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Investigation of ALL Immunophenotyping and *MEG 3* Gene Expression Related to the Risk of ALL in Sample of Iraqi Patients

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Abstract

Background. Acute lymphoblastic leukemia (ALL) is a childhood malignancy caused by lymphoblast accumulation in the bone marrow. *Long noncoding RNA maternally expressed gene 3 (MEG3)*, is gradually realized as a tumor suppressor in hematological malignancies. **Aim**. The study aimed to investigate the type of ALL (Immunophenotyping) and *MEG3* gene expression related to the risk of ALL. **Methods**. At the Central Teaching Hospital of Pediatric in Baghdad, blood samples were collected from 50 patients comprised 25 boys and 25 girls, the most common age group was 6-10 years and 50 healthy children as control group. Immunophenotyping was done by flow cytometry (FCM), while *MEG 3* gene expression was assessed by Real-time Polymerase Chain Reaction (RT-PCR). **Results**. The result of estimation WBCs count, Hb, platelet count for ALL patients was revealed that WBCs count, Hb, platelet count level (27.99 ±6.93 x 10^9/ L,7.85 ±0.34 g/dl and 92.90 ±14.80 x 109/ L) respectively in patients compared with control (6.66 ±0.18 x 10^9/L, 12.76 ±0.15 g/dl and 307.42 ±14.19 x 10^9/ L) respectively at high significant difference (P≤0.01). The most prevalent subtype of ALL was B-ALL. The estimation of *MEG3* gene expression showed significant lower in patient' s group at the time of diagnosis with ALL in comparison with healthy control and patients' group during treatment which consider the higher expression between other groups with high significant difference (P≤0.01). **Conclusion**. B-ALL is more common than T-ALL in children and gene expression of *MEG3* gene is downregulate at the time of diagnosis then upregulate after chemotherapy within few days.

Keywords: Immunophenotyping, acute lymphoblastic leukemia, gene expression, long non coding RNA, *MEG3* gene.

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Introduction

A class of diseases known as leukemia is defined by an overabundance of cancerous white blood cells in the blood and bone marrow. These abnormal cells infiltrate organs (such as the liver, spleen, lymph nodes, meninges, brain, skin, or testes) and produce bone marrow failure symptoms (such as anemia, neutropenia, and thrombocytopenia) (1). The cancer of B or T lymphoblasts known as ALL is distinguished by an uncontrolled proliferation of aberrant, immature lymphocytes and their progenitors. This ultimately results in the replacement of bone marrow components and other lymphoid organs, giving rise to the characteristic disease pattern of acute lymphocytic leukemia (2). Genetic and epigenetic alterations in patients can lead to treatment resistance in malignant diseases, such as leukemia. Mutations in the molecular

target of the treatment, significant cellular alterations, modifications in the drug's interaction with the tumor, abnormalities in the tumor microenvironment, and cancer cell remodelling. Among the molecular alterations that lead to innate or acquired treatment resistance are epigenomic landscapes (3-5). Although the exact cause and pathophysiology of childhood ALL are unknown, it is thought to result from a combination of genetic variations and environmental/lifestyle factors. However, there is growing evidence in recent years that suggests single nucleotide polymorphisms (SNPs) may be a significant factor in determining a child's risk for developing childhood ALL (6). However, it is unknown how many genetic variables play a role, and more research into the complicated genomic understanding of children ALL is desperately needed (7). Different clusters of differentiation (CD) biomarkers, such as CD10, CD19, CD22, CD79a, CD99, terminal deoxynucleotidyl transferase (TdT), and human leukocyte antigen DR (HLA-DR) isotype in B-cell lineages, were found to be highly expressed between the two subtypes of ALL. In contrast, CD2, CD3, CD5, CD7, CD13, CD117, and TdT are more specific to T-cell lineages (8). Long non-coding RNAs (lncRNAs) is RNAs that do not code for proteins and are longer than 200 nucleotides in length; lncRNAs has a various biological function including the regulation of gene expression, regulation of cell differentiation and development (9). *Long noncoding RNA maternally expressed gene 3 (MEG3),* located on chromosome 14q32.3 DLK1 locus, is gradually realized as a tumor suppressor in hematological malignancies, *MEG3* encodes a myelocyte-related lncRNA and is believed to be involved in the process of carcinogenesis and responsiveness to chemotherapy (10). *MEG3* is considered a tumor suppressor. Accumulating evidence has suggested that genetic variants in the *MEG3* gene predispose to cancer. However, the impacts of *MEG3* polymorphisms in childhood ALL remain unclear (11). The study aimed to investigate the type of ALL (Immunophenotyping to B-ALL or T-ALL) and *MEG3* gene expression related to the risk of ALL.

Materials and Methods

This is a case-control study. The current study was carried out in the laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies through the period from beginning December 2023 to the end of June 2024, At the Central Teaching Hospital of Pediatric in Baghdad, blood samples were collected from 50 patients with ALL and 50 healthy with similar age and sex (aged from 1 to 14, 25 female and 25 male). Five mL of blood was drawn from everyone who participated in this study. The blood sample was placed in: Two ml (2000 µl) of blood was put in an EDTA anticoagulant tube with a gentle mix for the complete blood count (CBC) for all study groups and then kept for the immunotyping by flow cytometry to investigate the type of ALL (for patients only). Amount 0.1 ml (100 µl) of blood directly placed in Trizol preservation (300 µl trizol) for RNA extraction and maintained at -20° C until employed for the investigation.

Inclusion/Exclusion criteria

Fifty patients (male and female) suffered from ALL with age ranged between 1 and 14 years, collected from hematology unit in Central Teaching Hospital of Pediatric in Baghdad, Samples taken at the time of diagnosis and after induction chemotherapy. Permission was taken from all Participants after they were told about the aim and advantages of this study. The diagnosis of cases was based on the clinical examination of consultant physicians in the hematology

unit of Central Teaching Hospital of Pediatric, Confirmative diagnosis and classification were performed by the hematologists at the same hospital according to the findings of peripheral blood, and bone marrow study (aspirate and biopsies). The demographic data information and clinical manifestation were collected from the medical records for each patient and clinical laboratory assessments were all performed. The exclusion criteria were the adult (more than 14 years), patients with other types of blood cancers such as hemophilia, hereditary blood diseases thalassemia, and Mediterranean pelvic anemia.

Hematological study Complete Blood Count

A fully automated hematology analyzer Sysmex XN was used to count the total white blood cells (WBC) for leukopenia, platelets for thrombocytopenia, and hemoglobin (HB) levels for anemia. Normal values of WBCs, HB and Platelets show in (Table 1) (12).

Table (1) Show Normal Values of WBCs, HB and Platelets.

Age / Parameter	1 year	2-6 years	6-12 years	12-14 years
WBC (x109/l)	11±5	10±5	9±4	4-10
Hb (g/dl)	12.6±1.5	12.5±1.5	13.5±2.0	Male (15±2.0) Female (13.5±1.5)
PLT(x109/l)	200-550	200-490	170-450	280 ± 130

Immunophenotyping by Flow Cytometry

Whole human blood sample lysed by Eight-color immunophenotyping panel using V450, V500, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Phycoerythrin-cyanine 5.5 (PE-Cy5.5), phycoerythrin-Cyanine7(PE-Cy7) Allophycocyanin (APC) and Allophycocyanin-H7(APC-H7) by ACSCanto II flow cytometer (BD Biosciences, USA). The device showed positive markers and this give specific type of ALL either B-ALL or T-ALL as showed in (Table 2) (13).

Table (2) The cluster of Differentiation CD Marker for Acute Lymphoblastic Leukemia in immunophenotyping by flow cytometry

marker in acute lymphoblastic leukemia	
Panel Type of ALL	**Positive Marker**
First line screening	CD34, TdT, HLA-DR, MPO, CD19, cCD79a, CD22, cCD3
Second line B-ALL	CD10, CD20, CD58, CD38,
Second line T-ALL	CD1a, CD2, cCD3, CD4, CD5, CD7, CD8, TCR

ALL: Acute Lymphoblastic Leukemia, CD**:** Clusters of differentiation, **c:** cytoplasmic, **a:** alpha, **TdT:** Terminal deoxynucleotidyl Transferase, **HLA-DR:** human leukocyte antigen DR isotype, **MPO**: myeloperoxidase, **TCR**: T Cell antigen Receptor.

MEG3 gene Expression:

To determine MEG3 gene expression by real-time PCR using the TransZol Up Plus RNA Kit (Transgen, China), 100 µl of blood was immediately put in 300 µl of Trizol preservation for RNA extraction and kept at -20° C. Nucleic acids concentration and purity was estimated via Nanodrop (Bioneer/ Korea). *MEG 3 gene* expression quantified employing a 2-steps qRT- PCR approach. In the 1st step, the conversion of RNA into complementary DNA (cDNA) utilizing the Add Script Reverse Transcriptase kit (Addbio/ Korea). according to a program consist of Priming (25 °C /11 min), Reverse transcription (42°C /35 min), RT inactivation (85 °C/ 5 sec) as shown in (Table 3).

Table (3): program of PCR for cDNA conversion.

Step	Temperature (°C)	Time (min)
Priming	25	00:11:00
Reverse transcription	42	00:35:00
RT inactivation	85	00:00:05

Afterwards, in the 2nd step, the qRT-PCR performed employing Primers with their sequences which used in this study were supplied by (Macrogen/ Korea) and design by used (Primer-BLAST on NCBI) are given in the Table (4).

Table (4): Primers of *MEG3* and *GAPDH* Reference Gene

primer	Sequence	primer sequence 5'- - 3'	Tm (°C)	GC%	size	Primer design
MEG3	F	TACACCTCACGAGGGCACTA	62.6	55	200bp	This study
	R	GCATAGCAAAGGTCAGGGCT	61.9	55		
GADHP	F	CACTAGGCGCTCACTGTTCTC	62	57	98bp	(YM,2023)
	R	AATCCGTTGACTCCGACCTT	61.4	50		

MEG3: ***maternally expressed gene 3*** ; ***GAPDH***: ***glyceraldehyde-3-phosphate dehydrogenase***

The Real-time PCR reaction was performed via SaCycler-96 Real Time PCR SYSTEM (Sacace/Italy) which programmed with thermo cycling protocol as following: 1 cycle of Initial Denaturation (94°C), 45 cycles of Denaturation (94°C) and Annealing (60° C), while the melting curve temperature was (90°C) as shown in (Table 5).

Table (5): Real-Time PCR Thermal Program for Gene Expression.

Step	°C	Time	Cycle
Initial Denaturation	94	30 sec.	1
Denaturation	94	5 sec.	45
Annealing	60	35 sec.	
Melting Stage	90	15 sec.	100

Statistical Analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of difference groups (patients and control) in study parameters. T-test and least significant difference (LSD) was used to significant compare between means. P-value was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of correlation coefficient between variables in this study.

Results

Based on the findings, 50 patients (newly diagnosed and then follow up) comprised 25 boys and 25 girls. The results of distribution of ALL patients showed that the group age (6-10) years had the greatest frequency in the research sample 25 (50%), followed by 14 (28%) at (0-5) years age group, while the age group of (11-14) years had lowers percent 11 (22%) with high significant difference ($P \leq 0.01$) as illustrated in table (6). The result of estimation WBCs count, Hb, platelet count for ALL patients was revealed that WBCs count, Hb, platelet count level (27.99 ±6.93 $x10^9$/l ,7.85 ±0.34 g/dl and 92.90 ±14.80 x 10^9/l) respectively in patients compared with control (6.66 ±0.18 x 10^9/l, 12.76 ±0.15 g/dl and 307.42 ±14.19 x 10^9/ l) respectively at high significant difference ($P \leq 0.01$). Which considered anemic status for Hb, Leukocytosis condition for WBC and thrombocytopenia situation for platelet in patients as showed in table (7).

Table (6): Distribution of ALL Patients According to Age

Age group (years)	Patients No. (%)	(P-value)
0-5	**14 (28%) b**	**0.0014 ****
6-10	**25 (50%) a**	
11-14	**11 (22%) c**	

Different letters in same column mean different significantly

Table (7): Comparison between Patients and Control Groups in WBC, HGB and PLT.

Group	Mean ±SE		
	WBC (10^9/L)	**HGB (g/dl)**	**PLT (10^9/L)**
Patients	27.99 ±6.93	7.85 ±0.34	92.90 ±14.80
Control	6.66 ±0.18	12.76 ±0.15	307.42 ±14.19
T-test	13.767 **	0.739 **	40.698 **
P-value	0.0027	0.0001	0.0001

The B-ALL was the most prevalent ALL subtype (74%) while T-ALL was (26%) as listed in table (8). clusters of differentiation (CD markers), including CD10, CD19, CD22, CD79a, CD99, TdT, and HLA-DR isotype, are highly expressed in B-cell lineages, while CD2, CD3, CD5, CD7, CD13, CD117, and TdT are more specific to T-cell lineages. The frequency of markers illustrated in table (9).

Table (8): Immunotyping for Patients According to Markers of ALL

Type of ALL	NO.	Percentage (%)
B-ALL	37	74.00
T-ALL	13	26.00
Total	50	100%
P-value	---	0.0007 **

Table (9): The Frequency of CD Markers used in flow cytometry in 50 Patients of Acute Lymphoblastic Leukemia

Markers used in flow cytometry	Positive markers			P -value
	B-ALL (n=37), n (%)	T-ALL (n=13), n (%)	Total (NO=50), n (%)	
cCD79a	37(100%)	4(30.7%)	41(82%)	0.0001 **
CD10	35(94.5%)	5(38.4%)	40(80%)	0.0001 **
CD19	37(100%)	0	37(74%)	0.0001 **
CD34	22(59.4%)	6(46.1%)	28(56%)	0.0009 **
CD38	26(70.2%)	0	26(52%)	0.0001 **
CD58	24(64.8%)	0	24(48%)	0.0001 **
cTdT	18(48.6%)	3(23%)	21(42%)	0.0001 **
CD20	18(48.6%)	0	18(36%)	0.0001 **
CD22	15(40.5%)	0	15(30%)	0.0001 **
cCD3	0	13(100%)	13(26%)	0.0001 **
CD7	0	13(100%)	13(26%)	0.0001 **
CD33	8(21.6%)	3(23%)	11(22%)	0.105
CD13	5(13.5%)	4(30.7%)	9(18%)	0.307
CD66c	9(24.3%)	0	9(18%)	0.0001 **
CD99	0	9(69.2)	9(18%)	0.0001 **
CD8	0	6(46.1%)	6(12%)	0.0296 *
CD5	0	5(38.4%)	5(10%)	0.0375 *
CD1a	0	4(30.7%)	4(8%)	0.0497 *
CD117	0	4(30.7%)	4(8%)	0.0497 *
CD45	2(5.4%)	1(7.6%)	3(6%)	0.603
CD2	0	3(23%)	3(6%)	0.071
CD4	0	3(23%)	3(6%)	0.071
CD9	2(5.4%)	0	2(4%)	0.284
HLA-DR	1(2.7%)	1(7.6%)	2(4%)	0.776
CD11b	1(2.7%)	0	1(2%)	0.779
CD36	1(2.7%)	0	1(2%)	0.779

* (P<0.05), ** (P<0.01) ; **n:** Number of each type, **ALL:** Acute Lymphoblastic Leukemia**, CD:** Clusters of Differentiation**, c:** cytoplasmic**, a:** alpha, **b**: beta, **TdT:** Terminal deoxynucleotidyl Transferase**, HLA-DR:** Human Leukocyte Antigen DR isotype**.**

The estimation of MEG3 gene expression showed significant lower in patient' s group at the time of diagnosis with ALL (0.66 ±0.12 fold) in comparison with healthy control (1.22 ±0.10 fold) and patients' group during treatment (after induction chemotherapy) was (4.29±0.31 fold) which consider the higher expression between other group as showed in table (10) and figures (1-4).

Table (10): Fold Change of *MEG3* Gene Expression in Patient and Control Groups

Group	*MEG3*	*GAPDH*	ΔCT	ΔΔCT	2^ΔΔCT	Fold change
Patients (at the time of diagnosis)	30.356	18.688	11.668	1.168	0.6620	0.66 ±0.12 c
Patients (after chemotherapy induction)	26.712	18.208	8.504	-1.996	4.2909	4.29±0.31 a
Control	28.288	17.788	10.50	0.00	1.2246	1.22 ±0.10 b
L.S.D. (P-value)	--	--	--	--	--	0.557 ** (0.0001)

** (P≤0.01).

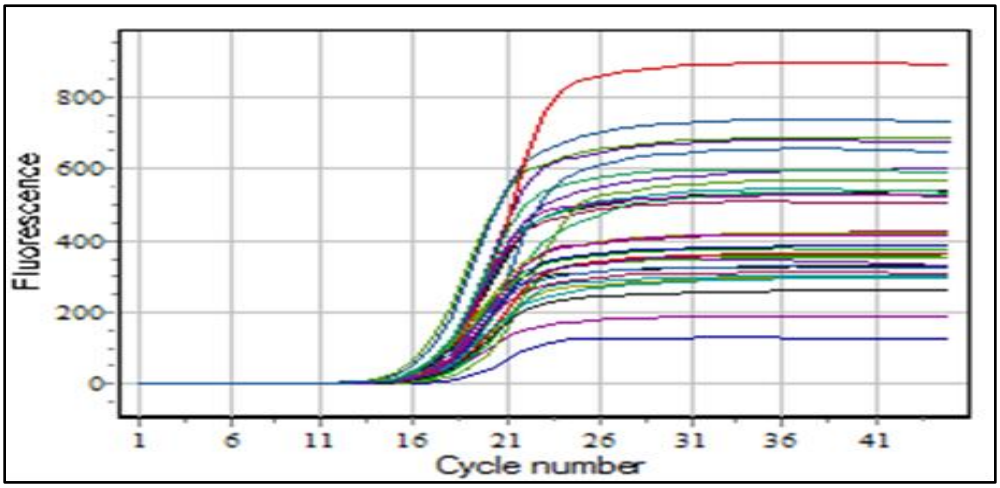

Figure (1): Gene Expression Curve for Reference Gene in Control and Patient.

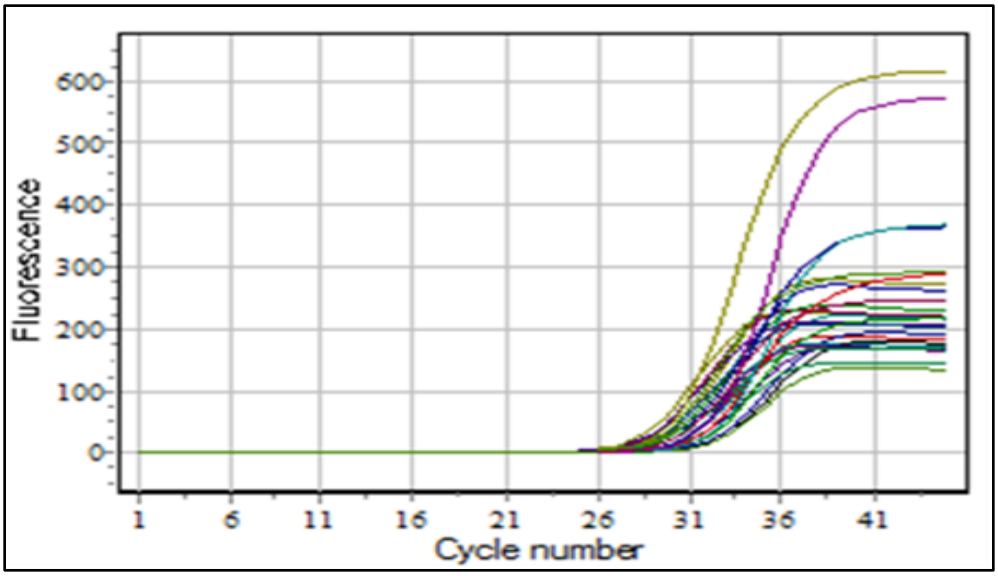

Figure (2): Gene Expression Curve for MEG3 Gene in Patients (at the time of diagnosis).

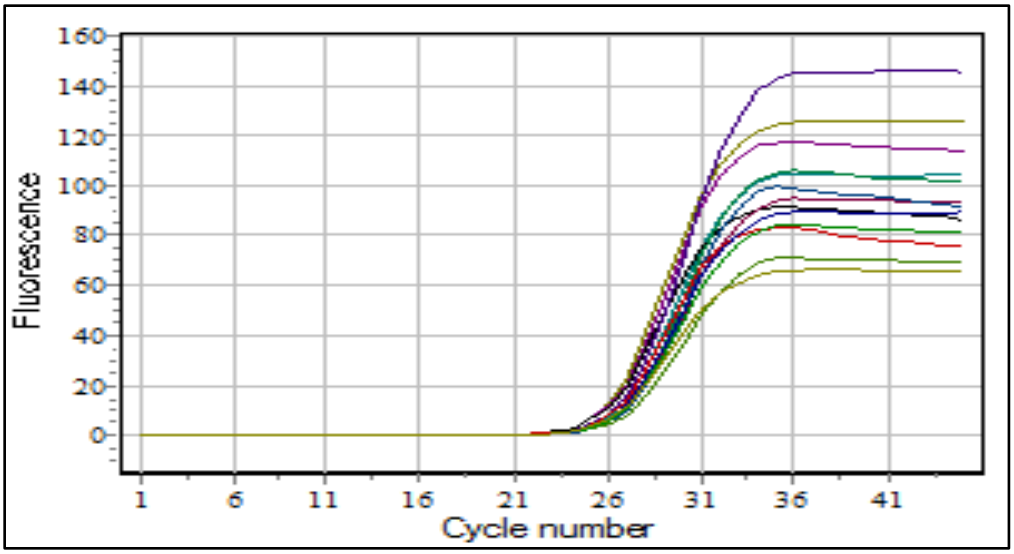

Figure (3): Gene Expression Curve for MEG3 Gene in Patients (after chemotherapy induction).

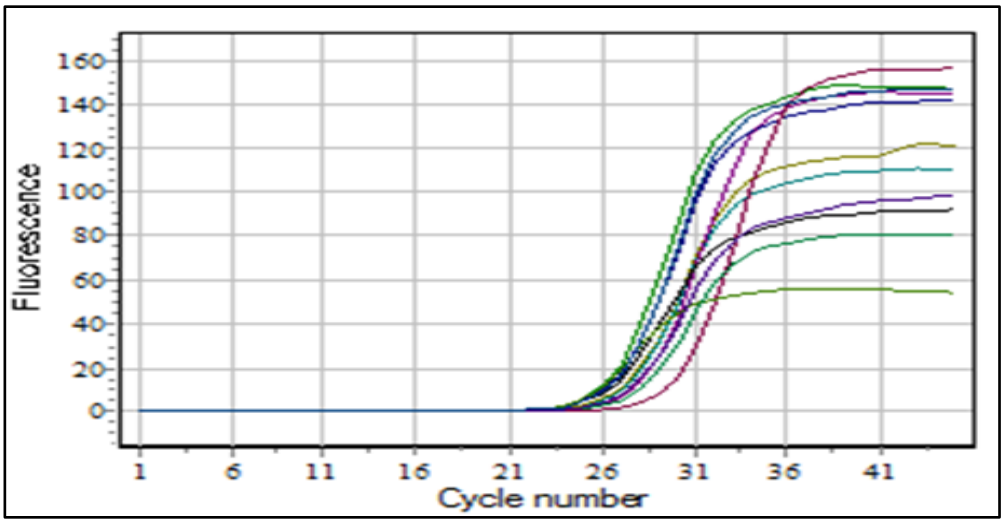

Figure (4): Gene Expression Curve for MEG3 Gene in Control.

Discussion

Studies evaluating the impact of sex on ALL are rare (14). Many Reports find in different locations where boys are more likely than girls to get leukemia in childhood (15-18). Leukemia is the most common type of cancer in children aged (0–14) (19). The previous age distribution was in agreement with the study of Ghanavat *et al*.,2024 showed that the most common age group was 2–10 years (71.4%). the study of Ahmed and Ahmed (2022) in Iraq found that most of the cases (75%) were between the ages of (1 -10) and only 25% were older than 10 years old. There is clear evidence of leukemia-related anemia, with a considerable decrease in hemoglobin in particular age ranges and a decrease in platelet counts in all age group. Similar to the Iraqi study of Ahmed and Ahmed's (2022), demonstrated that the average Hb concentration in was (7.93±2.2 g/dl) as well as anemia affected 90% of the patients. Another study (22) discovered that the average haemoglobin level was (7.41 ± 2.57 g/dL).

A person with leukemia may have less than 150,000 platelets in their blood. The result observed decrease in platelet count in

patients compared to controls and this is compatible with a study by (23). The result of WBC count matched with many other studies that highlighted the importance of initial WBC count as a predicted indicator for determining risk measures in ALL (24-25). The result matching study carried out in 2022 indicated that T-ALL had been detected in less cases (4.7%) than B-ALL (26). another study showed B-cell lymphoblastic leukemia accounted for 80.43% and T-cell was 19.57%. (27). Downregulation of *MEG3* may contribute to leukemogenesis by decreasing the activity of the P53 tumour suppressor gene, overexpression results in apoptosis and inhibition of proliferation. This polyadenylated lncRNA is downregulated in cancer cells but overexpressed in human pituitaries. The result was similar to the first investigation of *MEG3* gene expression in ALL patient before and after treatment (Gao, 2021) which hypothesized that *lnc-MEG3* might play a critical role in ALL patients. However, no relevant research has been conducted. They found that *lnc-MEG3* expression was decreased in ALL patients and its expression before treatment was correlated with good treatment response in ALL. Additionally, after induction therapy, *MEG3* expression was found to be elevated, and this increase was linked to a greater response to treatment in ALL patients (28). These conclusions are based on lnc-*MEG3*, a frequently investigated lncRNA that has been linked to a number of haematological diseases (29-30). For example, both acute and chronic myeloid leukemia cell lines have decreased *lnc-MEG3* expression. Furthermore, in terms of the clinical aspect, AML patients reveal lower levels of lnc-*MEG3* expression in comparison to controls (30).

Conclusion

Based on the results, it can be concluded that the group age (6-10) years are more susceptible to the ALL disease than other age groups. Male and female are equal in their development of ALL. The B-ALL are more prevalent than T-ALL and the most positive CD markers are CD79a, CD10 and CD19. The most prevalent hematological signs in patients are anemia and thrombocytopenia. The MEG3 expression decreased at the time of diagnosis and then increase during induction therapy is correlated with good treatment response.

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