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c-FLIP and microRNA 708-5p gene expression in newly diagnosed and chemotherapy in acute myeloid leukemia of Iraqi patient

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Abstract:

BACKGROUND: C-FLICE-like inhibitory protein (c-FLIP) is a protein that does not merely block apoptosis signaling but also adjusts further pathways of cell death. Acute myeloid leukemia (AML), a nonhomogeneous hematologic malignancy, is the highly common form of AML among adults and is described through the clonal enlargement of myeloid blasts in the bone marrow (BM), peripheral blood, and/or else tissues.

OBJECTIVES: The aims of this study were to investigate the role of c-FLIP and microRNA (miRNA) 708-5p as a prospective prognostic biomarker as well as the therapeutic goal in AML.

PATIENTS MATERIALS AND METHODS: This study includes two groups of patients (40) individuals newly diagnosed AML patients, (20) AML taking chemotherapy, and (50) apparently healthy volunteers. The study was conducted at the National Center of Hematology/Mustansiriyah University. The methods employed in the analysis include total RNA extraction, complementary cDNA synthesis, and quantitative real-time polymerase chain reaction (PCR) were used to evaluate the gene expression of *cFLIP* and *miRNA-708-5p*. Complete blood count to estimate some hematological parameters.

RESULTS: The expression of c-FLIP was notably higher in both newly diagnosed and patients under chemotherapy compared to controls with fold expression (3.291 and 2.92), respectively, with a highly significant (P = 0.0001). The increase in *miRNA 708-5p* expression in newly diagnosed patients with fold expression (5.345), whereas downregulation in patients under chemotherapy with fold expression (0.789) indicates that treatment may restore its levels, contributing to the suppression of c-FLIP and promoting apoptosis.

CONCLUSION: The c-FLIP and miRNA708-5p gene might be used as a biological marker for the AML initial diagnosis. The researches emphasize the role of miRNA 708-5p as a tumor suppressor, which negatively regulates the antiapoptotic protein c-FLIP, indicating its potential as a therapeutic target in AML treatment. By modulating the levels of miRNA708-5p, it may be possible to regulate the expression of c-FLIP, thus enhancing the effectiveness of apoptosis-inducing therapies. This suggests the promising development of miRNA-based therapies as part of AML treatment strategies. Furthermore, miRNA 708-5p can act as a prognostic indicator in AML, with its expression levels offering valuable insights into disease progression and patient response to treatment. Further research into miRNA 708-5p can lead to a better understanding of its role in AML pathogenesis and its potential applications in clinical practice.

Keywords:

Acute myeloid leukemia, cellular-FLICE-like inhibitory protein, gene expression, microRNA 708-5p, real-time polymerase chain reaction

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Introduction

Acute myeloid leukemia (AML) is a life-threatening disease that is a stem cell tumor, characterized by the presence of leukemia cells (immature myeloid cells) that infiltrate from the bone marrow and press on the normal blood components causing bleeding, fatal infection, and the presence or absence of an increase in the number of white blood cells in addition to the infiltration of organs.[1] Many countries are characterized by high rates of AML and low survival rates, and Iraq is one of these countries (Iraqi Cancer Registry).[2] In developed countries, AML has the highest incidence of acute leukemia in adults.[3] AML classification based on morphological and cytochemical results AML can be classified into subtypes M0, M1, M2, M3, M4, M4 eso (eosinophilia), M5, M6, and M7 using the FAB system.[4] The classification has been replaced by the WHO classification as follows AML with recurrent genetic abnormalities, AML with multilineage dysplasia, AML and MDS therapy-related, AML not otherwise specified, and AML spreads associated with Down syndrome.[5] Studies from Iraq, which are done in Mosul City, Baghdad, Sulaymaniyah Governorate, and Karbala Province, emphasize the frequency and features of AML. In Sulaymaniyah governorate, AML constituted 17% of all leukemia cases, affecting more males, and the incidence increased in the old age group.[6] In Karbala province, the AML also accounted for about 19.2% of all leukemia, with a median age of 36 years, and male predominance was also shown.[7] According to the statistics of Basra Governorate for cancer patients in 2017, the rate of leukemia was about 4.7 per 100,000 children, whereas it was 4.5 per 100,000 adults.[8] AML has varying occurrence rates globally; the USA has an age-adjusted incidence of 4 ANL for every 100,000 people. 1 per 100,000 and a prevalence of 69,700 incidences in 2019.[9] The incidence rate as 20 per 100,000 and the prevalence as 43,420 in 2018. It is, therefore, necessary to understand that multiple factors go into the development of the disease referred to as AML, and these factors may include genetic factors, epigenetic factors, and environmental factors. AML has been described as the sequential acquisition of somatically located mutations and the frequency of certain gene mutations contributing to leukemogenesis.[10] AML involves the abnormal propagation and differentiation of a clonal of myeloid stem cells. It may arise with basic hematological upset, or as a result of previous treatment, some abnormalities playing an essential role in the production of AML like translocation of chromosome *t* (15:17).[11] which can lead to a variety of complications. Therefore, a reduction in erythrocytes and platelets and a rise in leukocytes, with the latter being nonfunctional myeloblasts,[12] resulting from a disruption in the normal maturation process of myeloid cells. 50%–80% of AML patients still die even after initially effective therapy, despite the many advances in cancer detection and treatment over the previous several decades.[13] The most common mutated gene in adult AML patients is the nucleophosmin1 gene in 25%–35% of patients according to the FAB classification system.[14] The C-FLICE-like inhibitory protein (c-FLIP) is a catalytically sedentary caspase-8/10 homolog interfering with effective DISC creation in the extrinsic pathway straight at the level of the receptor. c-FLIP was defined as a delicate controller with pro- and antiapoptotic influences in progress as well as in usual adult tissues. The c-FLIP expression dysregulation was noted in autoimmune diseases and numerous kinds of cancer.[15] Furthermore, the c-FLIP is an apoptosis inhibitor that started through the death receptor ligation. In addition, to its participation in the death receptor-mediated apoptosis, the c-FLIP has been depicted to become a multifunctional protein, modulating the features, procedures, and pathways, such as endoplasmic reticulum morphology, autophagy, necrosis, and inflammation.[16] The microRNAs (miRNAs) are slight noncoding RNAs that adversely control the multiple gene expression either through producing the degradation of mRNA or creating translational silencing. Moreover, it has been decisively recognized that the miRNAs govern different procedures of key cellular, such as differentiation, apoptosis, differentiation, proliferation, development, and being applied into the human diseases, comprising the cancer. The miRNAs have been recognized that they work as tumor suppressor genes or traditional oncogenes. The epigenetic silencing or the genomic removal of a miRNA usually re-presses one or more oncogenes' expression might cause augmented oncogenic expression. Otherwise, intensification, overexpression, or the epigenetic silencing loss of a gene that encodes a miRNA targeting one or further tumor suppressor genes can prevent the antioncogenic pathway action. The profiles of aberrant miRNA have been observed in different cancers. The human miR-708 gene is situated at chromosomal position 11q14.1. miR-708 is an intronic miRNA that is situated in the initial coding gene intron, ODZ4 (odz, odd Oz/ten-m homolog 4 (Drosophila), which is recorded in the similar direction as miR-708. ODZ4 (or Teneurin-4) is a drosophila pair-rule gene homolog encoding a transmembrane protein included in intercellular signaling through the progress.[17] miR-708-5p is a miRNA uttered in a no. of diseases. miR-708-5p was initially recognized in usual tissues as well as tumor specimens from the patients with cervical cancer and reveals an elevated degree of order resemblance with the miR-28. The miR-708 (miR-708-3p) passenger strand evinces prospective biological purpose and is integrated into the RNA-created silencing compound. miR-708-5p is included in many diseases, comprising cardiovascular diseases cancer, and neurodegenerative diseases.[18] The study was designed to investigate the role of c-FLIP and microRNA (miRNA) 708-5p as a prospective prognostic biomarker as well as the therapeutic goal in AML.

Patients Materials and Methods

This study is a case–control study consisting of two groups with AML. The patients' ages ranged from 15 to 85 years; the first group included 40 newly diagnosed AML patients (males 17, females 23), and the second group consisted of 20 AML taking chemotherapy (males 8, females 12). The third group includes another 50 apparently healthy volunteers (males 30, females 20). The personal information is the age, sex, duration of disease, and complete blood count. The samples were admitted from the National Center of Hematology/Mustansiriyah University, and this study was conducted in Baghdad from October 2023 to March 2024.

Inclusion criteria

- Patients: Persons who have been diagnosed with AML, Aged ≥18 years old
- Healthy control: Apparent good health; no signs of AML or other cancers. They do not suffer from diabetes, thyroid disorders, or any other chronic illnesses.

Exclusion criteria

- Patients and control: Children <18 years old
- Healthy control: People, who have been not exposed to any form of cancer, have no family history of cancer, or have no chronic illnesses.

Ethical approval

The study's design was accepted by Applied Sciences/Biotechnology University of Technology according to order number 3524 dated November 20, 2023. Written informed consents were obtained from all patients and apparently healthy control group.

Collecting of blood samples

From each participating category (patients and control), about 3 mL of venous blood was collected using a disposable syringe (5 mL), and the blood was first collected in the tube as well as a gel tube of ethylene diamine tetra-acetic (EDTA), and then dispended in an Eppendorf tube. After that, 250 µL from EDTA type was added to 750 µL Trizole in an Eppendorf tube, and finally, the mix was reserved at (−23°C) till the extraction.

RNA extraction

Total RNA was extracted using a total RNA Purification Kit according to the manufacturer's instructions (TransZol Up Plus RNA Kit, Transgen Biotech, China). The miRNA was extracted using an EasyPure® miRNA Kit, (Transgen Biotech, China). RNA quality was assessed using a nanodrop spectrophotometer (One C, Thermo Fisher USA). cDNA was synthesized using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix, (Transgen biotech, China). The mRNA levels of c-FLIP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using TransStart® Top Green qPCR Super Mix (Transgen biotech, China) and a Qiagen rotor gene Q system (Qiagen, Germany).

GAPDH and U6 were used for normalization. The data were analyzed using the ΔΔCt method, and each assay was performed in at least triplicate. The c-FLIP as well as *miRNA708-5p* expression of newly diagnosed patients was determined, as well as patients under chemotherapy and controls using qRT-PCR. The primers designed used in study in Table 1.

Quantitative real-time polymerase chain reaction runs

The qRT-PCR was carried out using the QIAGEN Rotor gene Q Real-time PCR System (Germany). The expression levels and fold changes of the C-FLIPL, GAPDH, U6, and miRNA 708-5P genes were assessed using the TransStart® Top Green qPCR Super Mix kit and measuring the threshold cycle (Ct). Every reaction was performed twice. The needed volume of each component was determined using [Table 2].

Table 1: The study's designed primers

Primer	Sequence (5'→3' direction)	Primer size (bp)	Product size (bp)	Ta (°C)
C-FLIPL (gene expression)				
Forward	GGACCTTGTGGTTGAGTTGG	20	144	58
Reverse	TTCTGGATTTTTGTCTTCAGGTC	23		
GAPDH				
Forward	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reverse	GAGCCCCAGCCTTCTCCATG	20		
miRNA				
MiR 708-5P F.P.	AAGGAGCTTACAATCTAGCTGGG	23	70	58
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC	22		
miRNA-universal P.	CAGGTCCAGTTTTTTTTTTTTTTTVN			
U6	AGAGAAGATTAGCATGGCCCCT			

GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, C-FLIPL=Cellular-FLICE-like inhibitory protein, miRNA=MicroRNA

The cycling protocol was programmed for the following optimized cycles according to the thermal profile as shown in Tables 3 and 4.

Statistical analysis

The results used one-way ANOVA in the Statistical Package for the Social Sciences (SPSS 29) (IBM Corporation, Armonk, New York, USA) program. $P \leq 0.05$ were used to determine the statistical significance. Pearson correlation was used to determine the correlation between two quantitative variables, and receiver operating characteristic (ROC) was used to evaluate a test's overall diagnostic performance and to compare the results of two or more diagnostic tests. It is also used to find the best cutoff value for determining the presence or absence of a disease.

Table 2: The components of quantitative real-time polymerase chain reaction were employed in the cellular-FLICE-like inhibitory protein, glyceraldehyde 3-phosphate dehydrogenase, U6, and microRNA 708-5P gene expression experiments

Components	20 µL rxn
2×TransStart® top green qPCR super mix	10
Nuclease free water	6
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
cDNA	2

qPCR=Quantitative polymerase chain reaction, cDNA=Complementary DNA

Table 3: The thermal profile of glyceraldehyde 3-phosphate dehydrogenase and U6 gene expression

Step	Temperature (°C)	Time (s)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	58	15	
Extension	72	20	
Dissociation	55°C–95°C		1

Table 4: The thermal profile of cellular-FLICE-like inhibitory protein and microRNA 708-5P gene expression

Step	Temperature (°C)	Time (s)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	60	15	
Extension	72	20	
Dissociation	55°C–95°C		1

Results

The fold expression of c-FLIP in newly diagnosed patients and patients receiving chemotherapy is upregulated compared to the controls (3.291, 2.92, and 1.00), respectively, and the expression of *miRNA708-5p* in a newly diagnosed patient is more significant than under chemotherapy patient and control (5.345, 0.789, and 1.00), respectively, as shown in Table 5.

The c-FLIP expression levels showed highly significant differences among the patients (newly diagnosed) and under chemotherapy compared to the control in the current study [Figure 1].

The *miRNA708-5p* expression levels manifested highly significant differences among the patients (newly diagnosed) compared to the under chemotherapy and control in the current study [Figure 2].

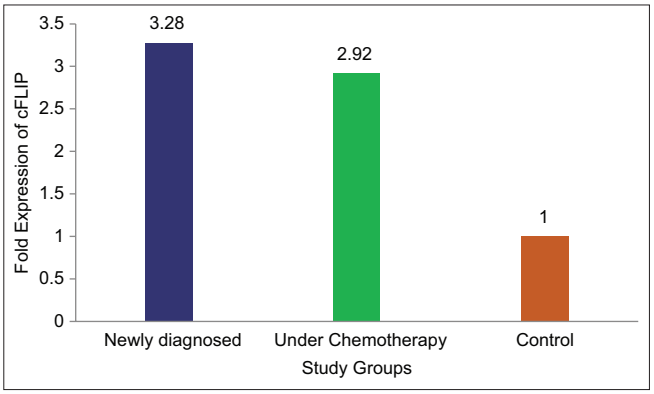

Figure 1: Fold of c-FLIP expression depending on the $2^{-\Delta\Delta Ct}$ method

Table 5: The fold expression of cellular-FLICE-like inhibitory protein and microRNA 708-5p according to the Δct calibrator method

Group	Mean target gene	Mean of reference gene	Δct	Δct calibrator	ΔΔct	2^-ΔΔct	Fold
c-FLIP gene							
Newly diagnosed	13.404	21.324	−7.92	−6.9306	−0.9894	1.985	3.291
Under chemotherapy	13.5583	21.309	−7.7507	−6.9306	−0.8201	1.765	2.92
Control	14.9328	21.135	−6.2022	−6.9306	0.7284	0.603	1.00
P	–	–	0.0001	0.0001	0.0001	0.0001	0.0001
miRNA 708-5p gene							
Newly diagnosed	15.403	11.05	4.353	7.737	−3.384	10.440	5.345
Under chemotherapy	18.193	11.081	7.112	7.737	−0.625	1.542	0.789
Control	17.864	11.0924	6.771	7.737	−0.965	1.953	1.00
P	–	–	0.0001	0.0001	0.0001	0.0001	0.0001

C-FLIPL=Cellular-FLICE-like inhibitory protein, miRNA=MicroRNA

The receiver operating characteristic curve

The analysis ROC [Table 6] can distinguish between (2) states of the patient, characteristically denoted as "diseased" as well as "nondiseased," so it may be utilized in clinical epidemiology for quantifying how accurately the medical diagnostic tests are.[19] Furthermore, the area under the curve (AUC) is an active and joined specificity and sensitivity measure that designates the diagnostic tests' intrinsic validity, and the x-axis or the self-governing parameter is the untrue optimistic rate for the prognostic test.[20]

The results in Table 6 indicated that the cutoff value between sensitivity (90%) and specificity (90%) of c-FLIP expression was (1.97). Moreover, the outcomes elucidated that the area below the c-FLIP expression curve was (0.953), as well as the difference was significant $P \leq 0.05$. When the value of the (AUC = 0.0001), the results demonstrated that the cutoff value between the sensitivity (66%) and specificity (92%) of miRNA708-5P expression was (5.30). Furthermore, the outcomes portrayed that the area below the miRNA708-5P expression curve was (0.843), and the significant difference was $P \leq 0.05$, if the value of the (AUC = 0.001). Figure 3 illustrate the ROC for cFLIP and *miRNA-708-5P*.[4]

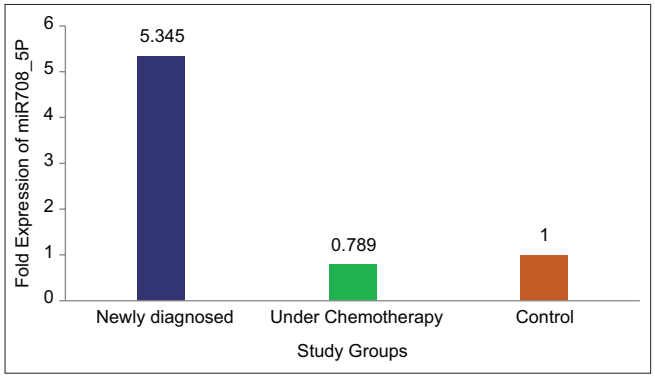

Figure 2: Fold of microRNA708-5p expression depending on the $2^{-\Delta\Delta Ct}$ method

Correlations between *c-FLIP, microRNA708-5P* expression, and hematological markers

This finding indicated a strong association between the *miRNA708-5P* expressions with the hemoglobin and also showed that there was a moderate negative correlation ($r = -0.499$) with a high significant ($P = 0.0001$), whereas a weak negative correlation with nonsignificant ($P = 0.2$) for the white blood cell was and no effect on age with ($P = 0.1$). Furthermore, the high significant ($P = 0.0001$) between the platelets and the hemoglobin indicated a positive correlation with a correlation coefficient (0.596), as depicted in Table 7.

Such findings indicated an inverse association between age and white blood cells, and a strong direct association between hemoglobin, and platelets, but a strong inverse relationship between hemoglobin and c-FLIP, miRNA708-5p, and there is an inverse correlation between platelets and c-FLIP, miRNA708-5p. On the other hand, there is a moderate direct association between *c-FLIP* and *miRNA708-5p*, with a correlation coefficient factor (0.544**) with a highly significant *P* value as illustrated in Table 7.

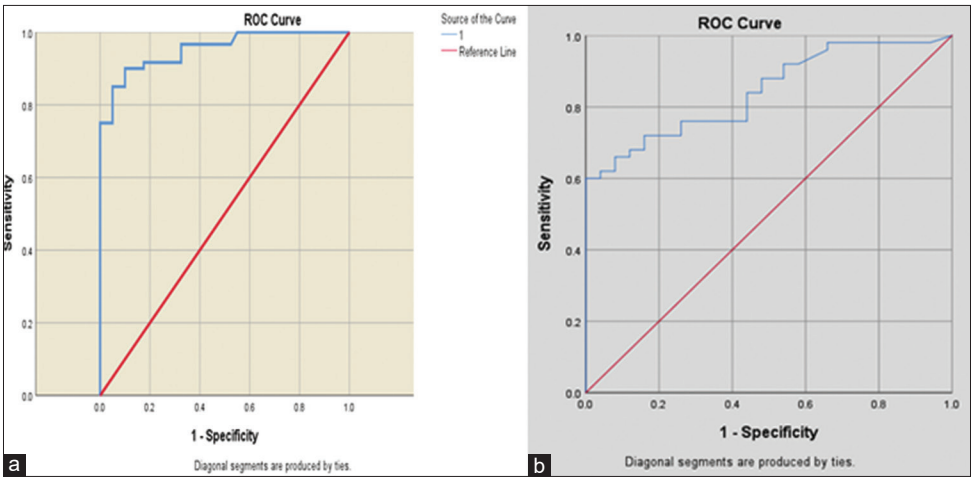

Figure 3: (a) c-FLIP receiver operating characteristic (ROC) curve, (b) microRNA708-5P ROC curve. ROC: Receiver operating characteristic

Table 6: The analysis of receiver operating characteristic curve for cellular-FLICE-like inhibitory protein and microRNA 708-5P expression

Parameters	AUC	Explanation	*P*	The best cutoff	Sensitivity (%)	Specificity (%)
c-FLIP	0.953	Excellent	0.0001	1.97	90	90
miRNA 708-5P	0.843	Good	0.001	5.30	66	92

C-FLIPL=Cellular-FLICE-like inhibitory protein, miRNA=MicroRNA, AUC=Area under the curve

Table 7: Correlations between cellular-FLICE-like inhibitory protein, microRNA 708-5P, and clinical marker hematology

Correlation	Age	Hemoglobin (g/dL)	WBC (×10⁹/L)	Platelets (×10⁹/L)	cFLIP	miRNA 708_5p
Age		0.021	−0.218*	0.028	0.175	0.145
		0.8	0.02	0.7	0.08	0.1
Hemoglobin (g/dL)	0.021		−0.048	0.596**	−0.427**	−0.499**
	0.88		0.6	0.0001	0.0001	0.0001
WBC (×10⁹/L)	−0.218*	−0.048		−0.149	0.062	−0.123
	0.02	0.6		0.139	0.5	0.2
Platelets (×10⁹/L)	0.028	0.596**	−0.149		−0.414**	−0.234*
	0.7	0.0001	0.1		0.0001	0.01
cFLIP	0.175	−0.427**	0.062	−0.414**		0.544**
	0.08	0.0001	0.5	0.0001		0.0001
miRNA 708_5p	0.145	−0.499**	−0.123	−0.234*	0.544**	
	0.1	0.0001	0.2	0.01	0.0001	

*The correlation is significant at (0.05) level (two-tailed), **The correlation is significant at (0.01) level (two-tailed). C-FLIPL=Cellular-FLICE-like inhibitory protein, miRNA=MicroRNA, WBC=White blood cell

Discussion

In this study, there was overexpression of the c-FLIP gene among newly diagnosed individuals. A relative downregulation in c-FLIP gene expression was found in patients receiving the treatment, indicating a relative improvement after administration. Treatment: C-FLIP expression varies significantly between newly diagnosed patients and those undergoing chemotherapy, making it a critical marker for evaluating patients' condition. The current study is consistent with another study conducted for patients with renal cancer. Kim *et al*.[21] have found that the c-FLIP gene expression is overexpressed in renal cancer. Moreover, the current study is consistent with other studies. McLornan *et al*.[22] indicated that high c-FLIP expression is associated with lower overall survival in adult AML patients, indicating a potential prognostic relevance in AML.

c-FLIP is a key regulator of apoptosis and is aberrantly expressed in many cancers. It also regulates other cell death pathways, such as autophagy and necroptosis. Therefore, c-FLIP may be a promising target for cancer therapy.[23]

This statement is supported by the results that appeared, which are an increase in c-FLIP gene expression in AML.

This study found that newly diagnosed AML patients had significantly greater miRNA708-5p expression levels than healthy controls, suggesting that these genes may play a role in disease diagnosis in AML. These data were in line with Trino *et al*.[24] The potential of miRNA708-5p as biomarkers for diagnosing and prognosing AML, as well as their promise as therapeutic targets, is highlighted in the paper.

The *miRNA-708-5p* emphasizes its developing actions as a miRNA included in multiple oncogenesis features. miR-708-5p is an oncogenic or a tumor exploitive in different kinds of tumors, either solid as well as hematological. The translational effort manifests that the miR-708-5p has prospective applications as a biomarker, a therapeutic goal, or a therapeutic in the clinic. After the cell of cancer, investigations are illuminating an innovative purpose for the miR-708-5p in the excepted responses.[25]

This statement is supported by the results that appeared, which are an increase in miRNA708-5p gene expression in AML, which could be a biomarker.

In this study, we discovered that the high miRNA708-5p gene expression was associated with high c-FLIP gene expression and poorer overall survival in AML patients, indicating its potential as a prognostic biomarker.

Conclusion

The c-FLIP and miRNA708-5p gene might be used as a biomarker for the AML initial diagnosis. The research highlighted the role of miRNA 708-5p as a tumor suppressor that negatively regulates the antiapoptotic protein c-FLIP, suggesting its potential as a therapeutic target in AML treatment. By modulating the levels of miRNA 708-5p, it may be possible to regulate the expression of c-FLIP, thus enhancing the effectiveness of apoptosis-inducing therapies. This highlights the potential for developing miRNA-based therapies as part of AML treatment regimens. miRNA 708-5p can serve as a prognostic indicator in AML. Its expression levels can provide insights into the progression of the disease and the patient's response to treatment.

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Conflicts of interest

There are no conflicts of interest.

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