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RESEARCH ARTICLE

ASSESSMENT OF BAALC EXPRESSION LEVEL IN ACUTE MYELOID LEUKAEMIA PATIENTS AND ITS PROGNOSTIC SIGNIFICANCE.

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BAALC:Brain and acute leukaemia cytoplasmic, EFS: event Free survival .

Abstract

Baalc expression is an indicator of aggressiveness in aml. Overexpression of this gene is associated to poor of clinical outcome.high baalc expression was independent associated with lower cr,shorterefs and shorter os.

Aim of work:the study aimed to is assess baalc expression levels in acute myeloid leukaemia patients and then evaluate its prognostic significance.

Subjects and methods:this study was conducted on 56 adult patients who were admitted to the clinical oncology unit including 35 males and 22 females.for all patients complete blood picture, ldh , bone marrow aspiration , immunophenotyping , cytogenetic analysis and assessment of baalc gene expression by real time pcr were done.**results:**the prognostic value for baalc gene expression was evaluated.the validity of baalc gene expression as a prognostic marker for aml showed that the sensitivity and specificity were 86.1% and 80% respectively. The relation between baalc gene expression and overall survival in aml patients revealed that patients with baalc ≥ 2.11 had shorter os than did those with lower level baalc.

Introduction:-

AML a malignant clonal disorder characterized by alterations and low production of healthy hematopoietic cells; these alterations inhibit differentiation and induce proliferation and accumulation of blasts. Blasts replace normal hematopoietic tissue, triggering cytopenias (Parikh et al., 2014).

Accumulation of immature cells begins in bone marrow, but in most cases quickly builds up in the blood, and sometimes spreads to other parts of the body such as the lymph nodes, spleen, liver, testes and the central nervous system (Parikh et al., 2014).

Several poor prognostic factors for AML have been reported, which include gene mutations in TET2, ASXL1 or DNMT3A and overexpression of ERG, EVI1, MN1 or BAALC (Döhner et al., 2010). In general, overexpression of BAALC is observed in one-fourth of AML patients (Baldus et al., 2003 &Langer et al., 2008), and many reports have shown its adverse impact on the survival of AML patients across all karyotypes (Damiani et al., 2013).

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Genomic profile has been used to identify prognostic factors of leukemia. ERG, BAALC, MN1 and WT1 genes are considered as categorization indices based on gene expression patterns; they can be used to distinguish therapeutic intervention and disease process in AML patients **(Port et al., 2014).**

BAALC overexpression mechanism has been reported in myeloid leukomogenesis. It's up regulation prevents myeloid blast differentiation and is associated with poor prognosis in AML and ALL patients. BAALC up regulation has been observed in glioblastoma, melanoma and gastrointestinal cancers; so BAALC oncogenic role was suggested (Aziz et al., 2015).

Although high BAALC expression is a well-characterized poor prognostic factor in AML, neither the exact mechanisms by which it drives leukemogenesis and drug resistance nor therapeutic approaches have been properly elucidated (Morita et al., 2015).

BAALC induces cell cycle progression of leukemia cells by sustaining extracellular signal-regulated kinase (ERK) activity through an interaction with a scaffold protein MEK kinase-1 (MEKK1), which inhibits the interaction between ERK and MAP kinase phosphatase 3 (MKP3/DUSP6) (Morita et al., 2015).

BAALC conferred chemoresistance in AML cells by upregulating ATP-binding cassette proteins in an ERK-dependent manner, which can be therapeutically targeted by MEK inhibitor (Morita et al., 2015).

Subjects and methods:-

Patients:

This study was conducted in the clinical pathology& medical oncology departments in Zagazig-University Hospitals during the period from January 2015 to January 2018.**A total of (56) patient was included in this study:**

Inclusion criteria:

De novo AML patients before receiving induction chemotherapy.2- Age > 18 years old.3- Adequate liver & kidney function tests.4- No concurrent malignancy.5- Adequate cardiac function.6- PS ≤ 2.

Exclusion criteria:

Previously treated AML patients.2-AML (M3) .3-Age <18 years old.

Patients group were subjected to the following:

Complete history taking about the onset & duration of the clinical course, age, history of easy fatigability, dizziness, fever, bleeding tendency & abdominal enlargement.

Clinical examination: was done for fever, purpura, ecchymosis, gum hypertrophy, lymphadenopathy & CNS manifestation.3-Routine laboratory investigation:a) CBC and blood smear examination.b) Liver, kidney functions and LDH.c) ESR.d) PT and APTT. e) Viral markers: HBsAg, HCVAb & HIV.4-Radiological investigation:a) ECHO for evaluation of cardiac function.b) Pelvi-abdominal sonar.c) Chest X-ray if needed.4-Specific laboratory investigation:a) Bone marrow aspiration & examination followed by cytochemistry and Immunophenotyping.b) Cytogenetic analysis.c) Reverse transcriptase PCR for BAALC gene expression.

Treatment plane:

Patient were treated by an induction regimen 3+7 regimen consisting of continuous infusion of cytarabine (25mg/m^2) daily for 7 consecutive days combined with 3 days of doxorubicin (30mg/m^2).Patients > 60 years was treated by 2+14 (cytarabine 10mg/m^2/12 hoursdaily for 14 days combined with 2 days of doxorubicin 25mg/m^2) vs low dose cytarabine 10 mg/m^2/12 hours for 14 days.

Patients follow up:

CBC and BM aspirate were performed on day 28 after receiving induction therapy to evaluate the remission state.
CR was defined as normocellular BM containing less than 5% blast cells and peripheral blood with at least $1X10^9$/L neutrophils and $\geq 100X10^9$/L platelets. Remission failure was classified as either partial remission (PR: defined as 5-15% blast cells or <5% blasts but with hypocellular BM), Resistant disease (RD: defined as > 15% blasts in BM), or induction death (ID: defined as related to treatment or hypoplasia).Patients were followed up for three yearsto evaluate OS.

Specimen collection:
1-10ml of venous blood was aseptically withdrawn from each patient by venipuncture and divided as follows: 1 ml was delivered into Sterile EDTA vacutainer for CBC, 2 ml was delivered into sterile sodium citrate vacutainer for PT & APTT, 3 ml was delivered into sterile plain vacutainer& left to clot at 37˚c & centrifuged at 3000 rpm then serum was tested for liver , kidney function tests & LDH, 2 ml was delivered into EDTA vacutainer for ESR. 2ml was used directly for RNA extraction.2-5 ml of bone marrow was aspirated & the sample was divided as follows: bone marrow smears, 1ml was delivered into EDTA vacutainer for Immunophenotyping by flowcytometry, 2 ml was delivered into lithium heparin vacutainer for cytogenetic analysis, 2 ml was delivered into EDTA vacutainer for RNA extraction.

Methods:-

Specific laboratory investigations including:
BAALC mRNA expression: was done using quantitative real time PCR including the following steps:1-RNA extraction:
RNA was extracted from whole blood by RNA extraction kit.2-cDNA synthesize: single stranded cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kits.3-Real time PCR amplification.

Molecular detection of BAALC RNA gene expression:
RNA extraction from whole blood:
RNA was purified from anticoagulated bone marrow sample using the PureLink RNA Mini Kit (Life technologies,USA).

Pure Link procedure:
The following protocol was used to purify total RNA from 0.2 mLfresh whole blood:

1. 200 µl of whole blood sample was placed to a 1.5 mL sterile eppendorf.
2. 200 µl of Lysis Buffer prepared with 2-mercaptoethanol was added.
3. The eppendorf was vortexed thoroughly, then centrifuged at 12,000 ×g for 2 minutes at room temperature.
4. The supernatant was transferred to a clean 1.5 mL sterile eppendorf.
5. 200 µl 100% ethanol was added to the microcentrifuge tube. Any precipitate was dispersed by vortexing or pipetting up and down several times.
6. The sample was transferred (including any remaining precipitate) to the Spin Cartridge (with a Collection Tube) and centrifuged at 12,000 × g for 15 seconds at room temperature. The flow-through was discarded.
7. 700 µL Wash Buffer I was added to the Spin Cartridge and centrifuged at 12,000 ×g for 15 seconds at room temperature. The flow-through and the collection tube were discarded. The Spin Cartridge was placed into a new collection tube.
8. 500 µl Wash Buffer II with ethanol was added to the spin cartridge.
9. It was centrifuged at 12,000 ×g for 15 seconds at room temperature. The flow-through was discarded, and the Spin Cartridge was reinserted in the same Collection Tube.
10. Steps 8–9 were repeated once .
11. The Spin Cartridge was centrifuged at 12,000 ×g for 1 minute at room temperature to dry the membrane with attached RNA. The collection tube was discarded and the spin cartridge was inserted into a recovery tube.
12. 30 µLRNase-Free Water was added to the center of the spin cartridge.
13. It was incubated at room temperature for 1 minute.
14. The Spin Cartridge and Recovery Tube was centrifuged for2 minutes at ≥12,000 × g at room temperature.
15. Purified RNA was then reverse transcribed cDNA.

RT-PCR of total RNA to cDNA:
Using the High Capacity cDNA Reverse Transcription Kits:To synthesize single-stranded cDNA from total RNA using the High
Capacity cDNA Reverse Transcription Kits:
The 2× Reverse Transcription Master Mix was prepared per 20 µl reaction:
The kit components were allowed to thaw on ice.2- 2× RT master mix was placed on ice and mix gently.3-The cDNA Reverse Transcription Reactions were prepared as follows:i. 10 µL of 2× RT master mix was pipetted into each well of a 96-well reaction plate or individual tube.ii. 10µL of RNA sample was pipetted into each well,

pipetting up and down two times to mix. iii. The plate was sealed.iv. Briefly the plate was centrifuged to spin down the contents and to eliminate any air bubbles.v.The plate was placed on ice until the thermal cycler is ready to be loaded

Performing Reverse Transcription:
The thermal cycler conditions using Veriti thermal cycler (Applied biosystems, Japan) were programed;

	Step 1	Step 2	Step 3	Step 4
Temp.(˚C)	25	37	85	4
Time	10 min	120 min	5 min	∞

1. The reaction volume was set to 20 µl.
2. The reactions were loaded into the thermal cycler.
3. The reverse transcription runwas started.

Real time RT-PCR: using Taqman gene expression assay:
Principle:
The fluorescent dye in the master mix intercalates into the amplification product during the PCR process and enables the rapid analysis of target DNA.

The reaction mix was prepared and the plate was loaded:The reagents were thawed and mixed.
The number of reactions were calculated:
The PCR reaction mix was prepared
The plate was loaded:
The real-time pcr reaction was run using stratagene mx3005p qpcr system.
QPCR measures PCR product accumulation during the exponential phase of the reaction and before amplification becomes vulnerable to limiting reagents and cycling variability. Fluorescent qPCR data provides accurate information on initial starting copy number.

Using qPCR, amplification and detection are combined in a single step and in a single closed tube. This eliminates the need for numerous post-qPCR manual steps, and reduces the possibility of introducing variability or laboratory contamination.

The Mx3005P system has an open format that allows closed-tube real-time PCR detection with many chemistries including SYBR Green dye and fluorogenic probe systems, detecting up to five different dyes simultaneously.

Interpretation of results
The transcription levels of target genes were normalized to those of B-actin which used as reference gene to account for the variability in the amount of cDNA in each sample.
1. Cycle Threshold (CT) value for each reaction was obtained.
2. The delta CT was calculated by subtraction of CT values for the reference gene (B-actin) form CT values of the gene of interest (BAALC gene).

ΔCT **= C**T **target – C**T **reference**
The delta-delta CT for each patient was calculated by subtraction of the average of control ΔCT from the patient ΔCT.
ΔΔCT = ΔCT sample – Average ΔCT control

The fold change for gene expression in the sample relatively to the control was calculated by the negative value of the delta-delta CT subtraction (-ΔΔCT) becomes the exponent of 2.

$$R = 2^{-\Delta\Delta CT}$$

(Livak&Schmittgen, 2001)
The expression of target genes was presented as fold change of gene expression relative to control.Samples were considered positive for BAALC expression if their expression level was one log higher than the mean level of expression reported for the control group hence mean level for control was 1 so samples have expression level more than 2 were considered positive **(spanaki,et al 2007).**

The Ct or threshold cycle value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest.

Statistical Analysis:-

The following statistical methods were used for analysis of results of the present study. Data were checked, entered and analyzed using SPSS version 22 (SPSS Inc., Chicago, IL) used in Windows 8 for data processing and statistic.All data were expressed as in terms of mean ± SD. Chi Square test and Fischer exact tests were used as tests of significance. Log Rank test was used to assess survival.Significance was adopted at $p < 0.05$.

Results:-

Table 1:-Cytogenetic risk among studied AML patients.

Variable	AML patients (No=56)	
	No	%
cytogenetics:		
Normal	39	69.6
Abnormal	17	30.4
Risk:		
Favorable:	**11**	**19.6**
t (8;21)	5	8.9
inv (16)	6	10.7
Intermediate:	**42**	
Normal	39	69.6
Tri 8	3	5.4
Adverse: t(11;12)	**3**	5.4

Table 1 shows the cytogenetic risk for AML patients. Normal cytogenetics was 69.6%, while abnormal cytogenetic was 30.4%.According to risk stratification: favorable, intermediate & adverse cytogenetics were as follows 19.6%, 69.6% and 5.4% respectively.

Table 2:-Comparison between cytogenetic risk group and BAALC.

Variable	**Normal**	**Favorable**	**Intermediate & adverse**	**Kruskal-Wallis value**	**P**
BAALC:					
Median	3.21	1.13	1.18	19.66	0.000*
Range	1.05-13.76	0.80-10.43	0.86-3.21		

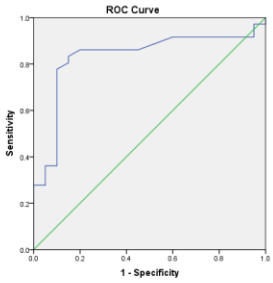
This table shows that in normal cytogenetic risk group median of BAALC was higher (3.21) in comparison with the two other groups. This was statistically significant.

Table 3:-Comparison between cytogenetic risk and BAALC byMannwhitney-U test.

Variable	Normal vs favorable	Normal & (intermediate & adverse)	Favorable & (intermediate & adverse)
BAALC	0.000*	0.00*	0.83

This table shows that there were statistical significant difference between normal and favorable group and also between normal & (intermediate and adverse) as regard BAALC gene expression.

ROC curve for BAALC as prognostic factor for survival in AML patients.



Area Under the Curve

Test Result Variable(s): BAALC

Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.832	.060	.000	.715	.949

AUC=0.83
Confidence interval=0.71-0.94.

Table 4:-Sensitivity and specificity of BAALC as prognostic factor for survival.

Crosstab					
			Survival		Total
			DIE	SURVIV	
baalc2.11	>2.11	Count	31	4	35
		% within baalc2.11	88.6%	11.4%	100.0%
		% within Survival	86.1%	20.0%	62.5%
	<=2.11	Count	5	16	21
		% within baalc2.11	23.8%	76.2%	100.0%
		% within Survival	13.9%	80.0%	37.5%
Total		Count	36	20	56
		% within baalc2.11	64.3%	35.7%	100.0%
		% within Survival	100.0%	100.0%	100.0%

Sensitivity =86.1%, Specificity=80%, Positive predictive value=88.6%, Negative predictive value=76.2%., Kappa0.65 and P=0.000.

Table 5:-Comparison between age, laboratory data and BAALC.

Variable	BAALC		t test	P
	> 2.11 No=36	≤ 2.11 No=20		
Age X±SD	38.03±12.76	42.24±9.96	1.29	0.202
Hb X±SD	5.51±1.26	7.33±1.77	4.46	0.000*
BM blasts: X±SD	85.20±6.09	61.29±6.27	14.06	0.000*

LDH: X±SD	924.74±164.47	580.19±79.66	8.96	0.000*
ESR: X±SD	121.72±17.26	119.85±35	0.38	0.699
TLC: Median: Range:	60 33-161	29 12-52	MW= 4.68	0.000*
Plt: Median: Range:	20 5-40	60 30-100	MW= 5.90	0.000*
PB blasts: Median: Range:	58 29-90	60 29-84	MW= 0.24	0.806

This table shows that AML patients with BAALC expression > 2.11 have statistically lower (Hb level & PLT count) and higher (TLC, BM blasts & LDH) in comparison to AML patients with BAALC ≤2.11.

Table 6:-Association between BAALC cut off and clinical data.

Variable	BAALC		χ^2	P
	>2.11	≤2.11		
Sex: Female (22) Male (34)	 14 21	 8 13	0.02	0.888
Fatigue: Yes (45) No (11)	 28 (62.2%) 7 (63.6%)	 17 (37.8%) 4 (36.4%)	Fisher Exact test	1
Fever: Yes (39) No (17)	 25 (64.1%) 10 (58.8%)	 14 (35.9%) 7 (41.2%)	0.141	0.708
Boneache: Yes (52) No (4)	 33 (63.5%) 2 (50%)	 19 (36.5%) 2 (50%)	0.287	0.592
Gum hypertrophy: Yes (41) No (15)	 34 (82.9%) 1 (6.7%)	 7 (17.1%) 14 (93.3%)	27.25	0.000*
Bleeding: Yes (40) No (16)	 29 (72.5%) 6 (37.5%)	 11 (27.5%) 10 (62.5%)	5.97	0.015*
Purpura: Yes (41) No (15)	 30 (73.2%) 5 (33.3%)	 11 (26.8%) 10 (66.7%)	7.4	0.006*
Splenomegaly: Yes (9)	 6 (66.7%)	 3 (33.3%)	0.07	0.778

No (47)	29 (61.7%)	18 (38.3%)		
Hepatomegaly: Yes (9) No (47)	 6 (66.7%) 29 (61.7%)	 3 (33.3%) 18 (38.3%)	0.07	0.778
LN: Yes (5) No (51)	 4 (80%) 31 (60.8%)	 1 (20%) 20 (39.2%)	0.71	0.397
Cytogenetics: Abnormal (17) Normal (39)	 2 (11.8%) 33 (84.6%)	 15 (88.2%) 6 (15.4%)	26.80	0.000*

This table shows that high BAALC AML patients were presented mainly with gum hypertrophy, purpuric eruption and bleeding. As regard association between BAALC and cytogenetics, 39 AML patients were of normal cytogenetics (33 from them were with high BAALC expression).

Table 7:-Association between survival , demographic and clinical data among studied AML patients.

Variable	Survival		χ^2	P	Relative Risk (95% CI)
	Die	Survive			
Sex: Female (22) Male (34)	 15 (68.2) 21 (61.8)	 7 (31.8) 13 (38.2)	0.24	0.625	1.1 (0.74-1.62)
Fatigue: Yes (45) No (11)	 29 (64.4%) 7 (63.6%)	 16 (35.6%) 4 (36.4%)	0.00	0.96	1.0 (0.61-1.66)
Fever: Yes (39) No (17)	 25 (64.1%) 11 (64.7%)	 14 (35.9%) 6 (35.3%)	0.00	0.96	0.99 (0.64-1.51)
Boneache: Yes (52) No (4)	 34 (65.4%) 2 (50%)	 18 (34.6%) 2 (50%)	0.38	0.53	1.3 (0.48-3.55)
Gum hypertrophy: Yes (41) No (15)	 31 (75.6%) 5 (33.3%)	 10 (24.4%) 10 (66.7)	8.54	0.003*	2.2 (1.08-4.73)
Bleeding: Yes (40) No (16)	 26 (65%) 10 (62.5%)	 14 (35%) 6 (37.5%)	0.03	0.86	1.04 (0.66-1.61)
Purpura: Yes (41) No (15)	 27 (65.9%) 9 (60%)	 14 (34.1%) 6 (40%)	0.16	0.68	1.09 (0.68-1.75)
Splenomegaly: Yes (9) No (47)	 6 (66.7%) 30 (63.8%)	 3 (33.3%) 17 (36.2%)	0.02	0.87	1.04 (0.62-1.73)
Hepatomegaly: Yes (9) No (47)	 6 (66.7%) 30 (63.8%)	 3 (33.3%) 17 (36.2%)	0.02	0.87	1.04 (0.62-1.73)
LN: Yes (5)	 4 (80%)	 1 (20%)	0.59	0.44	1.27 (0.78-2.07)

No (51)	32 (62.7%)	19 (37.3%)			
Cytogenetics: Abnormal (17) Normal (39)	 6 (35.3%) 30 (76.9%)	 11 (64.7%) 9 (23.1%)	8.93	0.000*	0.45(0.23-0.89)

This table shows that, there was a statistical significant association between survival and gum hypertrophy i.e, patients with gum hypertrophy carry the risk to die 2.2 more than those with no gum hypertrophy. There was also statistical significant association between survival and cytogenetics i.e, patients with abnormal cytogenetics carry less risk 0.45 to die than those with normal cytogenetics.

Table 8:-Relation between survival & laboratory data among studied AML patients.

Variable	Survival		Test	P
	Death (no=36)	Survive (no=20)		
Hb X±SD	5.72±1.4	7.05±1.82	t test= 2.97	0.004*
BM blasts: X±SD	81.06±11.01	66.10±10.5	t test= 5.20	0.000*
ESR: X±SD	121.72±17.26	119.85±35	t test= 0.38	0.699
LDH: X±SD	868.75±204.24	663.75±178.35	t test= 3.75	0.000*
TLC: Median: Range:	 56 14-161	 42.50 12-136	MW=-2.74	0.006*
PLT: Median: Range:	 24 5-95	 44 12-100	MW=-3.25	0.001*
PB blasts: Median: Range:	 58.50 29-90	 59 29-84	MW=-4.13	0.000*
BAALC: Median: Range:	 3.32 0.80-13.7	 1.21 0.86-6.88	MW=-4.07	0.000*

This table shows that there was statistically significant relation between survival and laboratory data. Patients with low haemoglobin level, increased bone marrow blasts, increased LDH level, higher TLC, lower platelet count, increased PB blasts and increased BAALC expression are at risk to die than those with higher haemoglobin level, lower blast cell count, lower (LDH, TLC, PLT and BAALC).

Table 9:-Mean survival time according to BAALC among studied AML patients by Kaplan Meier test.

baalc2.11	Total N	Death	Censored (still alive)		X± SD /week	95% confidence interval	Log rank test	P
			N	Percent				
>2.11	35	31	4	11.4%	45.85±5.1	35.66-56.04		
<=2.11	21	5	16	76.2%	123.61±4.9	113.93-133.29	31.20	0.000*
Overall	56	36	20	35.7%				

This table showed that mean survival time for patients with low BAALC (123.1±4.9) was longer than patients with high BAALC (45.85±5.1) which was highly statistically significant (log rank test= 31.20, P=0.000).

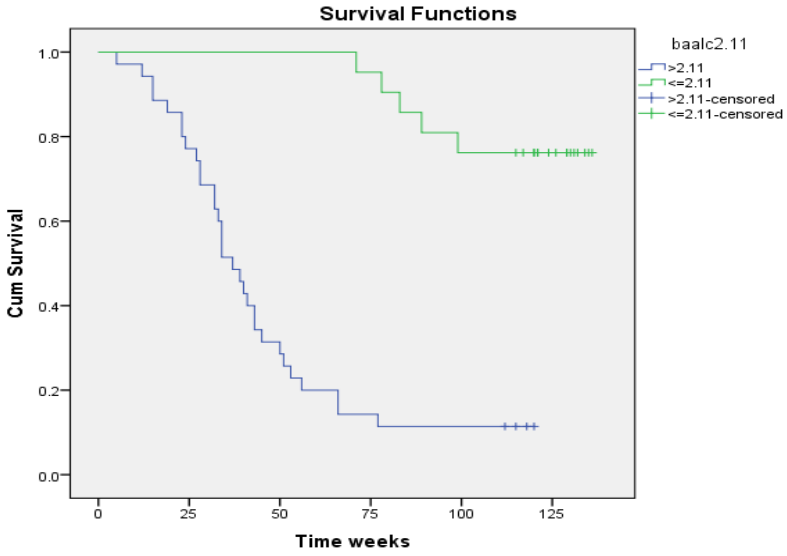


Table 10:-Mean survival time according to chemotherapy response among studied AML patients by Kaplan-Meier test.

Chemotheapy	Total N	Death	Censored (still alive)		X±SD	95% confidence interval	Log rank test	P
			N	Percent				
No CR	29	29	0	0.0%	34.2±2.8	28.74-39.73		
CR	27	7	20	74.1%	121.1±4.9	111.48-130.88	62.62	0.000
Overall	56	36	20	35.7%				

This table shows that mean survival time for patients who achieve complete remission (121.1±4.9) was longer than those who not achieve CR (34.2±2.8) and this difference was statistically highly significant (log rank test=62.62, p=0.000).

Table 11:-Mean survival time according to cytogenetic risk among studied AML patients by Kaplan-Meier test.

Cytogenetic	Total N	Death	Censored(Still alive)	X±SD	95% confidence	Log rank	P

			N	Percent		interval	test	
CN-AML	39	30	9	23.1%	60.25±7.13	46.28-74.23		
Favorable	11	4	7	63.6%	114.00±8.64	97.05-130.94	10.83	0.004
Inter &poor	6	2	4	66.7%	107.16±16.71	74.40-139.92		
Overall	56	36	20	35.7%				

This table shows that mean survival time for cytogenetically normal AML patients (60.25±7.13) was shorter than Favorable group (114.00±8.64) and (intermediate & poor) groups (107.16±16.71) this difference was highly significant (log rank test=10.83, p=0.004).

Disscusion:-

In our study, we analyzed BAALC gene expression in leukemic cells in 56 adult (between 18 & 60 years) patients with newly diagnosed AML before receiving induction chemotherapy. Our patients were diagnosed by complete laboratory workup; complete blood count, blood film, bone marrow aspiration, cytochemistry (peroxidase), immunophenotyping and cytogenetics. Specific investigation was done using;real time PCR for BAALC mRNA gene expression.

In our study, BAALC mRNA wasn't detected in normal persons. The difference between patients and control was statistically significant, which was in agreement also with what was found by **Baldus et al., (2003) and Baldus et al., (2006)** in their two large studies (86 and 307 adult de novo AML patients) by the same technique (real time RT-PCR).

To investigate whether BAALC gene expression is correlated with demographic data of leukemia, it was found that there was no significant relations between BAALC gene expression and age which was in agreement with the results encountered by **(Bienz et al., 2005 and Metzeler et al., 2009)** who found no significant difference between expression level of BAALC and age with a cut off of (60y) (median age of 49 and 59 years, respectively).

However, in a study of **Schwind et al.,(2010),** they found that older patients with favorable molecular risk factors, such as low BAALC if treated more intensively, might have outcomes comparable with those of younger patients with corresponding molecular features.

As regard age, **Rashed et al.,(2015)** disagree with our study as they found a significant correlation between high BAALC gene expression and age. High BAALC expressers were included more in the group of patients more than 45 years. So increasing sample size with more age variation may confirm or exclude such correlation.

Regarding gender, there was no significant difference between sex and BAALC gene expression. **Weber et al.,(2014), Rashed et al.,(2015) and Amirpour et al., (2016),** reported similar results.

When the patients were categorized according to FAB classification. The most frequent wsas M5 (37.5%), followed by M4 (32.1%), then M2 (26.8%) and finally M1 (3.6%) but this disagree with **Rashed et al., 2015** as it was found that those with M0 (16%), M1 (40%), M2(32%),M4 and M5 (2%).

It was proved that BAALC expression is restricted to CD34+ hematopoietic progenitor cells by immunohistochemistry, proving the granular cytoplasmic localization of the protein. BAALC is to be considered as a marker for lineage-committed and uncommitted hematopoietic progenitor cells**(Rashed et al., 2015).**

From the former data, it could be concluded that BAALC belongs to M0, M1, M2 FAB subtypes and less expressed in more differentiated subtypes of AML and may confirm partially the physiological expression of BAALC in CD34+ cells.

Zhou et al., (2015) has observed a significant difference of BAALC overexpression among AML subtypes of M0/M1/M2/M3. Thelow differentiated subtypes (M0/M1/M2) had a significantly higher frequency of BAALC

overexpression than the highly differentiated subtype (M3). **Nolte et al. 2013** observed a trend toward a lower BAALC expression in M3 samples as compared to healthy controls.

Our patients were classified according to cytogenetics into 39 patients with normal cytogenetics and 17 patients with abnormal cytogenetics. According to cytogenetic risk; 11 patients were with favorable risk, 42 patients with intermediate risk and 3 patients with adverse risk.

According to karyotype classification, the frequency of BAALC overexpression in patients with intermediate (42/70, 60 %) and poor karyotypes (9/11, 82 %) was significantly higher than that in patients with favorable karyotype (13/34, 38 %).

In agreement with **Zhou et al., (2015)**, the frequency of BAALC overexpression was higher in normal cytogenetic risk group and lower in the other two groups. They analyzed the clinical relevance of BAALC overexpression in Chinese de novo AML patients. Their results revealed that BAALC was upregulated in AML with a high frequency and high BAALC expression was a promising biomarker for discriminating AML, especially CN-AML from normal controls.

AML patients were stratified into different prognostic risk groups according to karyotypes **(NCCN 2012).** Normal karyotypes, classified in the intermediate prognostic category, constitute the largest cytogenetic subset of AML (approximately 45 %).Clinical outcome of CN-AML patients is quite heterogeneous. Less than half of CN-AML patients are long-term survivors (**Zheng et al., 2008)**. Therefore, identifying prognostic biomarkers that can more precisely predict patient outcome is essential for development of molecular risk-adapted treatment strategies that may improve the clinical outcome of AML.

In our study, CN-AML patients had lower (Hb level, PLT count) and higher (TLC, blast cell count and LDH level) which were statistically significant in comparison with the other both groups. It was revealed that median of BAALC was 3.21 in CN-AML patients and it was statistically significant compared to other both groups. This current finding was in agreement with **Zhou et al., (2015)** who reported that median of BAALC was 3.74.

As regard the diagnostic performance criteria for BAALC gene expression, at the cut off value >2.11, the sensitivity and the specificity were 86.1 and 80%, respectively. **Zhou et al., (2015)** revealed that at cutoff value more than 2.35, the sensitivity and the specificity were 55 and 100 %, respectively.

According to the cutoff value of 2.11, AML patients were divided into two groups: low BAALC expression (≤ 2.11) and high BAALC expression (> 2.11).

As regard age, there was no significant difference between BAALC low expressed and high expressed patients which was in agreement with **Solimanet al., (2016).**On the other hand **Rashed et al., (2015)andZhou et al., (2015)** reported the patients with high BAALC expression were significantly older than the patients with low BAALC expression.

It was demonstrated that high TLC was associated with high BAALC expression, which is close to other study, by **Solimanet al.,(2016).** The percentage of bone marrow blasts in high BAALC expression was significantly higher than in BAALC low expressed which was in agreement with **Qi et al., (2008)** and**Zhou et al., (2015).**However several studies didn't reveal significant difference in BM blasts between low BAALC expressed and high BAALC expressed groups (**Schwind et al., 2010 and Weber et al., 2014).** This discrepancy could be owedto the differences in ethnics and in AML subtype distribution.

As regard clinical data: gum hyperplasia, bleeding and purpuric eruptions were significantly associated with high BAALC expression. The early presenting symptom in AML patients mainly the monocytic subtypes is gingival hyperplasia due to gum infiltration by blast cells.

Abnormal activation of oncogenic signaling cascades is implicated in AML pathogenesis (**Blume-Jensen &Hunter 2001).** Many reports revealed that Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway is activated in a significant proportion of AML patients and expected as a potential therapeutic target (**Towatari et al., 1997).**Mitogen-activated protein kinase kinasekinase 1 (MEKK1) is a

well-known kinase as well as a scaffold protein of key elements in ERK pathway. Many reports have suggested an essential role of MEKK1 in anti-apoptotic function in myeloid cells and leukemogenesis (**Nakamura et al., 2005 and Jin et al., 2010).**On the contrary ERK can trigger tumor suppressor pathways as well, such as cellular senescence, apoptosis and differentiation in a context-dependent manner **Deschênes-Simard et al.,(2013).**

Krüppel-like factor 4 (KLF4), a member of Krüppel-like family transcription factors, is induced by ERK activation (**Chen and Tseng 2005).** Although KLF4 has been shown to enhance stem-cell like properties as one of four iPS factors, it has been drawing attention in AML as an essential factor for monocytic differentiation (**Feinberg et al., 2007).**

It was demonstrated that through interactions with MEKK1 and KLF4, BAALC potentiates oncogenic ERK pathway while inhibiting ERK-dependent differentiation (**Morita et al., 2015).**Chronic activation of ERK induced by constitutive active form of MEK1 leads AML cells to monocytic differentiation (**Miranda et al., 2002).**

So, it was demonstrated that this differentiation of AML cells depends on KLF4 induction, which is one of the other interacting partners of BAALC. In addition, it was shown that BAALC inhibits the nuclear localization of KLF4 by interacting with KLF4 in cytoplasm. It was reported that cytoplasmic sequestration of KLF4 confers oncogenic potential. Therefore, targeting KLF4 would be a rational choice for overcoming treatment-resistant BAALC-high AML (**Morita et al., 2015).**

To investigate the prognostic impact of BAALC expression in AML, survival analysis was performed in de novo AML patients with follow up data. It was found thatpatient with low BAALC expression achieve CR in comparison to patients with high BAALC, this was in agreement with (**Elsharnouby et al.,2010)** whofound that frequency of CR was higher and the relapse rate was significantly lower in patients with low BAALC expression than in patients with high expression. Also, patients with low BAALC expression had a significantly better clinical outcome than high expressors.

Using Kaplan-Meier,it was found that patients with high BAALC expression had significantly shorter overall survival than those with low BAALC expression (45 vs 123 weeks respectively) and this was in agreement with (**Zhou et al., 2015).**

In agreement with our study **(Bienz et al., 2005; Baldus et al., 2006; Langer et al., 2008; Langer et al., 2009; Schwind et al., 2010; Eisfeld et al., 2012).** In addition **(Langer et al., 2009)** stated that patients with high BAALC expression were almost twice as likely to die as those with low BAALC expression. It was found that together with **(Santamaria et al., 2009 andSchwind et al., 2010)** higher BAALC expression is associated with higher relapse rate and other factors as the multidrug resistance gene ABCB1 (MDR1) were identified as highly up-regulated genes in high BAALC expressers, which is consistent with the resistant disease associated with these patients.

Adenosine triphosphate (ATP)-binding cassette sub-family B member (ABCB1), also known as multidrug resistance 1 (MDR1), is an ATP-dependent transporter that is responsible for inhibiting the accumulation of chemotherapy drugs in multidrug resistant cells (**Gillet and Gottesman 2010).**It was shown that BAALC gene expression has a positive association with increased expression levels of ABCB1 in AML drug resistance. Additionally, the association of high mRNA expression levels of BAALC with increased levels of ABCB1 contributes to an increased risk of relapse in AML patients, as well as to a decreased rate of complete remission and worse overall survival rate in these patients (**Guo et al., 2014)**.

(Xu et al., 2012) demonstrated that BAALC silencing resulted in decreased proliferation and enhanced apoptosis in leukemic cells. Knockdown of BAALC expression in KG1a cells resulted in significant inhibition of cell proliferation. Enhanced apoptosis was also observed in the BAALC knockdown cells. The results indicate that BAALC expression contributed to the development of KG1a cells by a possible pathway of enhancing cell proliferation, inhibiting apoptosis. This provides evidence supporting the role of BAALC as an oncogene. It was shown that high levels of BAALC mRNA are related to higher proliferation of bone marrow blasts leading to higher peripheral white blood cell counts and an inferior clinical outcome.

However, (**Heuser et al., 2012),** identified that constitutive activation of BAALC did not promote proliferation or survival of HSC, but blocked myeloid differentiation and promoted leukemogenesis when combined with the self-

renewal promoting oncogene HoxA9. These results indicate that BAALC up-regulation may block myeloid differentiation at a particular stage and additional genetic alterations have to be acquired for the full-blown leukemia.

In conclusion, BAALC gene expression is increased in AML patients. High BAALC is associated with lower rates of CR and increase risk of relapse. It is a specific significant molecular marker in disease progression, response to treatment and survival. It might be of a potential use as target molecules in therapy.

In conclusion:-

BAALC gene mRNA was significantly expressed in bone marrow cells of de novo AML cases at diagnosis and before start of any chemotherapeutic regimen while very low and undetectable expression was found among healthy persons.

This result is going with the hypothesis that the BAALC gene can be used in the monitoring of MRD which would add to the disease follow up .Also the high expression of BAALC gene among de novo AML cases but not in normal cells of healthy persons suggests it as potential candidates for AML immunotherapy.

BAALC gene expression was higher among AML patients not achieving complete remission. These results are denoting association between BAALC gene and poor outcome in AML.