How to Cite:
Muhammid, H. S., Hassan, A. J., & Abdulla, H. N. (2022). Evaluation the role of MicroRNAs in diagnosis and prognosis of acute myeloid leukemia. *International Journal of Health Sciences*, *6*(S8), 276–293. https://doi.org/10.53730/ijhs.v6nS8.9713

Evaluation the role of MicroRNAs in diagnosis and prognosis of acute myeloid leukemia

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***Abstract*---**Acute myeloid leukemia (AML) is a clonal disorder of hematopoietic progenitor cells which are characterized by relevant heterogeneity in terms of phenotypic, genotypic, and clinical features. Among the genetic aberrations that control disease development there are microRNAs (miRNAs). The present study aimed to investigate the role of miRNAs in diagnosis and prognosis of AML and it was designed as cross sectional study in patients with acute myeloid leukemia and normal individuals as control group, we determined: RNA yield and quality; and quantification of miRNA-203, miRNA-143, and miRNA-495 expression by Quantitative Real-Time PCR (qPCR) in the serum of AML patients and control groups. The study was conducted in period between December 2020 and September 2021 at the University of Babylon , College of Science, department of biology . In this case-control study, blood samples were collected from 115 AML patients (38 male and 77 female) and their ages ranged between 18 and 66 years , then, 60 patients were selected based on the quantity and natural color of their samples (28 male and 32 female), in addition to the 30 samples from healthy apparently subjects as a control group (11 male and 19 female), and this group matched with the patients groups. According to the gender we found the rate of infection in female (58.3%) more than male (41.7%) , in addition we found the infection according the age of patients was a higher in age period between 30-40 years at mean of age (37.52 ± 1.76) ,as for the subtypes of disease we found the M3 sub type was a higher from other types at (38.3%), as for the subtypes of disease we found the M3 sub type was a higher from other types at (38.3%) depending on P-value

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 27 March 2022, Manuscript revised: 9 May 2022, Accepted for publication: 18 June 2022

>0.05. in the miRNA types was a high in miRNA-203 at (0.17 ± 0.03) in contrast to the miRNA-143 and miRNA-495 at (0.15 ± 0.02 and 0.04 ± 0.006) respectively. We noticed that the mean of age patients with AML subtypes according on miRNA types shows significant differences in miRNA-203, miRNA-143 and miRNA-495 at (0.001,0.023 and 0.001) ,in addition the highly mean of infection with miRNA-203 type was high in M5 at (0.35 ± 0.09), miRNA-143 was high in M4 at (0.24 ± 0.06), and miRNA-495 was high in M5 at (0.08 ± 0.03).

***Keywords*---**acute myeloid leukemia (AML), MicroRNAs, biomarkers.

Introduction

Acute myeloid leukemia (AML) is the most common leukemia among the adult population and accounts for about 80% of all cases , and it is characterized by clonal expansion of immature "blast cells" in the peripheral blood and bone marrow resulting in ineffective erythropoiesis and bone marrow failure (Bain and Bene ,2019). Depending upon the etiology, genetics , immune-phenotype , and morphology, there are many different classification systems for AML , but the most common risk factor for AML is myelodysplastic syndrome. Other hematological disorders that increase the risk of AML include myelofibrosis and aplastic anemia .In addition, several congenital disorders like Down syndrome and Bloom syndrome are increasing the risk of AML, which tends to present in the early 20s. Also, the environmental exposures like radiation, tobacco smoke and benzene are considered among the risk factors for AML . Finally, previous exposure to chemotherapeutic agents is also a risk factor for AML subtybes (Hartmann and Metzeler ,2019 ; Boddu and Zeidan ,2019).

Previously, AML was classified according to the French-American-British classification system using morphology and immune-phenotype/cytochemical criteria to define eight major AML subtypes (FAB M0 to M7) (Bonnet and ,1997). AML is a heterogeneous disease characterized by the increased proliferation and survival of immature myeloid cells and is the result of a number of genetic abnormalities, including chromosomal rearrangements and mutations (Ferrara and Schiffer,2013). Early studies characterizing the role of miRNAs in AML focused on identifying AML-specific miRNA expression patterns , therfore distinctive miRNA profiles were identified for many cytogenetic subtypes of AML (Dixon-McIver et al .,2008 and Li et al ,2008) .

MicroRNAs (miRNAs) are short non-coding single-stranded RNAs (~19–22 nucleotides) (Vitsios et al.,2017; Wallace and O'Connell,2017) that will negatively regulate mRNA stability (Svoronos et al,2016; Wallace and O'Connell,2017; Fernandez et al,2017) , and this biomarker play an important role in many biological functions, such as cell growth, differentiation, proliferation, and apoptosis (Pichiorri et al.,2011; Vitsios et al.,2017). Moreover, miRNAs can act as tumor suppressors or oncogenes , contributing to malignant transformation in solid and hematological tumors, including AML(Wong et al.,2010; Senyuk et

al.,2013; Svoronos et al.,2016) . Since , there are many studies showed that microRNAs act as diagnostic and prognostic biomarkers in AML (Maki et al.,2012; Lin et al.,2015; Caivano et al.,2017).

Materials and Methods

Subjects of the Study

The study subjects comprised of 115 AML patients(38male and 77female) ,and their ages ranged between 18 and 66 years .These patients were suffered from Acute myeloid leukemia and there samples were collected from the Baghdad medical city hospital during the period from (December) 2020 to (September) 2021 under the supervision of specialized hematopathologist , and according to the medical ethics of the hospital and consent form taken from all patients and volunteers group. Also , a questionnaire was taken from the patients and case sheets including: number, age, sex, subtypes of AML (M0. M1, M2 ,M3,M4 , M5 ,M6 .M7) and duration of disease. Then , 60 patients were selected based on quantity and natural color of their samples (28male and 32 female) , in addition to the 30 samples from healthy subjects as control group (11 male and 19 female) and this group matched with the patients grou

Blood collection

Three milliliters of venous blood were drawn from each of these groups using a disposable syringe using aseptic technique. After allowing the blood to clot for 15 minutes at room temperature, it was centrifuged at 2,000 x g for 10 minutes to obtain the serum. Next, 500 µl of serum was collected in an Eppendrof tube with 500 µl of Trizol and stored at -20 to be used for miRNA-203 and miRNA-143 miRNA-495 qPCR

Laboratory Assays

Total RNA extraction

Total RNA were extracted from serum samples by using (Tranzol UPreagent kit,Cat.No ET111,TransGen Biotech) and done according to the manual procedure of company instructions

Estimation RNA yield and quality

The extracted genomic RNA was checked by using Nanodrop spectrophotometer (THERMO. USA) that check RNA concentration and estimation of RNA purity through reading the absorbance in at 260 /280 nm as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times, then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestal.

3. The sampling arm was lowered and clicking OK to blank the Nanodrop, then cleaning off the pedestals.
4. Finally , the pedestals are cleaned and pipet 1µl of RNA sample for measurement.

DNase I Treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples o f DNase I enzyme kit and done according to method described by Promega company, USA instructions. Then , the mixture was incubated at 37C° for 30 minutes , after that 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

cDNA synthesis

cDNA synthesis for miRNA was done using GoScript™ Reverse Transcriptase Kit and performed according to the manual procedure described by Promega company, USA instructions.(Cat.No A5001).

Table1: miRNA primers and probes sequences used in this study

PRIMER	SEQUENCE **5′ - 3′**
miR-203 qPCR primer	F : GCGGTGAAATGTTTAGGAC R:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGTG
MiR-143 qPCR primer	F: TGTAGTTTTCGGAGTTAGTGTCGCGC R: CCTACGATCGAAAACGACGCGAACG
MiR-495 qPCR primer	F:GTCGTATCCAGTGCAGGGTCCGAGG R:TATTCGCACTGGATACGACCTGTCC
U6	F: GITTTGTAGTTITGGAGTTAGTGTTGTGT R: CTCAACCTACAATCAMMAACAACACAAACA

Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of miRNA-203, miRNA-143 and miRNA-495 expression analysis that normalized by housekeeping gene (U6) in serum and blood patients and normal samples by using Real-Time PCR technique and this method was carried out according to method described by Magdalena *et. al.* (2019) which include the following steps:.

A- qPCR master mix preparation

qPCR master mix was prepared by using GoTaq® qPCR Master Mix Kit that according on SYBER Green dye detection of gene amplification in Real-Time PCR

system and done according to method described by Promega company, USA instructions. .(Cat.No A6000).

B. qPCR Thermocycler conditions

The qPCR plate was loaded and the thermocycler protocol was followed:

Table 2: qPCR Thermocycler conditions

qPCR step	Temperature	Time	Repeat cycle
GoTaq® Hot Start Polymerase activation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extension Detection(scan)	60 °C	30 sec	

Data analysis of qPCR

The data results of qPCR for miRNA and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) by using The ΔCT Method Using a Reference that described by (Livak and Schmittgen, 2001) as following equations:First, normalize the CT of the target gene to that of the reference (ref) gene, for both the test sample and the control sample:
ΔCT(test) = CT(target, test) – CT(ref, test)
ΔCT(control) = CT(target, control) – CT(ref, control) .Second, normalize the ΔCT of the test sample to the ΔCT of the control: ΔΔCT = ΔCT(test) – ΔCT(control)
Finally, calculate the expression ratio: $2^{-\Delta\Delta CT}$ = Normalized expression ratio

Statistical Analysis

Statistical analysis was carried out using SPSS version 27. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SE). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. A p-value of ≤ 0.05 was considered as significant.

Result

Distribution of patients with acute myeloid leukemia

Table (1) showed that distribution of patients with acute myeloid leukemia(AML) according to socio-demographic characteristics including age and gender , in which the female patients percentage was 58.3% (35 out of 60), while it was 41.7% (25 out of 60) in male patients .

Table 3: The Distribution of patients with acute myeloid leukemia according to socio-demographic characteristics

Gender	Number	Percentage
Male	25	41.7%

Female	35	58.3%
Total	60	100%

Acute myeloid leukemia(AML) remains a rare but fatal malignancy. The current results found that the disease was more recurrent in females than in males according to the collecting samples , and there is a possible reason for this, which is the occurrence of some genetic mutations in the X chromosome in female ,which leads to the presence of disease genes in the newborn, then develop to the younger female that causes leukemia (Newell and Cook, 2021). In addition, Suresh *et. al.*, (2006) showed that myeloid cells in females and several pathophysiological mechanisms such as exposure to common environmental risk factors and repeated infections in female which might result in the development of this disease .

In the current study the age of patients distribution between 18-66 years, that may be related to the disease genes and some molecular features, that was most pronounced in the AML patients (Lindsley *et al.*,2015). The age distribution by sex of leukemia patients, which is characterized by demography, indicates an important influence of age composition, and this can make age-matching the optimal condition for comparison (Engen *et. al.*,2020). Wang *et. al.*, (2019) found that there is an excess of females with AML compared to males, and the disease was also concentrated among younger individuals more than adults, and the occurrence of many mutations in many genes whose representation is excess in females compared to males in the groups of the study .

Distribution of patients with acute myeloid leukemia according to subtypes

Figure (1) revealed that distribution of patients with according to subtypes including M0, M1, M2, M3, M4, M5, M6 and M7. Majority of patients presented with subtype M3 (N=23, 38.3%) of total patients , while M4 subtype represent 20.0%(N=12) of total patients, but M2 and M5 represent 15%(N=9), in addition M1 and M7 subtypes represents 6.7%(N=4) and 5%(N=3) respectively of total patients and there was no patients presented with subtypes M0 and M6 (0.0%)

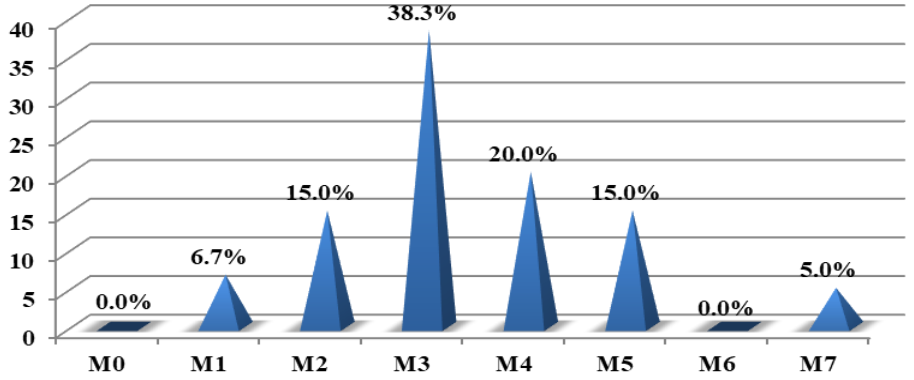


Figure 1: Distribution of patients with acute myeloid leukaemia according to subtypes (N=60)

The present results showed a large distribution between types of AML this was consistent with the results of Schoch *et al* (2003) that mention the differences between subtypes of AML which resulted from the epidemiological and pathogenetic heterogeneity in AML patients such as diseases and environmental exposures that appear to be increase the risk of disease to the individual by AML subspecies. In addition, the risk of AML is significantly increased in patients with other hematopoietic disorders, including myelodysplastic syndromes (MDS), some myeloproliferative neoplasms (MPNs), and aplastic anemia (Sritana *et. al.*,2008).In the current study the M3 subtype (38%) is more than the other subtypes of AML , while the M0 and M6 (0%) subtypes was less, and these results are consistent with Naghmi and Khalid (2013) who found the same subtypes are highly incidence in contrast to other subtypes. Also , in present study the most common subtype was M3 at (38%) followed by M4 at (20%) ,then equal number of M2 and M5 at (15%). In contrast to a study done in Japan on adult patients of AML, the most frequent subtype was M2 followed by M3 and M4 (Kuriyama *et. al.*,2001) . Furthermore , figure (1) shows a comparison of AML subtypes , and it was noted that AML-M3 subtype was the most common, while not a single case of M0 and M6 was seen , these finding comparable with a study of Naghmi and Khalid (2013); who found that the same type are highly incidence in contrast to other types.

Distribution of patients with acute myeloid leukemia according to Age

The present data (Table , 4) revealed that the mean age of patients was (37.52 ± 13.62) years with older patients was 66 years and younger patients was 18 years , while table (5) and figure(2) showed the mean differences of age (years) according to study groups (patients and control groups) , and there were no significant differences between means of age in studied groups.

Table 4: The Distribution of patients with acute myeloid leukemia according to the age

Age	Minimum	Maximum	M ± S.D
Male and Female	18	66	37.52 ± 13.62

Table 5: The mean differences of age according to the study groups (N=90)

Study variable	Study group	N	Mean ± SE	P-value
Age (years)	Acute myeloid leukemia	60	37.52 ± 1.76	0.549
	Control group	30	35.80 ± 1.97	

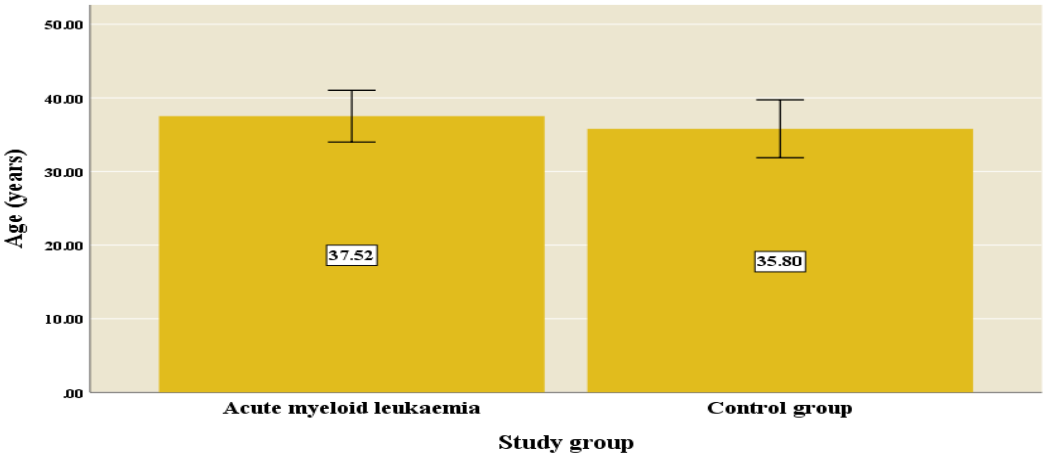


Figure (2): The mean differences of age (years) according to the study groups (N=90)

The use of growth factors to promote hematopoietic recovery has yield consistent reductions in treatment related morbidity or mortality . In addition , drug resistance by inhibiting drug efflux mechanisms or increasing sensitivity to cytotoxic agents, these strategies may be shown significantly effect on the age distribution in AML outcome (Sonneveld *et. al.*,2000).

The present study was undertaken to analyze different aspects of AML patients , in which the AML study found the more prevalent was in adults at mean of age (37.52) years than younger age, this results are consistent with Lowenber, (2000) who found a high incidence of AML with ≥ 30 age in contrast to other ages , in addition the disease increased in this age because the cytogenetic abnormalities that showed in many patients with AML , who mention by Grimwade *et. al.*, (2001) that observed the cytogenetic abnormalities were associated with a lower age rate (30%) compared with patients with intermediate findings (75%).

One of the most important reasons that lead to an average life expectancy of acute leukemia, which appeared in current results, can be attributed to the different ages of patients admitted in hospitals, as most of the samples we collected were in the ages 20-40 years compared to the young or old ages who receive treatment in other hospitals or in other places in the country, these results was agreement with Rodrigues *et. al.*, (2003), who mentioned that 66% of adult patients with AML admitted to Hospital São Paulo were over 30 years of age, as compared to the 34 % rate reported in other international series , furthermore , the elderly or small ages do not have proper access to health care, or whether comorbidities or social aspects prevent the ideal diagnostic procedures, among other causes (McMullin and Mackenzie, 2001). The rapid diagnosis of AML and health care or early treatment was also from the reasons that distributed disease in age periods more than 20 in contrast to another periods (Goldstone *et al.*,2000) , these finding was consistent with the present results that showed highly incidence in age 20-40 years.

Expression of miRNAs

Distribution of patients with AML according to the expression levels of miRNAs

Table (6) , and figures 3,4,and 5 showed the level expression of study biomarkers including miRNA-203, miRNA-143 and miRNA-495 in patients and control groups . Where all biomarkers shows a significant differences in relation with AML at p-value (< 0.001).

Table 6 : The mean levels of miRNAs expression in patients and control groups

Biomarkers	Groups	N	Mean ± SE	P-value
miRNA-203 (fold)	Patients	60	0.17 ± 0.03	<0.001*
	Control	30	12.02 ± 2.32	
miRNA-143 (fold)	Patients	60	0.15 ± 0.02	<0.001*
	Control	30	2.49 ± 0.22	
miRNA-495 (fold)	Patients	60	0.04 ± 0.006	<0.001*
	Control	30	0.87 ± 0.15	

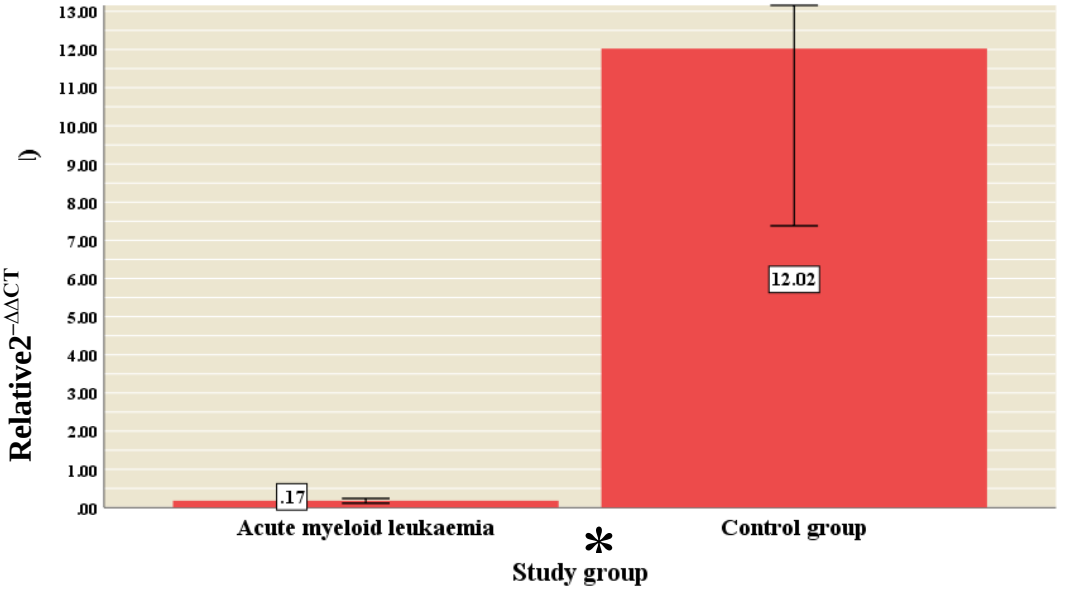


Figure (3): The mean levels of miRNA-203 expression (ng/µl) in patients and control groups(N.90) . (*): means significant

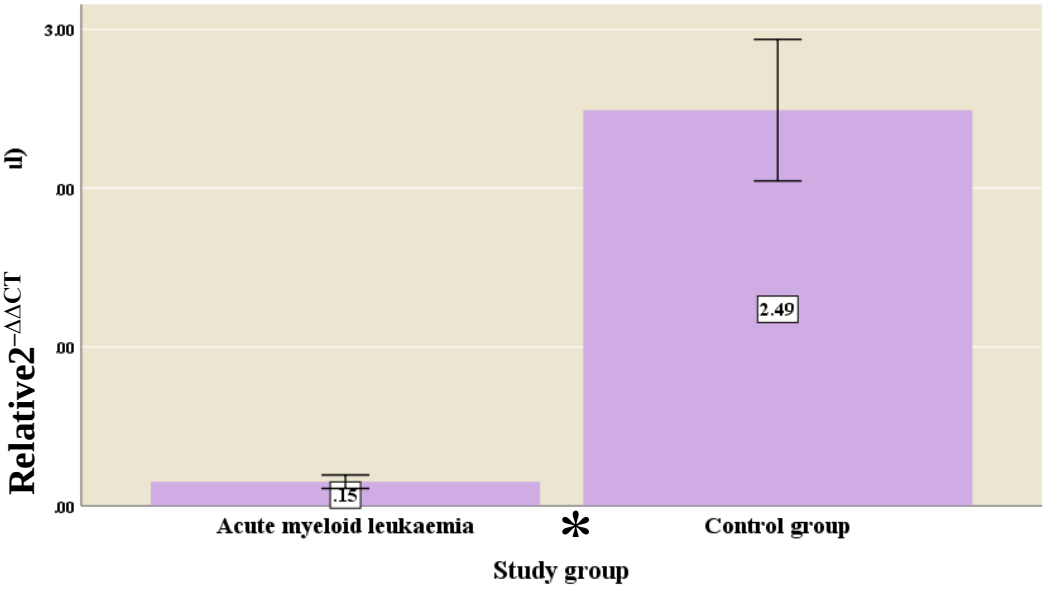
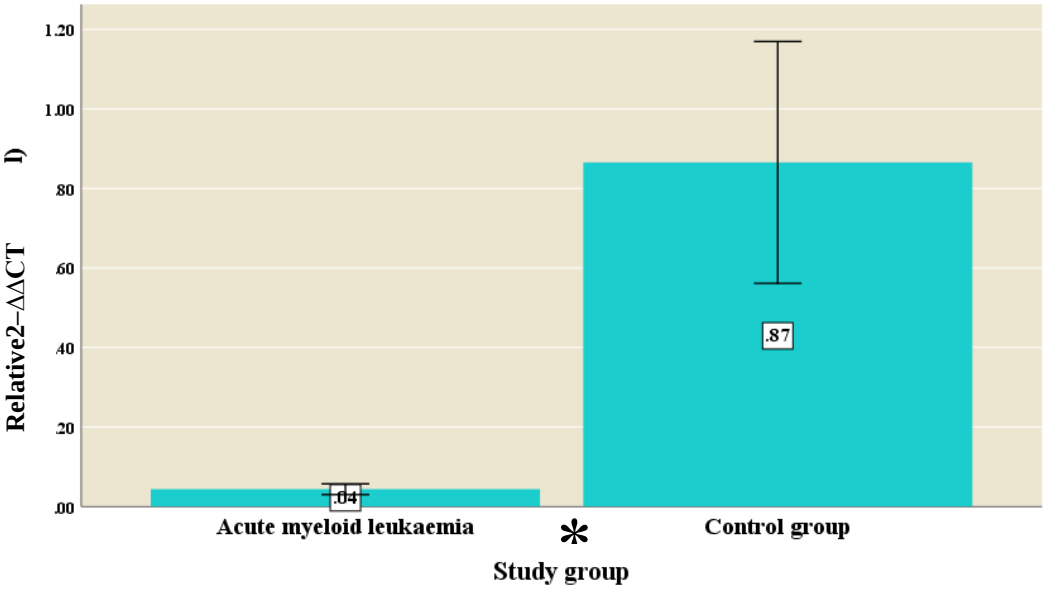


Figure (4): The mean levels of miRNA-143 expression (ng/µl) in patients and control groups(N.90) (*): means significant



Figure(5): The mean levels of miRNA-495 expression (ng/µl) in patients and control groups(N.90) . (*): means significant

MiRNAs, are class of regulatory found to be dysregulated in human cancers (Croce *et al.*,2005) , and mature miRNAs act as negative gene regulators and have been shown to function both as tumor suppressors and oncogenes (Zhang *et al.*,2007). In present study it was found a highly relation between miRNA and

AML patients . These results are consistent with the data of Chen *et. al.*,(2014) that indicated the role of miR-143 in myeloid differentiation and AML. In this study, the results found a strong induction of miR-143, miRNA-203 and miRNA-495 expression in patients with AML, which could be supported by publications of Donahue *et. al.*, (2009) and Batliner *et. al.*, (2012), furthermore, they found that the expression of miR-143 reached the highest levels in severe cases of the disease.

Figures 3,4, and 5 showed a highly miRNA-143 expression and significantly correlates with the survival of AML patients and is associated with good prognostic factors , also these data show high miRNA-143 expression as a favorable prognostic factor in AML and substantiate a general role for miR-143 in prognosis, which is supported by data in solid cancers from Krakowsky *et. al.*, (2018).

Distribution of Acute myeloid leukemia subtypes according to the age

The data in table (7) and figure (6) shows the mean differences of age (years) according to subtypes of AML including M1 and M2, M3, M4 and M5. The results revealed no significant differences between means of age according to subtypes of AML .

Table 7: The mean differences of age according to the subtypes of AML (N=57)

Study variable	Subtypes of AML	N	Mean ± SE	P-value
Age (years)	M1 and M2	13	40.69 ± 3.76	0.214
	M3	23	33.83 ± 3.20	
	M4	12	37.42 ± 2.48	
	M5	9	44.44 ± 4.95	

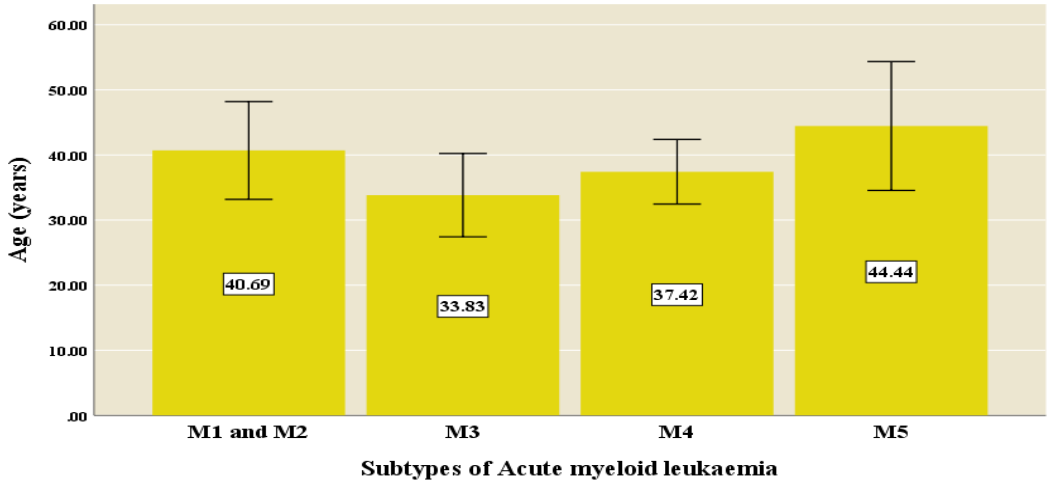


Figure (6): The mean differences of age (years) according to subtypes of AML (N=57)

In the current study it was found a highly number of patients in M3 subtype and at mean value 33.83 ± 3.20, while the high mean at 44.44 ± 4.95 was shown in M5 subtype with low number of patients (10) , in addition, the highest age of patients with AML was at 20-40 years , so that these correlation between age > 30 years and AML subtypes or abnormal mutation and other parameters such as cytogenetics, could be depending on it as prognostic parameter for diagnosis (Haferlach *et al.*,2003) .The age of AML patients presentation in current results showed increase between 20-40 years especially with the M3 subtype , and these result was consistent with Estey (2014) that showing age and AML subtypes mainly affected in severity and prognostic of disease .

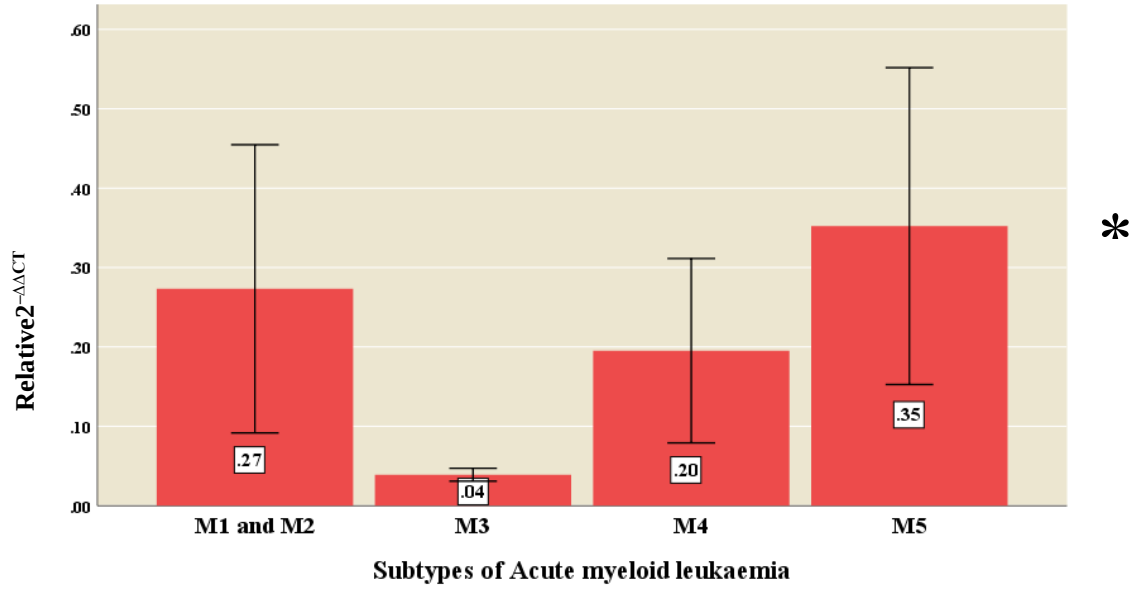
AML is closely related to age, as most researchers has focused on the relationship between age and disease due to the concept of age-related clonal hematopoiesis . Most of the patients were found in ages equal to 30 years due to the increase of receptors in the cells that get the disease and the AML increases with increasing age until the age of 60 years (Jaiswal *et. al.*,2014) ,these finding was consistent with present results where the highest incidence of patients was between the age group 20- 40 years. There are clinical differences according to age and gender in patients with AML and it would like to clarify that the focus of the disease among this age periods referred to previously may be because the ages are less than 20 years They may undergo less diagnostic procedures, such as morphological sub-classification and genetic evaluation , this explanation was consistent with the results Sorror *et. al.*, (2014).

The relationship between AML subtypes and miRNAs expression

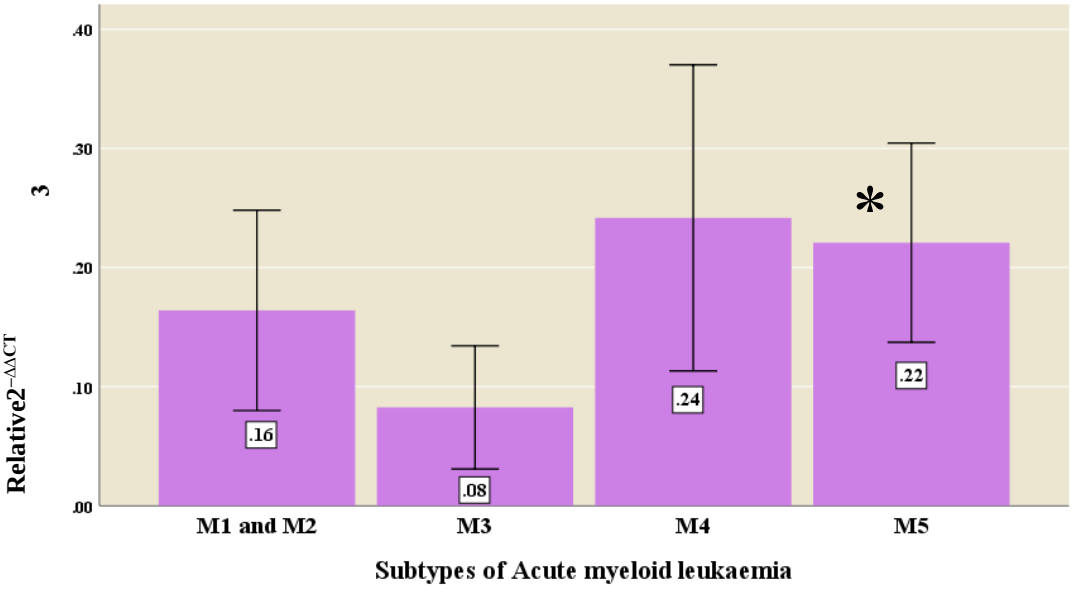
Table (8) , and figures7,8, and 9 showed that the mean levels of biomarkers including miRNA-203 (ng/µl), miRNA-143 (ng/µl) and miRNA-495 (ng/µl) according to AML subtypes (M1 and M2, M3, M4 and M5). There were significant differences between levels of miRNA-203 (ng/µl), miRNA-143 (ng/µl) and miRNA-495 (ng/µl) according to the subtypes of AML .

Table (8) : The mean levels of biomarkers according to the acute myeloid leukemia subtypes (N=57)

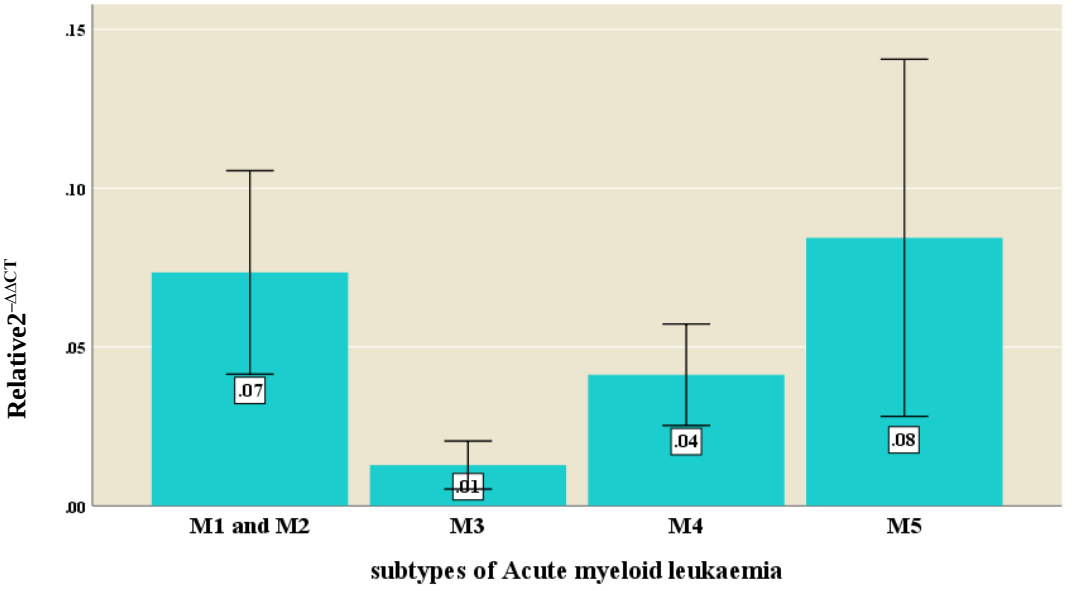
Study variables	subtypes of AML	N	Mean ± SE	F	P-value
miRNA-203 (fold)	M1 and M2	13	0.27 ± 0.09	6.030	0.001*
	M3	23	0.04 ± 0.004		
	M4	12	0.20 ± 0.06		
	M5	9	0.35 ± 0.09		
miRNA-143 (fold)	M1 and M2	13	0.16 ± 0.04	3.450	0.023*
	M3	23	0.08 ± 0.03		
	M4	12	0.24 ± 0.06		
	M5	9	0.22 ± 0.04		
miRNA-495 (fold)	M1 and M2	13	0.07 ± 0.02	7.596	<0.001*
	M3	23	0.01 ± 0.003		
	M4	12	0.04 ± 0.007		
	M5	9	0.08 ± 0.03		



Figure(7): The mean levels of miRNA-203 expression according to subtypes of AML.



Figure(8): The mean levels of miRNA-143 expression according to subtypes of AML



Figure(9): The mean levels of miRNA-495 expression according to subtypes of AML

The differences in the AML subtypes with the biomarkers under study in total sample, does not reflect the subtypes of AML patients in the another region , because the study did not include larger number of patients. The results confirmed that the correlation between AML subtypes and miRNAs as tool for monitoring the severity of disease and shorter overall survival, which is consistent with the results of Appelbaumm *et. al.*, (2006) . Different studies reported about the important role of miRNA expression that distinguish between AML and acute lymphoblastic leukemia (ALL) (Wang *et al.*,2010) ,on the other hand the study by Mi *et al* (2007) on miRNA types were sufficient to distinguish between AML and ALL with an accuracy of greater than 95%, miRNA-143as let-being significantly upregulated and miRNA-203,miRNA-495, downregulated in AML comparing to ALL . Together, the above work showed that these identified miRNAs could be new potential markers for ALL and AML classification and diagnosis (Zhi *et al.*,2013). In the current study it was observed that the high levels of miR-495, miR-203, expression , was compatible with Dixon-McIver *et al* (2008) and Jongen-Lavrencic *et al* (2008) who noticed increased in certain types of miRNA with AML .

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