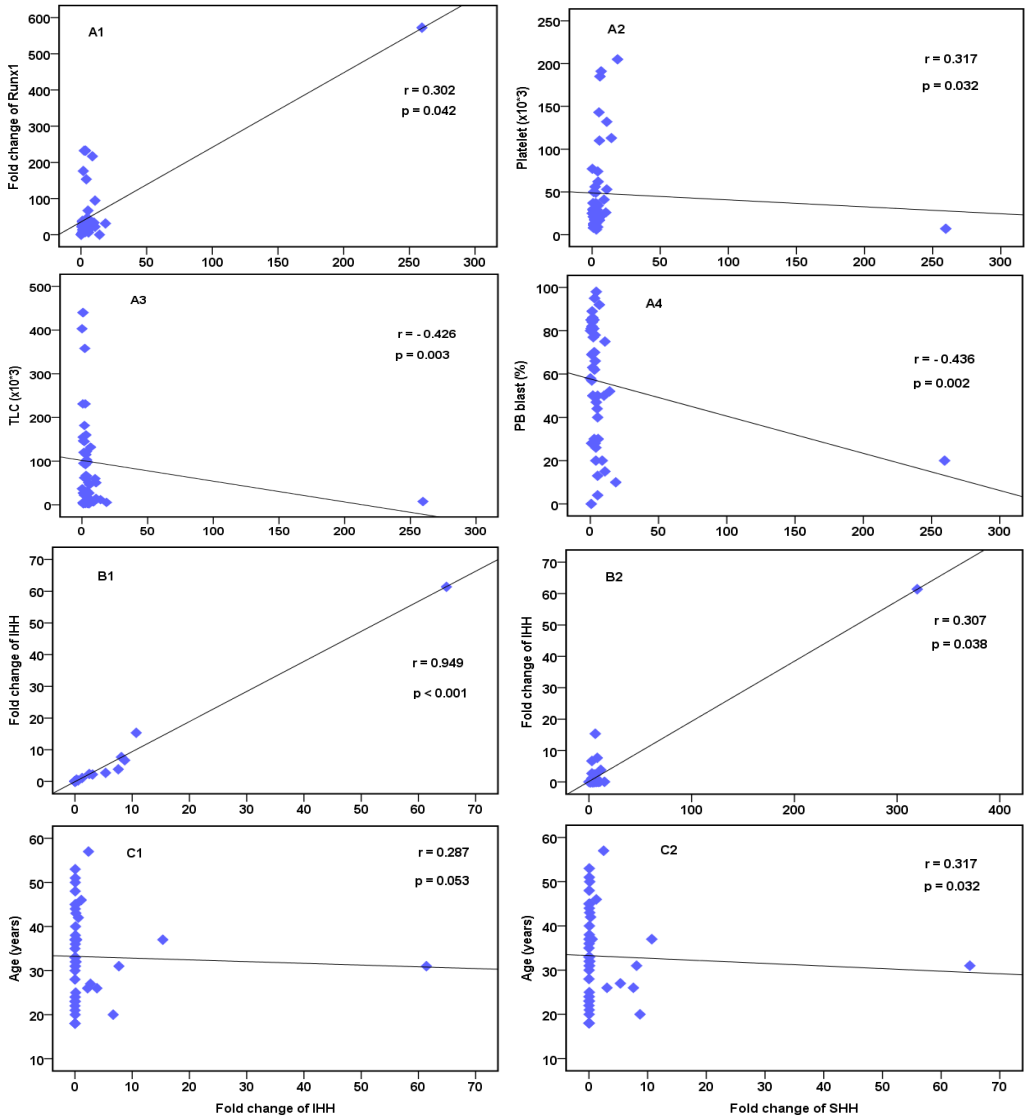

Figure 1. Spearman's correlation coefficient. A1.RUNX3 vs RUNX1, A2.RUNX3 vs PLT, A3.RUNX3 vs TLC, A4.RUNX3 vs PB Blast, B1.IHH vs SHH, B2.IHH vs runx2, C1.Age vs IHH, C2.Age vs SHH

In concordance with our finding the expression level of the three RUNXs were found to be increased in other studies including ONCOMINE datasets and GEPIA datasets [5]. RUNX1 gene expression was significantly positively correlated with that of RUNX3 (r=0.302, *P*<0.04). Many reports confirm the association between RUNX1 and RUNX3 [19]. This was also inconsistent with the GEPIA dataset whereby RUNX1 was both significantly positively correlated with RUNX2 and RUNX3 genes expression. Surprisingly, in LinkedOmics database, RUNX1 gene expression was negatively correlated with that of RUNX3[5] this could be due to racial or ethnic variability.

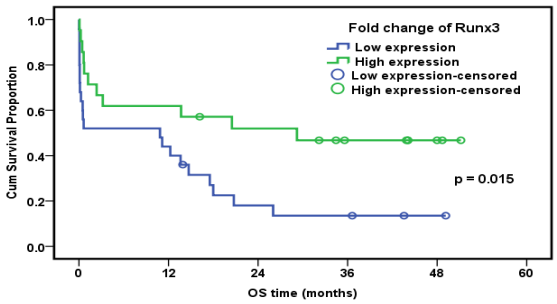

Figure 2. Prognostic value of mRNA level of RUNX3 in Egyptian Adult AML.

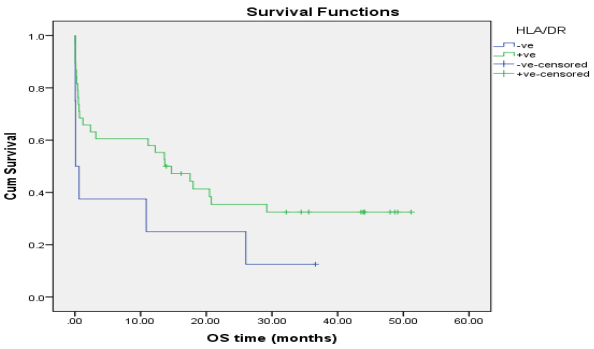

Figure 3. Prognostic value of HLA/DR in Egyptian Adult AML.

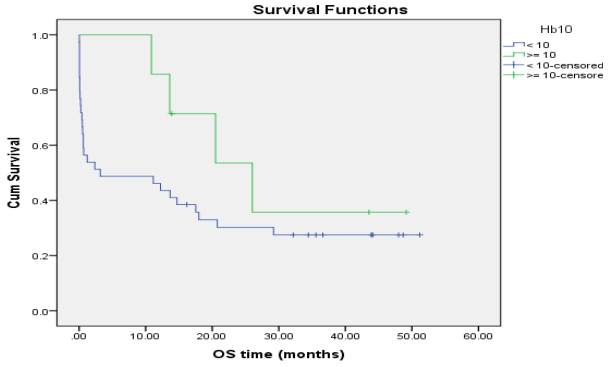

Figure 4. Prognostic value of Hb level in Egyptian Adult AML.

IHH & SHH were both down-regulated when compared to the control (*P*-value 0.018 and 0.02 respectively). The highest levels of both IHH & SHH co-expression were found in 8 cases (ectopic expression). However, up-regulation was in 50% of cases based on their median.

This is inconsistent with Bai et al., 2008 who found by immunohistochemical analysis 45% of cases positive for SHH expression in AML patients this could contribute to functional activity of HH signaling pathway [20]. In the present study, the expression of both these genes was increased with age (r=0.287, *P*=0.053), (r=0.317, *P*=0.032) respectively. RUNX3 expression was increased with age. Seventy-five percent of cases above or equal to 40 years were over-expressors of RUNX3, while about 65% less than 40 years of age were lower-expressors (*P*=0.018).

Ectopic expression of IHH & SHH was reported before in 20% of T-ALL[21]. The strongest positive correlation in the present study was between IHH & SHH (r=0.949 *P*<0.001) a similar strong correlation was found in the thymus, whereby Hedgehog signaling regulates the differentiation and proliferation of early thymocyte progenitors, T-cell receptor formation, CD4 vs. CD8 lineage commitment, and restricts late T-cell development [22-25] into CD4 positive. CD4 was originally described as T-cell antigen aberrantly expressed on AML blasts and used by most laboratories including ours for MRD detection[26]. Herein among the five studied genes, negative CD4 was significantly associated with the up-regulation of IHH, SHH and RUNX2 in non M3-AML blasts (*P*<0.022, *P*<0.057 and *P*<0.011 respectively). A condition reminiscent of IHH gene expression restricting CD4 expression on T-cells in thymus.

In this study, IHH gene over-expression had a tendency to decreased patient's TLC (*P* = 0.084). Seventy-two percent of overexpressors were having TLC<11000. In fact of three HH ligands IHH was found most specifically within hematopoietic cells[8]. Li et al., 2017 found that the relative expression level of SHH mRNA both in untreated and relapsed adult AML was significantly higher SHH than those in normal control and MRD negative AML[27]. HH signaling pathway has been shown to have a

potential role in leukemia maintenance through supporting leukemia stem cell (LSC) [28], but in the present study no significant correlation can be found between the expression of IHH, SHH genes and or CD34 AML blasts, this might be due to the lower fraction of leukemia expressing IHH and or SHH genes.

The link between HH pathway and RUNX gene family members in this study was provided by a significant positive correlation between RUNX2 and IHH genes expression (r= 0.307, *P* =0.038), up to our knowledge this is the first time to report such a link in AML. RUNX2 gene was previously reported to be expressed in metastatic breast cancer cells and was a direct major determined of metastatic spread [18]. Komori., 2018 confirmed that IHH induces RUNX2 which induces osteoblast and chondrocyte differentiation and plays an essential role in the bone metastasis of breast and prostate cancers by directly up-regulating of IHH and other genes[29]. Notably, our results exhibited a significant negative association between RUNX2 and BM cellularity (P=0.56), otherwise, the other 4 studied gene showed non-significant association with BM cellularity.

The current study is the first who demonstrated that RUNX3 is the dominant gene among the studied ones which has a remarkable impact on clinicopathological parameters in adult non-M3-AML patients. In our study AML with inv(16) were mostly 4/6 (67%) down-regulated. This is consistent with literature[30]. Transcriptional repression of RUNX3/AML2 gene by the inv(16) and t(8;21) was documented in AML[31]. Based on the univariate analysis, lower expression of RUNX1 and higher expression of RUNX2 indicated a better OS but with no significance. Likewise, LinkedOmics RUNX2 higher expression showed better OS also with non-significant association but their higher expression of RUNX1 was associated with better survival [5]. Multivariate analysis showed that RUNX3 and Hb level were the only significant independent prognostic factors affecting OS in Egyptian Adult AML patients (*P*=0.003, HR 3.3, 95% CI: 151-7.1)(*P*=0.041, HR 3.1, 95% CI:1.19.3) respectively, the higher expression of RUNX3 and high level of Hb were associated with better OS outcome without significant impact on EFS or DFS.

The significant impact of RUNX3 overexpression on OS in adult AML was demonstrated in this study (*P*=0.015). This improved survival advantage induced by RUNX3 overexpression was not reflected upon complete remission induction. The only parameter affecting CR significantly in our study was the Hb level.

Among the five studied genes RUNX3 stands out having no significant difference compared to the normal group. Then why RUNX3 overexpression was the only one which affects OS positively? Herein, this can be explained by four findings. First, a positive correlation was found between RUNX3 with platelet count (r=0.317, *P*=0.032). But, further studies will be required to establish whether the significant correlation between RUNX3 and platelet count was causative. Notably, there was no significant correlation between initial platelet count and all other 4 studied genes. Second, a stronger negative correlation was depicted between RUNX3 and both TLC(r=-0.426, *P*=0.003) and PB blast(r=-0.436, *P*=0.002) but not BM blast. That's to say cases with increased RUNX3 expression were having a significant reduction in circulating tumor burden (TLC P=0.005, PB blast P=0.001). 13/21 (81%) of our AML cases expressing elevated RUNX3 levels had significantly lower PB blast cell count ≤50, while 73% with lower RUNX3 expression had >50% PB blast count (P < 0.001).None of the other five studied genes had such influence. Third, 9/13 (69%) of our AML cases in the favorable cytogenetic group were higher expressers of RUNX3 while 21/33(64%) of the intermediate and adverse cytogenetic risk group were lower expressers of RUNX3 (P=0.044). Fourth, 20/21 (95.2%) cases with RUNX3 overexpression were also expressing HLA/DR class II (P=0.055) it's believed that the expression of HLA/DR on AML blast allow better presentation of various antigen to the host immune system.

Furthermore, no mutations were encountered in RUNX3 unlike RUNX1 in AML[32]. By contrast to solid tumors, methylation inhibition of the CpG islands in the promoter of RUNX3 was not documented in AML[31]. In contrast to our results, RUNX3 was significantly related to poor OS in leukemia in both the Linked Omics and GEPIA databases[5]. In fact, high RUNX3 expression was associated with poor EFS in

childhood AML[31]. Whether these databases included many children or not. Also, Spender et al., 2005 demonstrated that RUNX3 and RUNX1 expression were mutually exclusive in human B lymphoid cell lines[33]. In a recent Polish adult AML study[3] mortality rate was significantly higher in cases with RUNX3 overexpression this could be attributed to racial or ethnic differences. Also, in a cohort of patients with cytogenetically abnormal adult AML patients with wild-type FLT3, RUNX3 overexpression was an independent prognostic factor for poor OS[34]. This difference could be attributed to their abnormal cytogenetic. Particularly since RUNX3 overexpression in the present cohort was significantly associated with the favorable cytogenetic group (*P*=0.044).

In conclusion, we reported that, the strongest positive correlation was found between IHH and SHH expression. IHH was positively correlated only to RUNX2.. We reported an independent positive impact of RUNX3 overexpression and higher hemoglobin on OS in Egyptian adult non-M3-AML patients could make RUNX3 a promising prognostic biomarker for Acute Myeloid Leukemia.

Conflict of interest

The authors claim no conflict of interest.

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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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