Expression Profile of LncRNAs in Childhood Acute Lymphoblastic Leukemia: A Pilot Study

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Introduction

Acute lymphoblastic leukemia (ALL), also called acute lymphocytic leukemia, is a tumor type characterized by the prevalence of premature lymphocytes formed by the bone marrow. It is a form of blood and bone marrow malignancy that damages white and red blood cells, as well as platelets. Childhood ALL is highly prevalent in children and adolescents and accounts for a quartile of all childhood cancers. Leukemia is a type of hematological disorder. It is characterized by the oligoclonal expansion of hematopoietic cells that have been abnormally or poorly differentiated and hence can infiltrate the bone marrow and even invade the blood and other tissues.

Generally, there are four main subtypes of leukemia, based on whether they are acute or chronic, and myeloid or lymphocytic. Accordingly, chronic lymphoblastic leukemia (CLL), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and acute myeloid leukemia (AML) have been defined. Childhood ALL, as abovementioned, is a frequent malignancy in children and adolescents aged 1 to 14 years old and accounts for ≈25% of all childhood leukemia cases. Precursor B cell ALL (pre B-ALL) is a main type of ALL comprising 85% of ALL cases. Several studies have reported that the expression of some molecular markers may contribute to the classification of subtypes, as well as improving the ALL prognosis.

LncRNAs as a new type of molecules play controlling roles on several processes, including tumorigenesis and pluripotency. They are also identified with functional roles. Some RNAs (i.e., fRNAs, non-messenger RNAs (nmRNAs), or non-protein-coding RNAs (npcRNAs)), are practical RNA molecules which do not code proteins. Although the implication of IncRNAs has been verified in...
ALL initiation, progression, and treatment response, their
effect functional role has poorly been understood.\textsuperscript{15-17}
LncRNAs, exceeding 200 nucleotides in length, are
defined as regulators of different cellular processes such as
pluripotency, reshaping of chromosomes, transport of
intracellular molecules, oncogenesis, and transcription.
In this regard, the tissue specific expression of LncRNAs
has been described despite their predominantly low
abundance. Therefore, the study of tissue specificity of
LncRNAs could be interesting due to their role in cell
migration, proliferation, reaction to cytotoxic drugs, and
DNA damage especially in tumor cells. For example,
Ghazavi \textit{et al.} \textsuperscript{19} reported the changes in the expression of
LncRNAs in ALL cancer.

Another study also emphasized the association of LncRNAs
with leukemia development. Moreover, LncRNAs BALR-2
and BALR-6 were shown to be involved in cell endurance
or glucocorticoid reaction, both in human and mouse B
cells.\textsuperscript{11,19,20} Furthermore, it was revealed that transcription
profiles of LncRNAs can distinguish pre-B ALL subtype
precisely and can act as prognostic biomarkers.\textsuperscript{11,20} Further
investigations have explored the diagnostic potentials of
LncRNAs in ALL, as well as their biological and epigenetic
activities. Despite these efforts, the diversity of actions and
complex molecular interactions coupled with the contrasting
subtypes of LncRNAs still mask a complete comprehension
of therapeutic improvement.\textsuperscript{16,17,22} Intergenic LncRNA
RP11-68118.10 is located on chromosome 1. This LncRNA
is overexpressed in cardiac and skeletal muscles.\textsuperscript{23} Ouimet
\textit{et al.} \textsuperscript{24} showed that LncRNAs RP11-137H2.4 and RP11-
68118.10 were downregulated in pre-B ALL. The RP11-
137H2.4 gene is located on chromosome 10 and is an
antisense LncRNA type. LncRNAs RP11-624C23 and RP11-
203E8 are respectively intronic and intergenic, and their
genes are located on chromosome 8.

Previous studies have shown that overexpression of this
LncRNA in pre B-ALL cells can induce malignancy. In some
studies, it has been shown that three LncRNAs, RP11-446E9,
RP11-624C23, and RP11-203E8 were downregulated in pre
B-ALL.\textsuperscript{11,21} In this regard, our research focused on deciphering
the genetic changes and expression of LncRNAs RP11-
624c23.1, RP11-137H2.4, RP11-203E8, RP11-446E9, and
RP11-68118.10 in patients with ALL in order to understand
their role in disease pathogenesis and also their diagnostic
potential.

Material and Methods

\textbf{Study subjects}

In this study, we obtained 160 blood samples. Eighty
samples were obtained from patients, who had referred to
“Tabriz Pediatric Hospital” during the years 2018-2019,
and definitely diagnosed with leukemia. While, remaining
80 samples were obtained from healthy volunteers and
used as control. The study subjects were children between
the ages 1 and 14 who were pathologically diagnosed with
lymphoblastic malignancy. The samples were taken after
a written consent form was signed by the parents or legal
guardians of the children. For the inclusion criteria, patients
with no history of acute viral infection, autoimmune
diseases, and endocrine disorders were considered. The
control group included samples obtained from healthy
subjects. Approval for this study was obtained from the
Ethics Committee of Tabriz University of Medical Sciences.

\textbf{Sampling}

Intravenous blood samples were obtained from each
individual in both groups. Samples were put into Falcon
tubes containing EDTA as an anticoagulant. Then the
samples were transferred to the laboratory, where they
were preserved at -80°C freezing conditions.

\textbf{RNA extraction from blood samples and cDNA synthesis}

Total RNA was extracted from the blood samples using
Trizol reagent kit (Macherey-Nagel Co, Germany 740304),
according to the manufacturer's instructions. To test the
quality of extracted RNA, it was run on 0.5% agarose
gel. Completely dissolved RNA was appeared as a very
low molecular weight smear. , A UV spectrophotometer
(NanoDrop ND-1000; Wilmington, DE) with an
absorbance ratio of 260/280 was used to measure the
concentration of the extracted RNA. The extracted RNA
was then stored in RNase-free ultra-pure water at -70 °C.
Afterward, cDNA was synthesized from the extracted
RNA using the Quantitative Test Reverse Transcription Kit
(Qiagen, Catalog No: 205310), with all the steps performed
on ice as summarized below.

\textbf{RT-qPCR (quantitative reverse transcription-PCR)}

We used the RT-qPCR technique to identify gene
expression changes of LncRNAs RP11-203E8, RP11-
624c23.1, RP11-446E9, RP11-137H2.4, and RP11-68118.10
in ALL and normal samples. Specific primers for the target
genes were designed to examine their expression at the
RNA level in the Gene runner software (Version 3.05).
To avoid binding of designed primers to other sequences
similar to the target gene sequences, BLAST\textsuperscript{1} was run
(www.ncbi.nlm.nih.gov/BLAST). To detect changes in
each gene expression in ALL and control samples, RT-
qPCR was accomplished using the following monitored
primers:

\begin{itemize}
  \item for LncRNA RP11-624C23.1: \textsuperscript{1}
    \begin{itemize}
      \item forward: 5'-GGGTCTTACCCGACGTGAG-3'
      \item reverse: 5'-CGCGTGTTGAGGACTACAGA-3'
    \end{itemize}
  \item for LncRNA RP11-446E9: \textsuperscript{1}
    \begin{itemize}
      \item forward: 5'-GAGTTCCCTCCTCCCTCCTCCTCCTCCTCCTCCTCC-3'
      \item reverse: 5'-AGGTTCCCTCCTCCCTCCTCCTCCTCCTCCTCCTCC-3'
    \end{itemize}
\end{itemize}
LncRNAs in Childhood Acute Lymphoblastic Leukemia

The relative expression of lncRNAs was determined in ALL patients. The results revealed that lncRNAs RP11-624c23.1, RP11-203E8, RP11-446E9, RP11-137H2.4, and RP11-68118.10 expression levels were assessed with clinical immune-phenotype features of ALL patients (Table 1). The median amount ≥1 of the lncRNAs expression levels was considered as an upregulation cluster, and the median amount ≤1 was designated as a downregulation group. The association between these two variables was not significant. The expression profile of these five lncRNAs was meaningfully downregulated in ALL patients compared to the control group (P<0.0001, P=0.0007, P=0.0616, P=0.0292, P<0.0001) (Figure 1).

Moreover, the findings showed that the lncRNAs RP11-624c23.1 gene expression was significantly decreased in ALL samples compared to the control samples (P<0.0001) (Figure 2).

In addition, the relationship between the lncRNAs- RP11-624c23.1 expression changes and the immune-phenotype was studied in ALL patients. The results revealed that lncRNA RP11-624c23.1 expression changes did not significantly associate with the immune-phenotype in ALL patients (P=0.963) (Table 1).

In this study, the association of lncRNAs RP11-624c23.1, RP11-203E8, RP11-446E9, RP11-137H2.4, and RP11-68118.10 expression levels was assessed with clinical immune-phenotype features of ALL patients (Table 1).

| Regulation | RP11-203E8 | RP11-624c23-1 | RP11-446E9 | RP11-137H2-4 | RP11-68118-10 |
|------------|------------|---------------|------------|---------------|---------------|
| Value      | DR<sub>a</sub> | <1     | DR<sub>a</sub> | <1     | DR<sub>a</sub> | ≤1   | ≤1   | <1   | ≤1   | ≤1   | ≤1   | ≤1   | ≤1   |
| T. Cell    | 29.1       | 5.9    | 30.8       | 4.4    | 19.1          | 16.1 | 26.4 | 17.5 | 30   | -    | -    | -    | -    |
| Pro B Cell (Early B Cell) | 10.3       | 2.9    | 10.2       | 2.8    | 8.8           | 4.4  | 11.7 | 1.4  | 11.25 | -    | -    | -    | -    |
| Pre B Cell | 17.5       | 3.6    | 28.9       | 4.4    | 26.5          | 5.9  | 27.9 | 14.5 | 41.25 | -    | -    | -    | -    |
| B Cell     | 11.8       | 1.5    | 11.6       | 1.4    | 10.3          | 2.9  | 11.8 | 1.5  | 11.2  | -    | -    | -    | -    |
| Mixed      | 5.8        | 1.6    | 5.9        | 1.5    | 7.3           | 1.3  | 4.4  | 2.9  | 6.3   | -    | -    | -    | -    |
| P-value    | 0.161      | 0.963  | 0.286      | 0.55   | -             | -    | -    | -    | -    |

<sup>a</sup>Down regulation, <sup>b</sup>Up regulation

Figure 1. The relative expression of lncRNAs RP11-624c23.1, RP11-137H2.4, RP11-203E8, RP11-446E9, RP11-68118.10 in patients with ALL and in normal samples.

Table 1. Association of lncRNAs RP11-624c23-1, RP11-203E8, RP11-446E9, RP11-137H2-4, RP11-68118-10 expression levels with clinical immunophenotypic features of ALL patients.
The current study evaluated the expression levels of lncRNAs RP11-137H2.4, RP11-203E8, RP11-446E9, RP11-624C23, and RP11-68118.10 in patients who were positively diagnosed with Acute Lymphoblastic Leukemia (ALL) and their possible alterations compared to the normal cases.

ALL is one of the most prevalent cancers among children with an average age of <10 years and is defined by the aggressive multiplication of white blood cells and their mal-developed stem cells. ALL is categorized into several groups (mainly based on their CD cell surface markers) with respect to the immune-markers and clinical data, namely: T-cell, Pro B-Cell, Pre B-Cell, B-Cell, and Mixed. This categorization is done mostly based on blood lymphocyte markers (CD markers).

Considering high mortality rate associated with this disease, early diagnosis and appropriate treatment strategies can be very helpful. One of the most effective strategies for early diagnosis is the use of molecular biomarkers. Investigating the changes in the expression levels of the biomarkers indicates the importance of early diagnosis and treatment. LncRNAs are regarded as one of the most reliable and effective molecular biomarkers. These non-coding molecules, which play significant roles in modifying gene expression...
and epigenetic alterations, are expressed in different levels in different cancers such as breast tumor, lung cancer, prostate malignancy, and ALL. Therefore, evaluation of the expression levels of these molecules at different stages of cancer may present new biomarkers for cancer detection.

Changes in the expression of IncRNAs can subsequently lead to changes in the expression of target genes, in addition to stimulation of changes in the dependent signaling pathways. Thus, the study of IncRNAs can be of considerable importance while investigating the transcription level and subsequent translation. Furthermore, expression of IncRNAs can lead to changes in the induction of signal pathways such as RAS / MAP kinase, NFkB, AKT, b-catenin, and so on.

In addition to the abovementioned, various IncRNAs have shown different expression levels in various cancers, including breast, prostate, and gastric, as well as acute lymphoid carcinoma in children, and the association with cancer phenotypes such as migration, metastasis, and apoptosis has also been observed. A study examined the changes in the expression of IncRNAs in ALL cancer and proved it.

Fernando et al. displayed that CASC15 lncRNA could regulate the SOX4 gene in acute myeloid leukemia (AML). This gene confers a critical role in the development and evolution of B-cells. Furthermore, Sox4 plays an important role in the B-catenin signaling pathway, and consequently, altering the expression level of the IncRNA CASC15 can induce changes in this signaling pathway. It displays the importance of evaluating the investigated IncRNAs.

In a study by Wallaert et al. on important subclasses of IncRNAs for each of the T-ALL genetic subclasses, linked pattern of IncRNA expression in T-ALL subclasses with diverse stages of healthy T cell evolution in the thymus was evaluated. Similarly, Casero et al. in their study, concluded that the co-expression of protein-coding genes near IncRNA genes demonstrated development for oncologies associated with the lymphoid variation.

We chose IncRNAs RP11-68I18.10, RP11-137H2.4, RP11-446E9, RP11-624C23.1, and RP11-203E8 and investigated the expression profile of these five IncRNAs in ALL patients. Recent studies have shown that these five IncRNAs significantly affect different cellular processes in pre-B-ALL. The relationship between the immune-phenotype of ALL samples and the expression level of each IncRNA was also evaluated.

In the present study, we identified and analyzed the medical and pathological indication of immune-phenotype in patients with ALL, and assessed their association with the expression levels of the abovementioned IncRNAs one by one. Analysis of samples by classifying ALL in each case and comparing the expression level of each IncRNA showed no significant relationship between immune-phenotype of ALL samples and expression level of each IncRNA. To confirm the findings of the current study and the expression changes of IncRNAs in ALL and healthy samples, Fang et al. evaluated the expression levels of different IncRNAs in MLL-r cancer, and revealed changes in the expression levels of many of these IncRNAs. These changes in expression levels were compared between the control and unhealthy samples. The results of this study are consistent with those of our study in that they indicated a change in the expression of IncRNAs in both leukemia samples and control samples. The results of our study displayed a significant decrease in the expression of IncRNAs RP11-68I18.10, RP11-446E9, RP11-624C23.1, RP11-203E8, and RP11-137H2.4 in ALL samples compared to the healthy control samples. These expression changes can specifically reflect expression changes in a particular type of cancer.

Consistent with the present study, a study was conducted by Gioia et al. on 56 pre-B-ALL cancer samples to investigate the expression changes and roles of IncRNAs RP11-203E8, RP11-624C23.1, and RP11-446E9. These IncRNAs, which play a role in regulating metastasis and relocation of blood leukemic cells, were down-regulated in this cancer. Fernando et al., illustrated that the gene expression levels of IncRNAs RP11-446E9 and RP11-624C23.1 decreased in ALL. Our findings showed that the IncRNA RP11-624C23.1 expression was significantly decreased in ALL patients compared to the control subjects (P<0.0001). Additionally, we studied the association between IncRNA RP11-624C23.1 expression variations and immune-phenotype in these patients. Our findings revealed the alteration in the expression level of this IncRNA was not significantly associated with the immune-phenotype in ALL patients (P=0.963).

Our results revealed that IncRNA RP11-203E8 was downregulated in ALL patients compared to the control samples (P=0.0007), and the association between the expression level of this IncRNA and immune-phenotype in ALL patients was not significant (P=0.161). Our results also showed that the IncRNA RP11-446E9 was downregulated in ALL patients (P=0.616). The results of our study about IncRNAs RP11-203E8 and RP11-624C23.1 corroborate the results of Fernando et al. and is consistent with those of Gioia et al. Furthermore, the results of these two articles are in line with those of our study in terms of IncRNA RP11-446E9 expression in ALL cases.

Increased expression of IncRNAs RP11-203E8 and RP11-624C23.1 increases apoptosis and decreases phosphorylation of H2A.X, which is involved in response to DNA damage. Interestingly, the increased expression of both IncRNAs produced similar phenotypic effects, which indicate that the two IncRNAs contribute to the same molecular pathway.

Moreno et al. reported that the increased expression of IncRNA RP11-446E9 reduced the rate of migration and proliferation of leukemia cells, indicating the important role of this IncRNA in signaling pathways, which are dependent on the migration and proliferation. Another study demonstrated that an increased expression of IncRNAs RP11-624C23.1, RP11-446E9, and RP11-203E8
results in augmented apoptosis while facing genotoxic stress, which displays the contribution of these lncRNAs in apoptosis-dependent signaling pathways. Moreover, it was shown that RP11-203E8 and RP11-624C23.1 play important roles in regulating DNA damage response (DDR). LncRNA RP11-446E9 plays a role in activating cell death initiated by DNA damage, however it does not play a role in response to DNA damage. In addition, the increased expression of this lncRNA results in a decreased proliferation and cell migration.42

The studies of Ouimet et al. demonstrated that lncRNAs RP11-137H2 and RP11-68I18.10 were downregulated in pre B-ALL. Our study reflected a decrease in the expression level of lncRNA RP11-137H2.4 in ALL samples, which agrees with previously done research.10-24

In addition, our study indicated the relationship between the LncRNA RP11-137H2.4 expression changes and the immune-phenotype in ALL patients was not significant (P=0.55) (Table 1). The current study further revealed that lncRNA RP11-68I18.10 was downregulated in ALL patients compared to the control samples; this is consistent with a recent article. The association between this lncRNA expression variations and immune-phenotype in ALL patients also was not significant (Table 1). LncRNA RP11-68I18.10 is overexpressed in cardiac and skeletal muscles and this result is not in agreement with our study results.

Previous studies have displayed that overexpression of lncRNA RP11-137H2.4 in pre B-ALL cells can encourage malignant performances, for example, improved resistance to apoptosis, cell proliferation, and cell migration. In addition, genes of the MAPK signaling pathway are downregulated, resulting in RP11-137H2.4 silencing. The study of Ouimet et al. revealed that lncRNAs RP11-137H2 and RP11-68I18.10 were downregulated in pre B-ALL, and describing the precise roles of RP11-137H2.4 in cell cycle pathways and NRAS/BRAF/NF-xB MAPK cascade are significant enough to expand new therapeutic methods to overcome GC opposition in children treated for ALL. Furthermore, RP11-137H2.4 knockdown significantly increases apoptosis in the cells treated with camptothecin, prednisolone, and doxorubicin. Although suppressing RP11-68I18.10 was obligatory, it had no influence on apoptosis. Additionally, the levels of unusual effects on apoptosis are comparatively different, and they cause the deregulation of IncRNA in pre B-ALL. This effect determines specific lncRNAs despite having great effects on leukemia types. In this study, the expressions of aforementioned lncRNAs were reported to be reduced. Further studies are needed on the mentioned lncRNAs, and for defining the association of specificity and difference in lncRNA expression levels with ALL classes.

Conclusion

Results displayed a significant decrease in the expression levels of LncRNAs RP11-203E8, RP11-624C23.1, RP11-446E9, RP11-137H2.4, and RP11-68I18.10 in ALL patients compared to the control cases. Given the obtained results, an important prospective prognostic assessment is required to be done on these lncRNAs. They can also be used as a new diagnostic kit, as well as therapeutic tolerance in future. Moreover, studies on the mentioned lncRNAs and other lncRNAs are needed to identify the signaling pathways and the target genes of these lncRNA and their role in the tumor progression.

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Ethical Issues

The ethical approval was obtained from the Ethics Committee of Tabriz University of Medical Sciences (Code of Ethics: IR.TBZMED.REC.1398.732). The written informed consent form for participation in the study was also signed by the parents or legal guardians of the children.

Author Contributions

ZM and SG: Designed the study, ZM: Performed the experiments and wrote the paper with input from all authors. AR: Contributed to sample preparation. LR and BJ: Developed the theoretical framework and analyzed the data. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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