Role of Long Non Coding RNA H19 Gene Expression in Acute Lymphoblastic Leukemia: A Molecular Analysis

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Abstract:

Background: The malignant clonal development of lymphoid hematopoietic progenitors is a characteristic of acute lymphoblastic leukemia (ALL), a kind of hematological malignancy. Among the long non-coding RNAs (lncRNAs) is the H19 gene. Numerous physiological and pathological processes are significantly impacted by it.

Objective: The current study's objective was to use real-time PCR to measure the expression level of the H19 gene in ALL patients and to its relationship to the patients' clinical presentation and laboratory results.

Methods: This study was performed on 49 newly diagnosed ALL patients (39 B-ALL patients versus 10 T-ALL patients). Twenty subjects, age and sex matched, were included in the study as a control group. Real-time PCR was used to detect the expression level of H19.

Results: our study showed that H19 gene expression was significantly up regulated in ALL patients than in control group (P = 0.001). Statistically significant positive correlations among H19 expression level with WBCs count, percentage of peripheral blood blasts and also bone marrow blasts in B-ALL and T-ALL. Also, a statistical significance was found between H19 expression and 11q23 (MLL) gene rearrangement in B-ALL patients (P = 0.015).

Conclusion: H19 gene expression was higher in studied ALL patients' group compared with the control group. The higher WBCs count, percentage of PB blasts and BM blasts, the higher H19 expression level. So, further studies are recommended on a larger sample size to demonstrate the role of anti-H19 as a target therapy for ALL patients.

Keywords: acute lymphoblastic leukemia, H19 gene expression, real time PCR

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Introduction:

Acute lymphoblastic leukemia (ALL) is a haematological malignancy where lymphoid blasts show arrested maturation and differentiation. As a result, early lymphoid progenitors accumulate in the bone marrow, peripheral circulation, and extramedullary locations, disrupting hematopoiesis and causing extramedullary infiltrations [1]. It is the most prevalent pediatric cancer, accounting for 75%-80% of pediatric acute leukemias and 20% of adult acute leukemias, with the median age at diagnosis being 15 years old [2].

Upon its discovery, H19 became the first long non-coding RNA (lncRNA) to be submitted for genomic imprinting [3]. It is found on chromosome 11p15.5 and has four introns and five exons with 2.5 kb long [4]. The H19 gene is located inside the highly conserved imprinted H19/insulin-like growth factor 2 (IGF2) locus. Differential allelic expression of H19 from the maternal allele and IGF2 from the paternal allele is the result of these two genes' reciprocal imprinting [5].

H19 gene is involved in both embryogenesis and carcinogenesis. Additionally, it is essential for enhancing cell proliferation, differentiation, migration, invasion, and chemoresistance [6,7]. It has been demonstrated that H19 is an oncogene that is over-expressed in various types of cancers such as breast, liver, endometrial, lung, cervical, and esophageal cancers [6]. A similar pattern of H19 expression was observed in different types of leukemias, including chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) [8,9].

The aim of our study was to assess the expression level of H19 gene in the samples from ALL patients by real time PCR. In addition, to detect the relation between the expression level of H19 gene with the clinical presentation and laboratory data of these patients.

Patients and Methods:

The current study is a prospective study carried out in Clinical Pathology Department, South Egypt Cancer Institute, Assiut University in the period from July 2023 to October 2024. This study was performed on 49 of newly diagnosed ALL patients (group 1) based on 2022 WHO classification, 39 B-ALL patients (group 1a) versus 10 T-ALL patients (group 1b). Twenty subjects, age and sex matched were included in the study as a control group (group 2).

Sampling and routine investigations

All patients were subjected to complete clinical examination, complete blood count with examination of peripheral blood smears stained with Leishman stain for morphological identification of leucocytic differential count and detection of circulating blast cells, and bone marrow examination.

Immunophenotyping was done using multicolor flow cytometry (FACS Calibur, BD Biosciences-San Jose, CA, USA: E5140). Cytogenetic studies were performed using Zeiss Axio Imager Z2, fluorescent microscope (Germany: 3534001297) and Zeiss Axioscope2 mot plus, fluorescent microscope (Germany: 3305000202).

Molecular testing for detection of H19 expression:

Two ml of bone marrow samples via bone marrow aspiration were collected into EDTA tubes from ALL patients, and 2 ml of peripheral blood samples were collected from apparently healthy individuals (control group) via direct venous puncture under complete aseptic conditions.

Following the manufacturer's instructions, total cellular RNA was extracted using the QIAamp RNA Blood Mini Kit (Germany, cat. No.52304). Utilizing a Qubit 3 fluorometer (Malaysia, serial number 2321606754), the amount of RNA in each sample was measured. After that, cDNA was synthesized using Thermofisher Scientific's RevertAid First Strand cDNA Synthesis kit.

Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X), ROX Solution (K0251), and Applied Biosystems 7500 Fast Real-Time PCR Systems (Thermo Fisher Scientific, USA, serial number 275016697) were used to detect H19 gene expression using real-time PCR.

The following primers were used:

H19 sense: 5′-TGTTTCTTTACTTCCTCCACGG-3′
H19 antisense: 5′- TTCCTCTAGCTTCACCTTCCAG-3′
β-actin sense: 5′- TGAAGATCAAGATCATTGCTCCTC-3′
β-actin antisense: 5′- AGTCATAGTCCGCCTAGAAGC-3′

The relative quantification values were computed using the 2–ΔΔct method. Specificity of the product was confirmed by running of melting curve analysis of post PCR reaction as illustrated in figures (1) and (2).

Statistical analysis

All statistical calculations were done using SPSS (statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 28. Quantitative data were statistically described in terms of mean ± SD, mean ± SE and median (range). Qualitative data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages) when appropriate. The Mann Whitney U test was used to compare quantitative variables for data that was not regularly distributed. Using the Chi square ($\chi 2$) test, categorical data were compared. Fisher When the estimated frequency was less than five, the exact test was utilized instead. Diagnostic accuracy of H19 expression was assessed by receiver operator characteristics (ROC) curve. Correlation between H19 expression and other variables was determined by Spearman coefficient correlation. Univariable and multivariable logistic regression were performed to determine the predictive factors of ALL. P value set significant at 0.05 level.

Results:

Demographic data of both groups as illustrated in table (1).

The age of the patients ranged from 1 to 72 years old. According to sex 25 (51%) of patients were males and 24 (49%) were females. There was no significant difference between patients and controls as regarding demographic data of the studied groups (age, and sex), P = 0.086 and 0.65, respectively.

Clinical presentations of the studied patients are presented in table (2).

Regarding the clinical presentation, there is no significant difference between B-ALL and T-ALL cases.

Comparison of laboratory data between both studied ALL groups is illustrated in table (3):

By comparing the laboratory data between the two patients' groups studied, there was no statistically significant difference between B-ALL and T-ALL regarding to hemoglobin level, WBCs count, platelets count, and percentage of peripheral blood blasts and bone marrow blasts.

H19 gene expression:

Diagnostic accuracy of H19 gene expression levels were assessed by ROC curve. According to the ROC curve analysis, 1.015 was determined as the optimal cut off value for H19 expression levels, with a sensitivity and specificity of 71.4% and 75%, respectively. H19 expression had 72.46% overall accuracy in the diagnosis of ALL, with the area under curve (AUC) was 0.75 and significant value (P = 0.001) as illustrated in table (4) and figure (3). Using this cutoff value, we divided the studied ALL patients into down regulated group; RQ (relative quantification) was less than 1.015 and up regulated group; RQ was more than 1.015 (14 and 35 cases, respectively).

The studied gene was significantly more up-regulated in ALL group than in the control group with P < 0.001 as presented in table (5).

Correlation of H19 gene expression with other variables of ALL patients:

By studying the correlation between the expression level of H19 gene, age of the patients, WBCs count, hemoglobin level, percentage of bone marrow and peripheral blood blasts, there was significant strong positive correlation between H19 gene expression and WBCs count of B-ALL ($r = 0.985$, $P < 0.001$) and T-ALL patients ($r = 0.976$, $P < 0.001$). Other significant strong positive correlations were observed between H19 gene expression and peripheral blood blasts ($r = 0.829$, $P < 0.001$ in B-ALL patients and $r = 0.798$, $P = 0.01$ in T-ALL patients) and between H19 gene expression and bone marrow blasts ($r = 0.964$, $P < 0.001$ in B-ALL patients and $r = 0.988$, $P < 0.001$ in T-ALL patients) as shown in table (6), figure (4), (5), (6), (7), (8) and (9).

Comparison of H19 gene expression and cytogenetic analyses in the studied patients:

Cytogenetic analyses revealed that 11q23 (KMT2A/MLL) rearrangement and H19 gene expression differed significantly in B-ALL patients ($P = 0.015$), as shown in table (7).

The predicted factors of ALL were identified through the use of both univariate and multivariate logistic regression. According to table (8), the univariable analysis showed a statistically significant correlation between H19 gene expression and ALL (OR = 1.807, 95% CI = 1.092 - 2.992, P =.021). Following the control of other confounding variables, only the H19 expression in the multivariable analysis retained its statistical significance as a single factor linked to ALL (OR=1.833, 95% CI= 1.099-3.059, P =0.02), as shown in table (9).

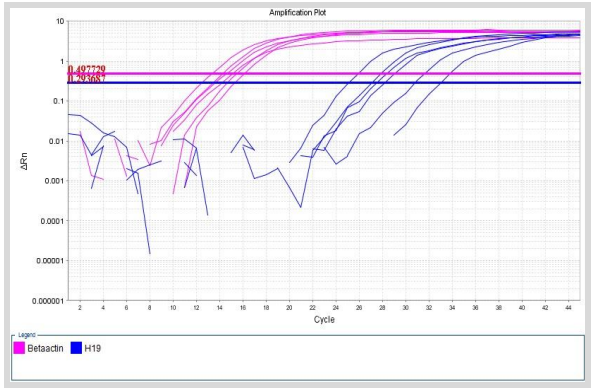

Figure 1: Amplication plot of *H19* and β-actin genes by Real time PCR

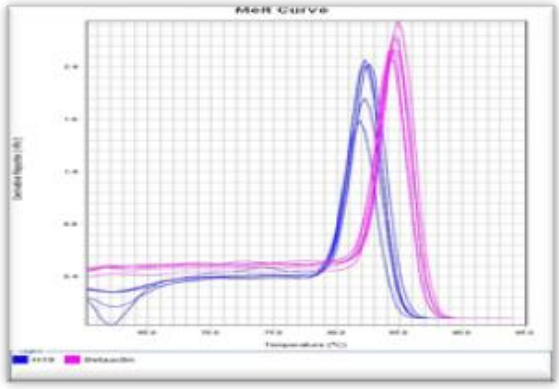

Figure 2: Melting curve of H19 and β-actin genes by Real time PCR

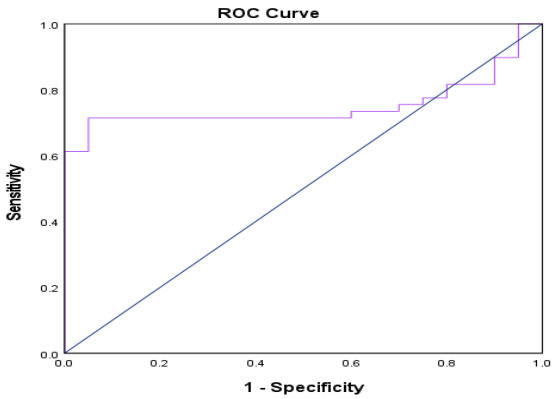

Figure 3: ROC curves for diagnosis of ALL cases. Area under the curve = 0.750, P value = 0.001 for *H19* gene expression.

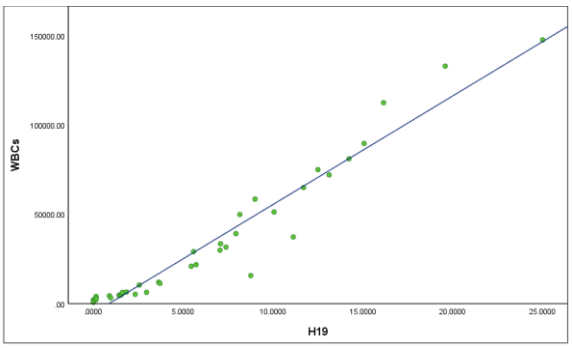

Figure 4: Scatter plot showing strong positive correlation between H19 expression and WBCs in B-ALL patients.

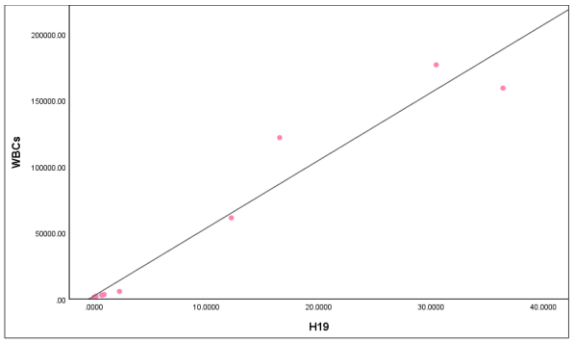

Figure 5: Scatter plot showing strong positive correlation between H19 expression and WBCs in T-ALL patients.

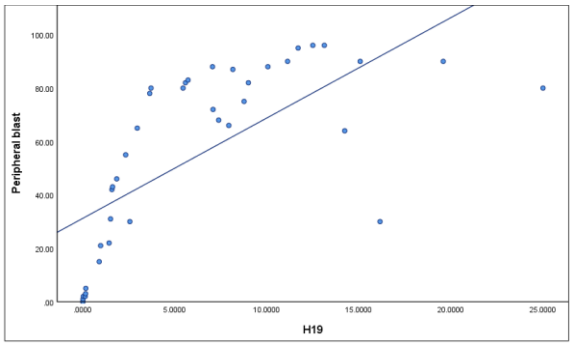

Figure 6: Scatter plot showing strong positive correlation between H19 expression and peripheral blood blasts in B-ALL patients.

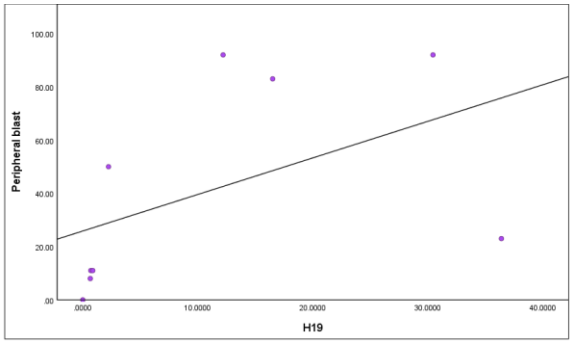

Figure 7: Scatter plot showing strong positive correlation between H19 expression and peripheral blood blasts in T-ALL patients.

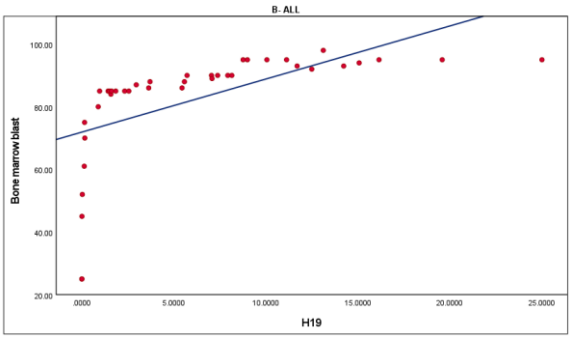

Figure 8: Scatter plot showing strong positive correlation between H19 expression and BM blasts in B-ALL patients.

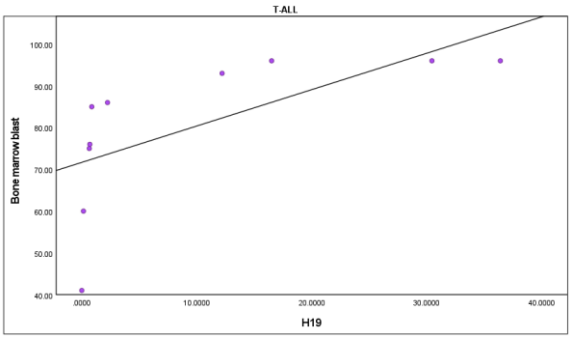

Figure 9: Scatter plot showing strong positive correlation between H19 expression and BM blasts in T-ALL patients.

Table 1: Age and sex distribution of the studied groups

Variable name	Control group (n= 20)	ALL group (n= 49)	*P* value
Age (years)			
➢ Mean ± SD	17.00 ± 13.97*	14.27 ± 17.26*	0.086
➢ Median (range)	12 (3-55)	8 (1-72)	
Sex			
➢ Male	9 (45%)**	25 (51%)**	0.65
➢ Female	11 (55%)**	24 (49%)**	

Data expressed as frequency (percentage), mean (SE), median (range). P value was significant if < 0.05. *Mann-Whitney test, **chi-square test.

Table 2: Clinical presentations of the studied patients

Clinical manifestations	B- ALL (N=39)	T-ALL (N=10)	Total (N=49)	*P* value
Anemic manifestations	25 (64.1%)	5 (50%)	30 (61.2%)	0.48
Hepatosplenomegaly	21 (53.8%)	4 (40%)	25 (51%)	0.496
Fever and repeated infections	17 (43.6%)	6 (60%)	23 (46.9%)	0.483
Bleeding tendency	15 (38.5%)	2 (20%)	17 (34.7%)	0.459
Lymphadenopathy	12 (30.8%)	5 (50%)	17 (34.7%)	0.285
Bone pain	9 (23.1%)	1 (10%)	10 (20.4%)	0.663

Data expressed as frequency. *P* value was significant if < 0.05. Chi-square test was used.

Table 3: Laboratory data of the studied patients

Laboratory data	B-ALL (N=39)	T-ALL (N=10)	Total (N=49)	*P* value
Hemoglobin (g/dl)				
• Mean ± SD	9.13 ± 2.06	8.64 ± 2.96	9.03 ± 2.25	0.243
• Median (range)	9.1 (3.9-15.9)	8.35 (5.7 - 15.5)	8.8 (3.9 - 15.9)	
WBCs (10^3/ul)				
• Mean ± SE	33.08 ± 6.164	53.86 ± 22.718	37.322 ± 6.719	0.779
• Median (range)	15.7 (0.7-147)	4.65 (1.5-177)	12 (0.7-177)	
Platelets (10^3/ul)				
• Mean ± SD	44.769±34.836	73.4 ± 66.337	50.612 ± 43.838	0.384
• Median (range)	36 (4 – 177)	37 (16 – 207)	36 (4 – 207)	
Peripheral blood blasts (%)				
• Mean ± SE	54.95±5.42	41.11±12.88	52.35±5.02	0.499
• Median (range)	66 (0 – 96)	23 (0 – 92)	64(0-96)	
Bone marrow blasts (%)				
• Mean ± SE	82.59±2.83	80.4±5.73	82.14±2.52	0.951
• Median (range)	88(25-98)	85.5(41-96)	87(25-98)	

Data expressed as frequency mean (SE), median (range). *P* value was significant if < 0.05. Mann-Whitney test was used.

Table 4: Accuracy of H19 expression in diagnosis of ALL

	H19
Sensitivity	71.4%
Specificity	75%
Positive predictive value	87.5%
Negative predictive value	51.72%
Accuracy	72.46%
Cutoff point (%)	> 1.015
Area under curve	0.75
P value	0.001

P value was significant if < 0.05. Roc curve was used.

Table (5): H19 expression in the studied groups

Gene expression		Control group (n=20)	ALL group (n=49)	P value
H19	Down regulation	15(75%)	14(28.6%)	< 0.001
	Up regulation	5(25%)	35(71.4%)	

Data expressed as frequency (percentage). *P* value was significant if < 0.05. chi-square was used.

Table 6: Correlation of H19 expression with other variables of ALL patients

Variable name		H19 expression	
		B- ALL (N=39)	T-ALL (N=10)
Age (years)	R	-0.117	0.037
	P	0.477	0.920
Hemoglobin (g/dl)	R	0.195	0.049
	P	0.235	0.894
WBCs (10^3/ul)	R	0.985	0.976
	P	<.001	<.001
Platelets (10^3/ul)	R	0.126	0.334
	P	0.446	0.345
PB blasts (%)	R	0.829	0.798
	P	<.001	0.01
BM blasts (%)	R	0.964	0.988
	P	<.001	<.001

Date expressed as r value (p value). P value was significant if < 0.05; LDH: lactate dehydrogenase. Spearman's rank correlation was used.

Table 7: comparison of H19 gene expression and cytogenetic analyses in the studied patients

Cytogenetic studies	B- ALL (N=39)		T-ALL (N=10)	
	Down regulation (N=9)	Up regulation (N=30)	Down regulation (N=5)	Up regulation N=5)
t (9,22) BCR::ABL				
Negative	6(66.7%)	25(83.3%)	5(100%)	4(80%)
Positive	0	3(10%)		
Not done	3(33.3%)	2(6.7%)	0	1(20%)
P value	0.083		1	
t (12,21) ETV6::RUNX1				
Negative	6(66.7%)	28(93.3%)	5(100%)	4(80%)
Not done	3(33.3%)	2(6.7%)	0	1(20%)
P value	0.07		1	
11q23 (KMT2A/MLL) rearrangement				
Negative	5(55.6%)	28(93.3%)	5(100%)	4(80%)
Positive	1(11.1%)	0		
Not done	3(33.3%)	2(6.7%)	0	1(20%)
P value	0.015		1	
t (1,19) TCF3				
Negative	6(66.7%)	28(93.3%)	5(100%)	4(80%)
Not done	3(33.3%)	2(6.7%)	0	1(20%)
P value	0.07		1	

Data expressed as frequency. P value was significant if < 0.05. chi-square was used.

Table 8: Factors predictive of ALL using Univariable Logistic Regression

Variables	B	S.E.	Wald	df	Sig.	Exp(B)	95% confidence interval	
							Lower	Upper
Age	-0.010	0.016	0.399	1	0.527	0.990	0.960	1.021
Gender								
Male	Ref.							
Female	-0.241	0.533	0.206	1	0.650	0.785	0.277	2.231
H19	0.592	0.257	5.301	1	0.021	1.807	1.092	2.992

CI indicates confidence interval. *P* value was significant if < 0.05. Binary logistic regression was used.

Table 9: Factors Predictive of ALL using Multivariable Logistic Regression

Variables	B	S.E.	Wald	df	Sig.	Exp(B)	95% confidence interval	
							Lower	Upper
Age	0.009	0.017	0.260	1	.610	1.009	0.976	1.043
Gender								
Male	Ref.							
Female	-0.185	0.623	0.088	1	0.766	0.831	0.245	2.817
H19	0.606	0.261	5.385	1	0.020	1.833	1.099	3.059

CI indicates confidence interval. *P* value was significant if < 0.05. Binary logistic regression was used.

Discussion:

LncRNAs may function as tumor suppressors or oncogenes, making them a potential diagnostic and prognostic biomarker in cancer [10]. One of these lncRNAs is H19 gene which plays an important role in many types of cancers [6].

This study was conducted on 49 newly diagnosed ALL patients; 79.6% of them were B-ALL and 20.4% were T-ALL. This is consistent with Gupta et al., (2019) and Jamal et al., (2021) who recorded the predominance of B-ALL cases distribution over T-ALL cases [11, 12].

The median age of the studied ALL patients was 8 years old and ranged from 1 to 72 years old. Pattnaik et al., (2020) who studied 105 patients with ALL reported that the median age was 10 years old (range 0.6–55 years old), and this agrees with our study in this finding [13].

In our study, male patients with ALL had an increased incidence compared to female patients, with 25 (51%) compared to 24 (49%) patients, respectively. This is agreed with previous studies by Jamal et al., (2021) and Ain Bashir et al., (2022) who confirmed a male predominance in ALL patients [12, 14].

The present study revealed that anemic manifestations were the most common clinical presentation, presented in 61.2% of cases followed by hepatosplenomegaly in 51% of cases. Other clinical presentations included fever, and recurrent infections (46.9%), bleeding tendency (34.7%), lymphadenopathy (34.7%) and bone pain (20.4%).

Yasmeen & Ashraf, (2009) found that 86% of patients presented with anemia and this is consistent with our study [15]. However, Kakaje et al., (2020) mentioned that lymphadenopathy and hepatosplenomegaly were the most common clinical presentations presented in 82.9% and 73.2%, respectively [16]. The discrepancy between the studies may result from the small number of patients in the present study (49 cases) compared to the previously mentioned study that was done on a more significant number of ALL cases (202 cases).

According to blood picture abnormalities, T-ALL patients were presented with a higher initial total leucocytic count than B-ALL patients. Many previous studies reported that T-ALL patients were often presented with a higher initial leucocytic count than B-ALL patients [17, 18].

In our study, the mean hemoglobin level was 9.13 ± 2.06 g/dl in B-ALL patients and 8.64 ± 2.96 g/dl in T-ALL patients with no statistically significant difference between both types of ALL patients. This is consistent with Kavyanjali et al., (2021) who found that the mean Hb level was 6.87 ± 2.24 g/dl in B-ALL cases and 7.46 ± 2.2 g/dl in T-ALL cases with no significant difference between both types of ALL in Hb distribution [18].

Regarding platelets count in the present study, most patients presented with thrombocytopenia with mean 44.769 ± 34.836 x 103/ul in B-ALL patients and 73.4 ± 66.337 x 103/ul in T-ALL patients with no significant difference between both types of ALL. In agreement with this study, Kavyanjali et al., (2021) evaluated the clinical and hematological features in 68 ALL Indian patients and reported that there was no significant difference in platelets count between B-ALL and T-ALL cases [18].

In our study we found no statistically significant difference as regards the percentage of peripheral blood blasts between B-ALL and T-ALL cases. This matched with Naeem & Bukhari, (2015) who assessed the hematological parameters in 50 Pakistani ALL patients at presentation and found no statistically significant difference in blast percentage between B-ALL and T-ALL [19].

In this study we evaluated the median of H19 gene expression level for our ALL patients' groups which was 3.71 (range: 0.006 - 36.38). It was significantly higher than the median of control group gene expression (P value: 0.001). The median of H19 gene expression was 5.45 (range: 0.006 - 25) in B-ALL cases and 1.56 (range: 0.007 - 36.38) in T-ALL cases. A significant up regulation of H19 gene expression in ALL cases was also recorded in 35 of 49 patients.

Similar to our study, Asadi et al., (2023) studied the expression level of H19 gene in 25 newly diagnosed Iranian ALL patients and found that H19 expression was higher in the B-ALL (median: 2.51, ranged from 1.81 to 4.12), ($P < 0.05$) and T-ALL (median: 4.72, ranged from 2.71 to 9.48), ($P < 0.01$) samples in comparison to the control group [6].

There was no statistically significant correlation among age, sex and H19 gene expression. This result agrees with Yörüker et al., (2018) who studied the expression level of H19 in gastric cancer patients [20].

In contrary, Zhang et al., (2018) who studied H19 expression in AML patients found that there was a statistically significant correlation among high H19 expression, sex ($P = 0.075$) and older age ($P = 0.004$) [9]. The discrepancy between the studies may result from the different age groups and different types of cancers included in both studies.

There was no significant correlation between H19 expression and clinical presentations of the patients in our study. No other studies detected this result in ALL patients as far as we know. Several studies should be done with a larger sample size to evaluate this finding.

There was no statistically significant correlation among hemoglobin level, platelets count and H19 gene expression. This is consistent with Zhang et al., (2018) who studied the expression of H19 in 161 AML patients by real-time quantitative PCR [9].

Zhang et al., (2018) found an association between high H19 expression and WBCs count in AML patients [9]. This is in agreement with our study as we found a significant strong positive correlation between H19 expression and WBCs count in B-ALL and T-ALL patients.

In the present study, there were strong positive correlations between H19 expression and peripheral blood blasts and between H19 expression and bone marrow blasts in B-ALL and T-ALL patients. This may be due to the pathogenesis of H19 as an oncogene, [8,9] as it enhances cell proliferation, differentiation, migration, invasion, and chemoresistance [6,7], but a correlation with this result was not examined in previous studies.

Cytogenetic analyses revealed that 11q23 (KMT2A/MLL) rearrangement and H19 gene expression differed significantly in B-ALL patients (P: 0.015) but no previous studies found this correlation. While Asadi et al., (2023) reported that H19 expression is related to various genetic abnormalities, particularly c-Myc, P53 and HIF-1α. It has been shown that c-Myc promotes carcinogenesis, cell proliferation, and H19 expression. Additionally, Asadi et al. (2023) discovered a negative correlation between H19 expression and P53. Samples from ALL patients showed a marked increase in H19 expression, which may be related to hypoxic conditions in BM. The ALL cell lines verified that P53 was suppressed and H19 was simultaneously up-regulated as a result of the hypoxic environment and HIF-1α activation [6].

Conclusion:

In our study, H19 gene expression was significantly up regulated in ALL patients. A statistically significant positive correlations were found among H19 expression level with WBCs count, percentage of peripheral blood blasts and also bone marrow blasts percentage in B-ALL and T-ALL patients; the higher WBCs count, increased percentage of PB blasts and BM blasts, the higher H19 expression level.

Also, we found a statistical significance between H19 expression and 11q23 (KMT2A/MLL) gene rearrangement. So, further studies are recommended on a larger sample in other centers to demonstrate the role of anti-H19 as a target therapy for ALL patients.

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