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Original Research Article

Association of long non-coding RNAs and ABO blood groups with acute lymphoblastic leukemia in Egyptian children

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most prevailing cancer among children. Despite extensive studies, ALL etiology is still an unsolved puzzle. Long non-coding RNAs (lncRNAs) emerged as key mediators in cancer etiology. Several lncRNAs are dysregulated in ALL, leading to oncogenic or tumor-suppressive activities. Additionally, a relation between ABO blood groups and hematological malignancies was proposed. The current study intended to explore the association of lncRNAs, ANRIL and LINC-PINT, and their downstream targets, CDKN2A and heme oxygenase-1 (HMOX1), with the incidence of ALL and treatment response, and to determine the distribution of blood groups across different childhood ALL phenotypes. Blood samples were taken from 66 ALL patients (at diagnosis and at the end of remission induction phase) and 39 healthy children. Whole blood was used for blood group typing. Expression of ANRIL, LINC-PINT and CDKN2A was analyzed in plasma by qRT-PCR. Serum HMOX1 was measured using ELISA. ANRIL and CDKN2A were upregulated, while LINC-PINT and HMOX1 were downregulated in newly diagnosed patients. All of which showed remarkable diagnostic performance, where HMOX1 was superior. HMOX1 was independent predictor of ALL as well. LINC-PINT and HMOX1 were significantly upregulated after treatment. Notably, ANRIL and LINC-PINT were associated with poor outcome. No significant difference in the distribution of ABO blood groups was observed between patients and controls. In conclusion, our results suggested an association of ANRIL and LINC-PINT with childhood ALL predisposition, at least in part, through altering CDKN2A and HMOX1 production. Furthermore, the impact of remission induction treatment was newly revealed.

1. Introduction

Leukemia is a group of hematological cancers characterized by proliferation of hematopoietic cells causing marrow function turmoil and marrow failure [1]. Leukemia was estimated to be the 15th in cancer incidence and the 11th most frequent cause of global cancer-related mortality, accounting worldwide for 474,519 cases and 311,594 deaths in 2020 [2]. It is the most common type of cancer in children who are more likely to experience the acute than the chronic form [3]. Indeed, acute lymphoblastic leukemia (ALL) is the most prevailing hematological and overall malignancy in children, accounting for 30 % of cancer diagnoses and over 80 % of all pediatric leukemias [4]. Despite the high cure rate observed in childhood ALL, still, 10–20 % of patients have resistant forms of disease which represents a prime cause of cancer-associated mortality in children [5].

Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into proteins. They are classified into different categories, including long non-coding RNAs (lncRNAs), short interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs) [6].

LncRNAs are RNA strands exceeding 200 nucleotides in length and are one of the most extensively researched ncRNA types [7]. According to recent estimations, the human genome contains more than 15,000 lncRNAs [8]. LncRNAs are crucial in regulating gene expression at the epigenetic, transcriptional, and post-transcriptional levels. Since they control the expression of genes implicated in molecular processes like cell proliferation/differentiation, apoptosis, and metastasis, a number of lncRNAs have even been advocated to serve as tumor suppressors or oncogenes [9,10]. Besides, the expression of lncRNAs is tissue- and stage-specific, demonstrating their significance in the mechanisms of cell differentiation [7].

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List of abbreviations

ABCC1	ATP - binding cassette subfamily C member 1
AdipoR1	adiponectin receptor 1
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ANRIL	antisense noncoding RNA in the INK4 locus
ASPP2	apoptosis stimulating proteins of p53-2
CCLE	Cancer Cell Line Encyclopedia
CDKN2A	cyclin-dependent kinase inhibitor 2A
COAD	colon adenocarcinoma
CNS	central nervous system
EGIL	European Group for the Immunological Characterization of Leukemias
EZH2	enhancer of zeste homologue 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HDAC1	histone deacetylase 1
HMOX1	heme oxygenase 1
IPT	immunophenotyping
LINC-PINT	long intergenic non-coding RNA p53-induced transcript
lncRNA	long non-coding RNA
miRNA	microRNA
MRD	minimal residual disease
ncRNA	non-coding RNA
NF-κB	nuclear factor kappa-B
piRNA	PIWI-interacting RNA
PRC	polycomb repressor complex
siRNA	short interfering RNA
SIRT1	Sirtuin 1
snRNA	small nuclear RNA
TGF-β1	transforming growth factor-β1

Antisense noncoding RNA in the INK4 locus (ANRIL) is transcribed from the INK4b-ARF-INK4a gene cluster which has been recognized as a genetic susceptibility locus linked to human diseases, particularly cancers [11,12]. It was reported to be overexpressed in acute leukemia patients [13]. ANRIL promoted proliferation and inhibited programmed cell death of adult T-cell leukemia cells through collaborating with enhancer of zeste homologue 2 (EZH2) to activate nuclear factor kappa-B (NF-κB) pathway [14]. Furthermore, ANRIL has been found to recruit and bind Combinex7 subunits of polycomb repressor complex 1 (PRC1) and suppressor of zeste 12 (SUZ12) subunits of polycomb repressor complex 2 (PRC2), which regulate cellular proliferation through regulation of CDKN2A/B locus [15,16]. CDKN2A is localized on chromosome 9p21 close to ANRIL [17]. It is frequently inactivated in a number of hematologic malignancies, where one of the most frequent genetic events is homozygous deletion or promoter hypermethylation [18].

Long intergenic non-coding RNA p53-induced transcript (LINC-PINT) is an lncRNA that operates as a tumor suppressor in a variety of malignant processes. It is downregulated in several solid and hematological cancers [19,20]. Moreover, LINC-PINT expression was associated with advanced clinical tumor stages and bad prognosis [21]. LINC-PINT may exert its action through inducing heme oxygenase-1 (HMOX1) transcription [22]. HMOX1 is an inducible enzyme concerned with heme degradation to produce carbon monoxide and biliverdin by which HMOX1 mediates its advantageous effects as antioxidant and anti-inflammatory [23].

Based on the aforementioned, ANRIL and LINC-PINT may be used as cancer diagnostic and prognostic biomarkers. The lncRNAs' regulatory mechanisms may also be a new target for cancer treatments.

ABO blood group antigens, on the surface of erythrocytes, display distinct phenotypes and genetically determined structures. Numerous studies have extensively scrutinized the significance of the ABO blood group in cancer biology [24–26]. However, the research results were controversial regarding ABO antigens and the risk and prognosis of various types of tumors. Recent studies showed that increased risk of gastric and pancreatic cancer was observed in patients with blood group A [27,28]. Blood type AB carriers had higher risk of developing ALL [29]. While higher percentage of O blood type was observed in patients with acute leukemia [30]. Others reported no statistically significant difference between controls and ALL patients with regard to ABO blood group distribution [31].

In this context, the aim of the current study was to investigate the possible association between the two lncRNAs, ANRIL and LINC-PINT, and their downstream targets - CDKN2A and HMOX1, respectively - with ALL in children. The differential distribution of ABO blood groups among ALL patients was also studied. This work was further extended to elucidate the possible correlation of ANRIL, LINC-PINT, CDKN2A, HMOX1 and ABO blood groups with the patients' clinicopathological data. The novelty of this study was investigating the impact of initial treatment till the completion of remission induction phase on the studied parameters.

2. Subjects and methods

2.1. Subjects

The present case-control study included 66 pediatric ALL patients who were treated at the National Cancer Institute-Cairo University and Dar El-Salam Cancer Center between December 2020 and March 2022.

The inclusion criteria for selected patients were: being newly diagnosed with ALL and ≤18 years of age. The exclusion criteria were: i) other types of leukemia; ii) patients who died before end of induction; iii) relapsing ALL; iv) patients receiving steroids for any reason before establishing the diagnosis of ALL.

In addition to the diagnosed patients with ALL, another group of 39 healthy children was enrolled during the same period, and were all matched for age and sex. None of the healthy donors had a personal or a family history of hematopoietic or lymphoid tissue malignancies.

Each participant's guardian provided written consent at enrollment after discussing the experimental procedure and the beneficial expectancies. The study protocol was endorsed by the Faculty of Pharmacy-Research Ethics Committee for experimental and clinical studies, Cairo University, Cairo, Egypt (approval number: BC 2458) and attuned to the 1975-Helsinki declaration that was revised in 2008.

2.2. Clinical assessment

2.2.1. Diagnosis and risk stratification

Diagnosis of ALL was made by professional physicians based on the following work up to detect blast cells and stratify the disease risk.

1. Complete blood picture with differential count.
2. Bone marrow aspirate examination, to study bone marrow cellularity and detect leukemic cells.
3. Immunophenotyping (IPT) using flow cytometry; the gold standard for cell lineage identification and sub-classification. According to the European Group for the Immunological Characterization of Leukemias (EGIL), a threshold of 20 % blast cells is used to define a positive reaction of blast cells to a given monoclonal antibody. The most important markers for the diagnosis of B-lineage ALL are CD19, CD20, CD22, CD24, and CD79a, and for diagnosis of T-cell ALL are CD1, CD2, CD3, CD4, CD5, CD7 and CD8 [32].

4. Cytogenetic studies to define the clinical pattern and to detect specific chromosome aberrations e.g. t (9; 22) and (BCR-ABL1) [33].
5. Cerebrospinal fluid (CSF) analysis and central nervous system (CNS) status that is defined as CNS-1 (no blast cells in CSF sample), CNS-2 (<5 leukocytes and <10 erythrocytes/mm^3 with blast cells), CNS-3 (≥5 leukocytes and <10 erythrocytes/mm^3 with blast cells), or traumatic lumbar puncture (≥10 erythrocytes/mm^3 with blast cells) [34].

2.2.2. Prognosis

Minimal residual disease (MRD) test was used to predict the treatment efficacy and the relapse risk. MRD was monitored at the end of the induction of remission phase (day 43–46) using EPICS XL-MCL flow cytometer and reagent systems (Coulter, Hialeah). Surface staining fluorescent labelled monoclonal antibodies against T or B antigens and isotypic controls were provided by Becton Dickenson (Mountain View, California). Intracellular staining was done using INTA Prep permeabilization reagent from the Beckman Coulter where cells were fixed with reagent 1 (containing formaldehyde). After washing, permeability was induced with reagent 2 (containing saponin) and the rest of the RBCs were lysed. Monoclonal antibodies used for 4 color combinations for the detection of MRD were as follows.

- BCP-ALL: TdT/CD10/CD19/CD 45; CD10/CD20/CD19/CD 45; CD34/CD38/CD19/CD 45; CD34/CD22/CD19/CD 45; CD19/CD34/CD45; CD10/CD20/CD22/CD 45.
- T cell-ALL: TdT/CD1/cyt CD3; TdT/cyt CD3/CD7; CD4/CD8/CD3/CD45.

A minimum of 3×10^5 ungated events were collected and examined. The ability to spot 30 clustered MRD events in 3×10^5 total cellular events (0.01 %) is designated as minimum target sensitivity for quantifying MRD, i.e. cut off point for MRD positive test was 0.01 %.

2.3. Chemotherapy protocol

Modified Total-XV protocol was the chemotherapy given to patients and adopted from St. Jude Children's Research Hospital [35]. Generally, treatment is tailored according to the initial risk stratification of the disease as well as the disease response to chemotherapy. The treatment plan includes three main phases; remission induction phase, to knock down the leukemia cells in the blood and bone marrow to achieve remission (a return to normal blood cell counts), consolidation (for all disease risk) and re-intensification treatment (only for high-risk ALL) phase, to eradicate the remaining cells that could begin to grow and cause the leukemia to relapse, and continuation therapy phase, to destroy any cancer cells that might have survived the previous phases.

Patients were consequently allocated to one of three risk groups (low, standard and high) depending on their presenting clinical features and biological attributes of their leukemic cells. All patients, regardless of their risk stratification, received the same induction chemotherapy before being assigned to different treatment phases according to their response to remission induction therapy. The main drugs given to patients during this phase were prednisone, daunorubicin, vincristine, and asparaginase. Patients having ≥1 % MRD on day 19 were administered 3 additional doses of asparaginase. Ensuing induction therapy included cyclophosphamide, mercaptopurine, and cytarabine. On hematopoietic recovery (days 43–46), the MRD was determined, and subsequent consolidation phase of therapy was initiated.

2.4. Sample collection and biochemical measurements

Initial peripheral blood sample (5 ml) was collected from each patient by trained technician before the start of any treatment at the time of patient's routine investigations, another sample was collected at the end of remission induction phase. Similarly, 5 ml blood samples were taken from controls at enrollment. Each blood sample was divided into three portions.

The first 2 portions were collected into EDTA-coated vacuette tubes (Greiner Bio-One, Germany), the first one was used for blood group typing using commercially available antibodies against type A and B blood (Anamol labs, India).

The second one was centrifuged at 3000×*g* for 15 min and plasma was separated and instantly used for total RNA extraction and subsequent detection of LINC-PINT, ANRIL and CDKN2A expression levels.

The third portion was collected into red-top vacuette tube (Greiner Bio-One, Germany), allowed to clot and was then centrifuged to separate serum to measure protein level of HMOX1 by Human HMOX1 ELISA Kit provided by WuhanFine Biotech Co.,Ltd, China (Cat # EH3234).

2.4.1. Genomic RNA isolation and qRT-PCR

The relative expression levels of lncRNAs LINC-PINT, ANRIL together with gene expression level of CDKN2A, were analyzed using quantitative real time polymerase chain reaction (qPCR).

Total RNA was extracted from plasma using QIAzol based miRNeasy purification Kit (Qiagen, Germany) complying with the manufacturer's instruction. The RNA concentration and quality were assessed using NanoPhotometer™ N60 (Implen, Germany).

Subsequently, complementary DNA (cDNA) was constructed from genomic RNA using Quantitect Reverse Transcription kit (Qiagen, Germany). Gene expression was assessed using StepOne Real-Time PCR System (Thermo Fisher Scientific, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). The PCR amplification program was as follows: 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 15 s, 53 °C for 30 s, 72 °C for 30 s). The primers used were designed using NCBI primer Blast (**Table 1**) and then custom-made by Invitrogen (USA). The internal reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was applied. Gene expression relative to the internal control ($2^{-\Delta Ct}$) was calculated, then the fold change was determined using $2^{-\Delta\Delta Ct}$.

2.5. Statistical analysis

Data were displayed as mean ± standard deviation (SD), median (interquartile range) or number (percentage) when relevant. Regarding continuous datasets, normally distributed data were examined for significance using unpaired Student's two-tailed t-tests for unpaired data, paired *t*-test for paired data and ANOVA then Tukey's post hoc test to compare data of more than two groups. For non-parametric data, Mann-Whitney test for unpaired data, Wilcoxon test for paired data and Kruskal-Wallis test then Dunn's post hoc test for data of more than two groups were used. On the other hand, categorical data were analyzed by Chi-square test. Correlations between parameters were determined by Spearman correlation. Receiver operating characteristic (ROC) analysis were performed for data in ALL group *vs.* normal control to assess the diagnostic power of the study parameters. Logistic regression analyses were conducted to pinpoint predictors of childhood ALL. Data that were

Table 1
Primer sequences used in qPCR.

Item	Primer Sequence
ANRIL	Forward 5′ CGC TGC CCA ATA CCT GTT CT 3′
	Reverse 5′ GCC AAT AGT CAC TCC GCA TTC 3′
CDKN2A	Forward 5′ GCG CGT ACA GAT CTC TCG AAT 3′
	Reverse 5′ AGG CTA AGT CCC AGC ACA T 3′
LINC-PINT	Forward 5′TCC CAA TGC ATG TGT TAG AGA GAT3′
	Reverse 5′ GCA CTA CAC CTC CAC CCC TA 3′
GAPDH	Forward 5′ GGC CCT GAC AAC TCT TTT CAT C 3′
	Reverse 5′ CTG GTT GAG CAC AGG GTA CT 3′

ANRIL, antisense noncoding RNA in the INK4 locus; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **GAPDH,** Glyceraldehyde 3-phosphate dehydrogenase; **LINC-PINT**, long intergenic non-coding P53 induced transcript.

significant according to univariate analysis were then selected for multivariate analysis to identify the independent variables that affected the response. Probabilities below 0.05 ($p < 0.05$) were regarded statistically significant, with a 95 % confidence interval. All data were analyzed using GraphPad Prism 8.0 (GraphPad Software).

3. Results

3.1. The demographics and clinical characteristics of the study subjects

A total of 66 ALL patients (40 boys and 26 girls) and 39 healthy controls (26 boys and 13 girls) were included in this study. The median age was 8 years (range, 1−17 years) and 7 years (range, 1−15 years) for ALL patients and control group, respectively. Age and sex distributions in ALL patients matched those of controls.

Seventy-six percent of patients were classified as standard risk group while the rest were low risk and none were classified as high-risk category. Eighty-three percent of patients were classified as CNS-1, 12 % as CNS-2 and the rest as CNS-3. Around 70 % of patients had TLC below 50 $\times$ 10^9/L. IPT analysis dichotomized ALL patients to B-cell or T-cell lineage subsets in a ratio of about 4:1 respectively. Finally, four-fifths of patients responded to treatment at the end of remission induction therapy with MRD <0.01 (Table 2).

3.2. Relative gene expression levels of ANRIL, LINC-PINT and CDKN2A and protein level of HMOX1 in ALL patients

As depicted in Fig. 1, newly diagnosed ALL patients showed significantly higher levels of plasma lncRNA ANRIL and CDKN2A reaching 293 and 1223 % of controls; respectively (Fig. 1A and B). ANRIL demonstrated a downturn at the end of remission induction that did not reach statistical significance compared to its initial level at diagnosis with no statistical difference compared to control samples. CDKN2A also showed slight decline at the end of remission induction therapy to a level that did not differ significantly from its initial level at diagnosis. Whereas, LINC-PINT was significantly downregulated to 18.5 % in newly diagnosed ALL patients as compared to controls, and then markedly upregulated by 2 folds at the end of remission induction in comparison to newly diagnosed levels. This upregulation brought LINC-PINT to a level approaching control's level (Fig. 1C). In addition, serum level of HMOX1 in newly diagnosed ALL patients significantly decreased to 31 % of control level, then significantly increased at the end of remission induction therapy to 200 % its level at diagnosis, but was still significantly lower than controls (Fig. 1D).

Table 2
Demographic and clinical data of the study population.

		Controls	Patients	P value
Sex n (%)	**Boys**	26 (66.67 %)	40 (60.61 %)	0.68
	Girls	13 (33.33 %)	26 (39.39 %)	
Age (years)		6.86 ± 3.58	7.63 ± 4.9	0.52
Risk group	**Low**	–	16 (24.24 %)	–
	Standard	–	50 (75.76 %)	
	High	–	0 (0 %)	
CNS status	**CNS-1**	–	55 (83.33 %)	–
	CNS-2	–	8 (12.12 %)	
	CNS-3	–	3 (4.55 %)	
TLC	**<50×10^9/L**	–	46 (69.70 %)	–
	>50×10^9/L	–	20 (30.30 %)	
IPT	**B-cell**	–	52 (78.79 %)	–
	T-cell	–	14 (21.21 %)	
MRD Day 43	**<0.01 %**	–	36 (78.26 %)	–
	>0.01 %	–	10 (21.74 %)	

Age is expressed as mean ± SD and other data are presented in numbers (percentage).
CNS, central nervous system; **IPT**, immunophenotyping; **MRD**, minimal residual disease; **TLC**, total leucocytic count.

Concerning the blood group distribution among the studied cohort, notably, blood group A was the most observed blood group (36.36 %), followed by O (31.83 %), then B (24.24 %) and finally AB (7.57 %). However, no statistical difference was detected between controls and ALL patients with regard to the distribution of the different ABO blood groups (Fig. 2).

3.3. Correlations between ANRIL, LINC-PINT and their downstream targets in the study subjects

Significant positive correlations were found between ANRIL and CDKN2A ($r = 0.2586$, $P = 0.0223$) (Fig. 3A), and between LINC-PINT and HMOX1 ($r = 0.2520$, $P = 0.0241$) (Fig. 3B).

3.4. Diagnostic performance of ANRIL, CDKN2A, LINC-PINT and HMOX1 in ALL patients

To assess the diagnostic performance of ANRIL, CDKN2A, LINC-PINT and HMOX1 in discriminating ALL patients from controls, receiver operating characteristic (ROC) curve analysis was conducted (Fig. 4). Results revealed that ANRIL was able to distinguish ALL patients from controls (AUC = 0.64, 95 % CI = 0.5192–0.7669, P = 0.0211) with a sensitivity of 70 % and a specificity of 58.98 % at a cutoff >1.9 (fold). CDKN2A distinguished ALL patients from controls (AUC = 0.69, 95 % CI = 0.5727 to 0.7993, P = 0.0032) with a sensitivity of 58.82 % and a specificity of 72.22 % at a cutoff >6.915 (fold). LINC-PINT also distinguished ALL patients from controls (AUC = 0.72, 95 % CI = 0.6051–0.8245, P = 0.0007) with a sensitivity of 60.38 % and a specificity of 71.43 % at a cutoff <0.2576 (fold). Furthermore, serum HMOX1 showed superior ability to discriminate ALL patients from controls (AUC = 0.92, 95 % CI = 0.8693–0.9741, P = <0.0001) with a sensitivity of 77.42 % and a specificity of 96.77 % at a cutoff <1.51 ng/ml.

3.5. Logistic regression analysis

As shown in Table 3, logistic regression analyses were carried out to identify the predictor parameters associated with ALL. The expression levels of LINC-PINT and HMOX1 were detected as significant predictors for childhood ALL in the univariate analysis. In subsequent multivariate analysis, HMOX1 turned out to be significant independent variable.

3.6. Association of ANRIL, LINC-PINT, CDKN2A and HMOX1 with sex and ALL clinicopathological features in ALL patients

As shown in Table 4, there was a significant positive association between ANRIL with both risk stratification and MRD at end of remission induction phase. Surprisingly, LINC-PINT showed a positive significant association with CNS status as well as MRD at end of remission induction phase, while HMOX1 was positively associated with risk stratification and IPT. Of note, no significant association was detected between ANRIL, LINC-PINT and HMOX1 with other data including age, sex or TLC. Regarding CDKN2A, no significant association was observed with any of the ALL clinicopathological features, except for sex; showing higher expression levels among boys.

3.7. Association of ABO blood groups distribution with clinical data of ALL patients

As shown in Table 5, no significant association was observed between any of the ALL clinicopathological features and ABO blood groups distribution.

4. Discussion

Over the past decade, many novel lncRNAs have been identified as important regulators of acute leukemia progression as well as potential

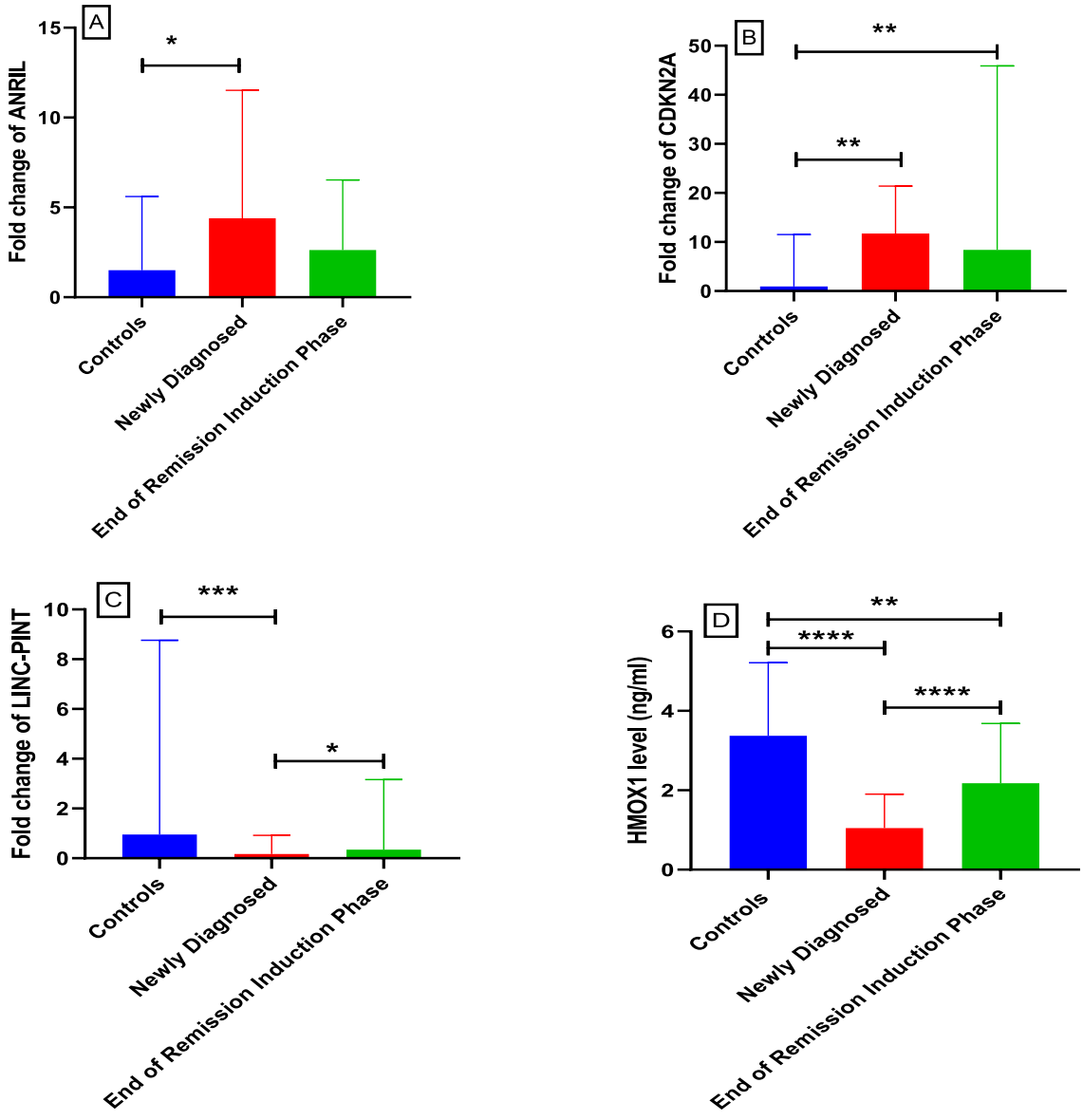

Fig. 1. Relative gene expression levels of RNAs ANRIL, CDKN2A and LINC-PINT (A, B and C) and protein level of HMOX1 (D) in ALL patients.
Data are presented as median with interquartile range for (A), (B) and (C), and as mean ± SD for (D). *Significant difference at $P < 0.05$, **Significant difference at $P < 0.01$, ***Significant difference at $P < 0.001$, ****Significant difference at $P < 0.0001$. **ANRIL**, antisense noncoding RNA in the INK4 locus; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **LINC-PINT**, long intergenic non-coding P53 induced transcript.

independent biomarkers [36]. The current study investigated the rational employment of the 2 lncRNAs, ANRIL and LINC-PINT, as diagnostic and prognostic tools for ALL in pediatric age group. Further, the study introduced some of their downstream associated genes, CDKN2A and HMOX1, respectively, as plausible participating intermediates. The newness of this study was exploring the impact of the first line treatment, at the end of remission induction phase, on the studied parameters. Further, the association of the latter with sex and clinical data of ALL patients was also newly unveiled.

Regarding ANRIL, the present study revealed that ANRIL was significantly elevated in newly diagnosed pediatric ALL. At the end of remission induction therapy, ANRIL showed a moderate decrease in its expression level that, promisingly, didn't significantly differ from normal controls. It seems likely that longer treatment duration might achieve a more profound control on ANRIL's expression.

In concordance, in ALL, ANRIL expression level was significantly elevated in the bone marrow tissues of T-ALL patients compared with healthy cases [37]. ANRIL was significantly overexpressed in newly diagnosed and relapsed pediatric B-cell precursor ALL as well [38].

Consistent with the current study results that revealed higher ANRIL levels in standard risk patients, a previous study showed that ANRIL was associated with poorer risk stratification in patients with acute myeloid leukemia (AML) [39], a notion that further anchors the possible association between ANRIL and ALL pathogenesis.

Additionally, ANRIL was upregulated in AML patients upon diagnosis and downregulated in patients with complete remission; it has been shown to modulate the adiponectin receptor 1/AMP-activated protein kinase/Sirtuin 1 (AdipoR1/AMPK/SIRT1) glucose metabolism related pathway to enhance AML cell survival [40]. ANRIL was also found to sponge miR-34a to upregulate histone deacetylase 1 (HDAC1), and HDAC1 overexpression hindered the recruitment of E2 F1transcription factor to apoptosis stimulating proteins of p53-2 (ASPP2) gene promoter, hence mediating the epigenetic suppression of ASPP2, which leads to AML cell proliferation, migration, and invasion [41]. The

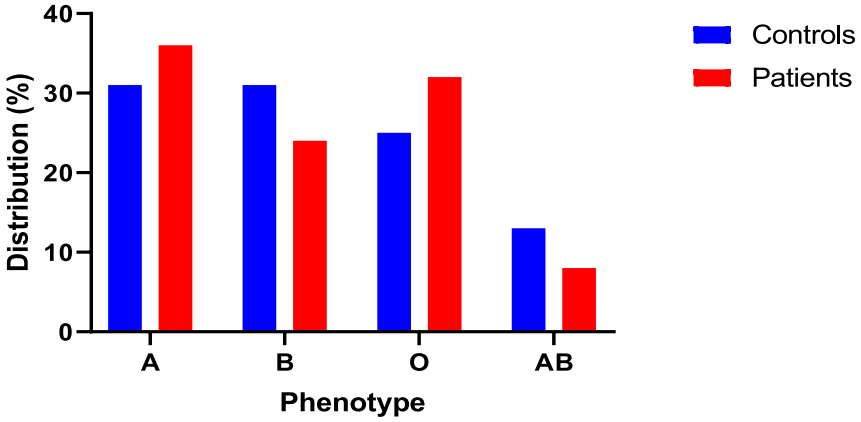

Fig. 2. Distribution of blood groups in controls and ALL patients.

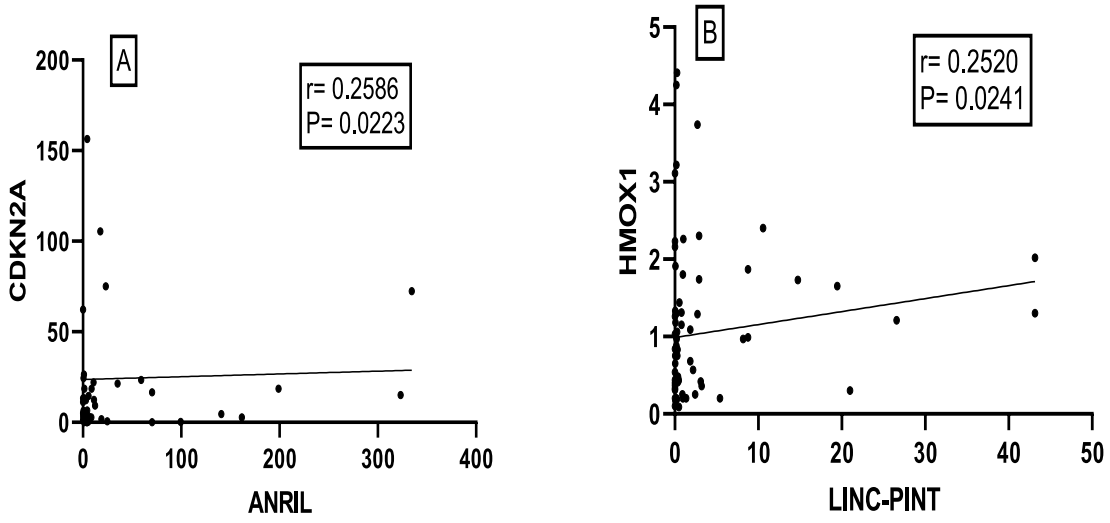

Fig. 3. Correlation analysis between (A) ANRIL and CDKN2A, (B) LINC-PINT and HMOX1 in the study population.
ANRIL, antisense noncoding RNA in the INK4 locus; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **LINC-PINT**, long intergenic non-coding P53 induced transcript.

deregulation of ANRIL-miRNAs and PRC2/PRC1–miRNAs interacting networks has also been incriminated in ANRIL-associated carcinogenesis [42,43].

ANRIL deregulation in cancer is still mostly unclear, and only a few mechanisms have lately been unveiled. ANRIL and p16-CDKN2A promoter methylation status was found to regulate ANRIL/p16- CDKN2A and ANRIL/p14-ARF transcription [44,45]. ANRIL transcription was also observed to be activated by E2F1 and *c*-Myc transcription factors [46,47].

The MRD values at the end of remission induction are known to be highly prognostic for treatment response [48]. In the present study, ANRIL was overexpressed in children with MRD >0.01 % at the end of remission induction therapy indicating that ANRIL might be associated with treatment resistance. The possible reason is that ANRIL seems to cause chemoresistance by inducing ATP - binding cassette subfamily C member 1 (ABCC1) through regulating Let -7a miRNA [49]. The ABC transporters protein family are suggested as contributors to the development of drug chemoresistance [50]. Reportedly, relatively high expression of ANRIL was significantly correlated with poor clinical prognosis and decreased overall survival rate in colorectal cancer [49]. In consistency, knockdown of ANRIL played an effective role in combating drug resistance in triple-negative breast cancer by inhibiting glycolysis through the miR-125a/enolase 1 pathway [51].

The ANRIL gene locus, INK4b-ARF-INK4a, located on human chromosome 9, are frequently associated with different types of cancer [52,53]. CDKN2A (INK4a), located in the same locus, is one of the most researched genes having tumor suppressor properties. It encodes the p16-INK4a protein that regulates cell cycle progression, as well as, cell differentiation, senescence, and apoptosis [54,55].

As was the case with ANRIL, there was a significant upsurge in CDKN2A expression among ALL patients. Notably, a positive correlation was observed between ANRIL and CDKN2A. The present results are in accordance with Kinan et al. who reported the overexpression of ANRIL, p16-CDKN2A, p15-CDKN2B, and p14-ARF in a great majority of the 17 tumor types in multitumor panel [56]. In agreement, an earlier study showed transcriptional co-activation of these 4 genes via bidirectional ANRIL–CDKN2A promoter induction in cell lines of colon cancer [44]. Others reported that the expression level of ANRIL was remarkably positively correlated with the level of p16-CDKN2A but not with that of p15-CDKN2B in Cancer Cell Line Encyclopedia (CCLE) database [44].

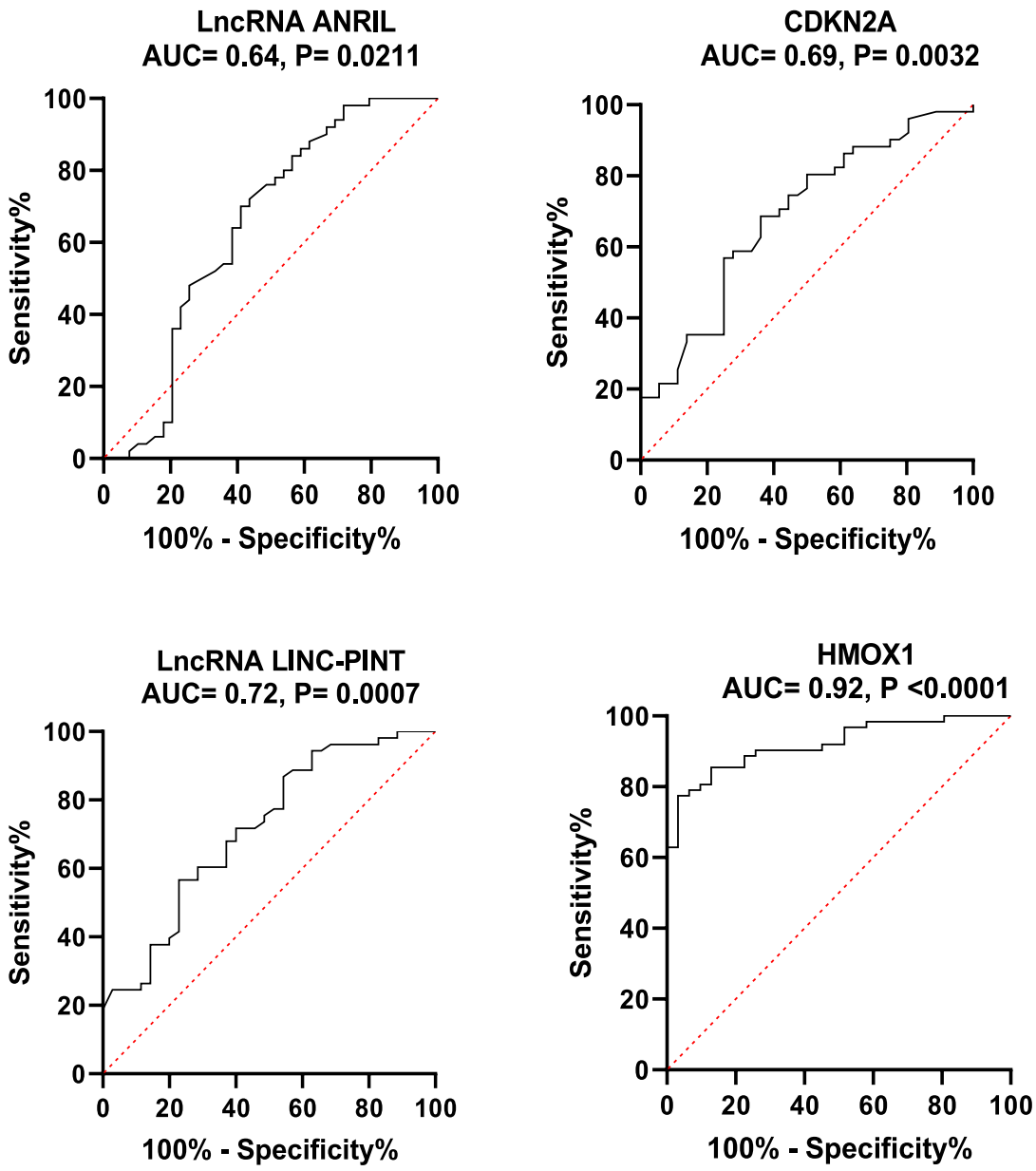

Fig. 4. Diagnostic performance of ANRIL, CDKN2A, LINC-PINT and HMOX1 in ALL patients
ANRIL, antisense noncoding RNA in the INK4 locus; **AUC**, area under the curve; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **LINC-PINT**, long intergenic non-coding P53 induced transcript.

The aforementioned findings may imply a dual role of ANRIL in the context of carcinogenesis, where its oncogenic potential is partly counterbalanced by concomitant tumor suppressor action of co-activated CDKN2A. However, the current results contradict earlier data from literature suggesting that ANRIL overexpression in prostatic carcinoma was mainly coupled with transcriptional inactivation of the p16-CDKN2A/p15-CDKN2B/p14-ARF locus through the two polycomb complexes PRC1 and PRC2 repressive action [57].

As of yet, no study has investigated the correlation between the current study parameters expression pattern and sex in human. The current results showed higher expression levels of CDKN2A among ALL boys. Whether this correlation impacts ALL susceptibility and pathogenesis in boys or not, warrants further investigations. Coincidentally, an experimental study deduced by linkage analysis that the CDKN2A/B locus was a positional candidate gene for sex-linked barring in chickens [58].

Concerning LINC-PINT, although it is considered a vital lncRNA that primarily functions as an onco-suppressor in various cancers, its clinical use is still extremely limited. However, studies have shown that LINC-PINT is considered as a promising biomarker for cancer prognosis [21, 59]. In the present study, LINC-PINT was significantly downregulated in newly diagnosed pediatric ALL patients then markedly upregulated again at the end of remission induction therapy. Furthermore, it was able to distinguish patients with ALL from healthy subjects and could significantly predict pediatric ALL. This could be explained by the potential of LINC-PINT to reduce the proliferation rate via apoptosis induction and cell cycle arrest in G2 phase [60]. Consistent with the current results, it was reported that LINC-PINT was downregulated in

Table 3
Logistic regression analyses of the studied parameters.

Parameter	Coefficient	SE	P value	Odds ratio	95 % CI
Univariate analysis					
ANRIL	−0.0061	0.0043	0.1605	0.9939	0.9855–1.0024
CDKN2A	0.0200	0.0119	0.0924	1.0202	0.9967–1.0443
LINC-PINT	−0.1303	0.0534	**0.0147**	0.8778	0.7906–0.9747
HMOX1	−1.6785	0.3513	**<0.0001**	0.1871	0.0940–0.3726
Multivariate analysis					
LINC-PINT	−0.0700	0.0586	0.2325	0.9324	0.8312–1.0459
HMOX1	−1.4671	0.3557	**<0.0001**	0.2306	0.1148–0.4630

Bold values denote statistical significance.
ANRIL, antisense noncoding RNA in the INK4 locus; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **CI**, confidence interval; **LINC-PINT**, long intergenic non-coding P53 induced transcript; **SE**, standard error.

early-stage esophageal squamous cell carcinoma patients [61]. Likewise, another study reported that LINC-PINT expression was lower in plasma samples from pancreatic cancer patients than from healthy individuals [59].

White blood cell counts in CSF and microscopy of a cyto-centrifuged CSF sample are typically used for CNS staging in order to morphologically identify leukemic blasts. This is done to categorize patients as CNS-1, CNS-2, or CNS-3 [62]. Of note, higher CNS stages are associated with increased risk of relapse [63]. To our knowledge, no available publications have characterized the expression level of LINC-PINT and different CNS status. Herein, patients with CNS-2 exhibited higher LINC-PINT expression than those with CNS-1. Higher expression of LINC-PINT in CNS-2 patients could be a defense mechanism by the body against CNS involvement owing to LINC-PINT tumor suppressor properties.

Interestingly, in the present study, poor responders showed significant increase in expression level of LINC-PINT. In agreement with this result a study revealed that colon adenocarcinoma (COAD) patients with low expression of LINC-PINT showed better responses to chemotherapy together with enhanced survival. This was explained on the basis that LINC-PINT exhibited a strong inverse correlation with immune indicators, indicating that LINC-PINT is negatively correlated with better immune response [64]. Consistent with this result a study showed that LINC-PINT level was negatively correlated with disease-free survival and overall survival as it induced cell proliferation, and repressed apoptosis via EZH2 targeting in clear cell renal cell carcinoma [65]. The twist in LINC-PINT expression pattern being negatively associated with ALL initiation then positively associated with poor prognosis may imply that LINC-PINT underlying processes may differ in different disease stages.

Worthy of note, LINC-PINT has a positive impact in regulating the gene expression of many genes as HDC, OASL, C17, VEGFA and HMOX1 [60]. In accordance, a positive correlation was found between LINC-PINT and HMOX1 protein level in the current study. Transforming growth factor-β1 (TGF-β1) signaling activation supresses tumor cell growth at early stages of tumor development [66,67], and might be one of the plausible links in the LINC-PINT/HMOX1 crosstalk. LINC-PINT overexpression was reported to promote TGF-β1 expression in PDAC cells [68]. Whereas, TGF-β_1 was capable of strongly inducing the expression of HMOX1 in human pulmonary epithelial cells [69]. LINC-PINT/miR-218–5p/HMOX1 axis could also be an additional link, as LINC-PINT is a competing endogenous RNA for miR-218–5p [70], and the latter was found to inhibit HMOX1 [71]. Thus LINC-PINT upregulation could suppress miR-218–5p overturning its inhibitory effect on HMOX1.

The HMOX1 coding gene is located on chromosome 22q13.1 [72]. Its expression is induced by several drugs and chemicals as statins, aspirin, niacin, oxidants, inflammatory mediators, heme [73], and physical stimulation (e.g. ultraviolet radiation), among others [74]. An earlier study suggested that activation of the HMOX1 and heme catabolic pathway may have beneficial effects on disease prevention [73]. The present study showed a significant decrease in HMOX1 level in newly diagnosed pediatric ALL. HMOX1 could be a superior diagnostic marker and a significant independent predictor of ALL. The current results are consistent with a study which reported ALL cells diminished growth after HMOX1 overexpression [60]. In contrast, HMOX1 level was elevated in many tumors as hepatoma, pancreatic and prostate cancers, to name a few, and further increased in response to irradiation and chemotherapy [75]. In addition, the current study revealed that the HMOX1 level mounted up at the end of remission induction therapy compared to patients' admission level. This observation can be explained on the basis of HMOX1 expression induction by LINC-PINT and in response to chemotherapy [60,75]. Analogously, HMOX1 was overexpressed in melanoma cell lines following treatment with arsenic trioxide or bortezomib [76,77].

In the current report, standard risk patients showed higher level of HMOX1 than those of low risk. This is consistent with the regulatory effects of HMOX1 on the apoptosis, proliferation, differentiation and drug resistance of leukemia cells of myeloid origin, where patients with higher levels of HMOX1 in AML cells had poorer survival outcomes compared to those with lower HMOX1 levels [78]. In agreement, HMOX1 expression was directly associated with patients' risk

Table 4
The association of ANRIL, LINC-PINT, CDKN2A and HMOX1 with sex and clinical data of ALL patients.

Parameter		ANRIL		CDKN2A		LINC-PINT		HMOX1	
Variable		Relative expression	P value	Relative expression	P value	Relative expression	P value	ng/ml	P value
Sex	**Boys**	5.603 (1.7–12.43)	0.4916	12.47 (3.29–74.93)	**0.0495**	0.1746 (0.03–1.59)	0.5448	0.9815 ± 0.8	0.4460
	Girls	3.827 (1.05–11.01)		1.506 (0.1–11.63)		0.1693 (0.01–0.72)		1.155 ± 0.95	
Age	**1–10 yr**	4.396 (1.26–11.21)	0.9791	11.71 (1.2–20.4)	0.8632	0.1746 (0.03–0.8)	0.6714	1.024 ± 0.85	0.7471
	>10 yr	4.396 (1.55–17.58)		8.023 (1.17–42.41)		0.2001 (0.01–3.13)		1.104 ± 0.91	
Risk group	**Low**	1.913 (0.39–5.6)	**0.0487**	0.844 (2.19–36.95)	0.9127	0.1746 (0.02–0.35)	0.8688	0.78 ± 0.8	**0.0401**
	Standard	5.526 (1.91–12.43)		12.47 (1.12–20.02)		0.1746 (0.02–1.72)		1.628 ± 0.96	
CNS status	**CNS-1**	23.76 (0.89–11.21)	0.546	27.39 (2.54–21.78)	0.3327	0.1459 (0.01–0.47)	**0.0022**	1.099 ± 0.9	0.4479
	CNS-2	30.25 (1.85–59.05)		18.88 (0.62–7.88)		1.843 (0.36–3.21)		0.8100 ± 0.59	
	CNS-3	20.50 (3.11–3.11)		26.00 (0.04–252.5)		–		0.6100 ± 0.3	
TLC	**< 50×10^9/L**	4.396 (0.83–12.43)	0.8464	12.47 (2.23–49.48)	0.1031	0.1459 (0.02–0.82)	0.4512	1.063 ± 0.89	0.8100
	> 50×10^9/L	4.396 (1.91–8.79)		3.437 (0.55–14.01)		0.2001 (0.02–2.06)		1.004 ± 0.8	
IPT	**B-cell**	3.556 (1.18–13.72)	0.9013	12.38 (1.21–25.87)	0.1922	0.1746 (0.04–0.95)	0.2823	0.8817 ± 0.63	**0.0043**
	T-cell	5.526 (1.67–6.22)		3.58 (1.22–12.47)		0.0873 (0.01–1.07)		1.609 ± 1.3	
MRD	**<0.01 %**	2.511 (0.57–5.02)	**0.0423**	12.47 (1.45–74.67)	0.3736	0.1033 (0.01–0.75)	**0.0073**	1.055 ± 0.96	0.9326
	>0.01 %	7.504 (2.15–7.5)		18.63 (12.47–42.97)		2.385 (0.17–30.52)		1.084 ± 0.67	

Data are presented as median (25 % −75%Percentiles) for ANRIL, CDKN2A and LINC-PINT and as mean ± SD for HMOX1.
Bold values denote statistical significance.
ANRIL, antisense noncoding RNA in the INK4 locus; **CDKN2A**, cyclin-dependent kinase inhibitor 2 A; **CNS**, central nervous system; **IPT**, immunophenotyping; **LINC-PINT**, long intergenic non-coding P53 induced transcript; **MRD**, minimal residual disease; **TLC**, total leucocytic count.

Table 5
The association of ABO blood group distribution with clinical data of ALL patients.

Parameter		A	B	AB	O	P value
Age	**1–10 yr**	17 (37 %)	9 (19.6 %)	5 (10.8 %)	15 (32.6)	0.3836
	>10 yr	6 (33.33 %)	6 (33.33 %)	0 (0 %)	6 (33.33 %)	
Risk group	**Low**	6 (37.5 %)	4 (25 %)	2 (12.5 %)	4 (25 %)	0.7931
	Standard	17 (35.42 %)	11 (22.91 %)	3 (6.25 %)	17 (35.42 %)	
CNS status	**CNS-1**	20 (37.74 %)	13 (24.5 %)	5 (9.44 %)	15 (28.4 %)	0.7968
	CNS-2	2 (25 %)	2 (25 %)	0 (0 %)	4 (50 %)	
	CNS-3	1 (50 %)	0 (0 %)	0 (0 %)	1 (50 %)	
TLC	**< 50×10^9/L**	15 (34.1 %)	11 (25 %)	3 (6.8 %)	15 (34.1 %)	0.9462
	> 50×10^9/L	7 (36.84 %)	4 (21.05 %)	2 (10.53 %)	6 (31.58 %)	
IPT	**B-cell**	20 (40 %)	10 (20 %)	5 (10 %)	15 (30 %)	0.2454
	T-cell	3 (21.43 %)	5 (35.71 %)	0 (0 %)	6 (42.86 %)	
MRD (day 43)						0.3577
	<0.01 %	13 (39.39 %)	9 (27.27 %)	2 (6.06 %)	9 (27.27 %)	
	>0.01 %	5 (62.5 %)	0 (0 %)	1 (12.5 %)	3 (37.5 %)	

Data are presented as numbers (percentage).
CNS, central nervous system; **IPT**, immunophenotyping; **MRD**, minimal residual disease; **TLC**, total leucocytic count.

stratification, as high-risk AML patients expressed greater HMOX1 mRNA and protein levels compared to lower risk groups [79].

ALL includes two subset groups, named B- or T-lymphoblastic leukemia [80]. Presumably, patients with T-cell ALL have poorer tolerance to chemotherapy and have increased risk of relapse, as the prognosis for T-cell ALL is inferior to that for B-cell ALL in most studies [81].

In the current study, HMOX1 level was found higher in T-cell ALL patients than in B-cell ALL patients. This might explain the HMOX1-associated poor clinical response and chemoresistance, as Zhe et al. reported that HMOX1 expression was higher in drug-resistant cell lines, and overexpression of HMOX1 decreased sensitivity to chemotherapy in primary AML cells [82].

As for exploring ABO blood groups distribution and their possible association with ALL etiopathology, the majority of the patients were of the A-blood type while the AB blood group was the least common. In congruence, previous studies showed that the AB-blood group type in hematologic malignancies was the least common [83,84]. Other study showed that the most common blood group of leukemia patients was B [25]. Additionally, the current study reported no statistically significant difference between controls and ALL patients regarding ABO blood group distribution. In line, several studies failed to find any statistically significant difference in the ABO blood group distribution in patients with acute leukemia when compared to the respective controls [25,85]. Contrastingly, a former study showed significant correlation between ALL and ABO blood groups, where blood group AB was associated with ALL higher risk [86]. This discrepancy may be attributed to different study populations and magnitude of patients with different ALL clinical subtypes.

Our results revealed the absence of association between blood groups and any of the patients' clinical features. The results also showed the lack of association with the initial treatment outcome. This may indicate that A and B antigens play no appreciable role in the pathways controlling ALL development.

The present study has some limitations. To begin with, patients in the current study were only recruited from National Cancer Institute-Cairo University and Dar El-Salam Cancer Center, which warrants conducting comparable multi-centered studies to gain more data and compare outcomes. Furthermore, the impact of COVID-19 pandemic affected our ability to collect more samples as it was the timeframe of the study. Future studies with larger sample size need to be conducted to replicate the current results.

5. Conclusion

The current study sheds light on the diagnostic potential of ANRIL, LINC-PINT, CDKN2A and HMOX1 in ALL discrimination, and introduces LINC-PINT and HMOX1 as possible predictors of the disease, with HMOX1 as a superior diagnostic marker and a prominent independent predictor. Moreover, being associated with poor ALL outcome, both ANRIL and LINC-PINT could serve as a valuable guide in ALL prognostics. Further, the study denotes that the lack of correlation with ALL initiation or progression disqualifies ABO blood groups in assessing the disease development. Finally, it could be inferred that initial curative effect of chemotherapy is attributable, in part, to restoring the levels of the oncogenic ANRIL and the tumor suppressors, LINC-PINT and HMOX1. The current findings can open new research avenues in the field of epidrugs that could target ANRIL or LINC-PINT to halt ALL development. Additionally, future invitro studies could assess the impact of these lncRNAs on specified chemotherapies and their associated curative effects so as to help in the selection of tailored drugs with optimum clinical outcomes.

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CRediT authorship contribution statement

Noha H. Sayed: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Formal analysis. **Mahmoud Hammad:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Safeya A. Abdelrahman:** Writing – original draft, Visualization, Resources, Methodology, Formal analysis, Conceptualization. **Hanan M. Abdelgawad:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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