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Research Article

Evaluation of Methylation at Promoter Regions of Long Non-coding RNAs in Patients with Acute Lymphoblastic Leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a fetal hematologic disorder that is mostly observed in children. Both B and T lymphocytes have been reported to play a role in ALL etiology. Long non-coding RNAs (lncRNAs) are large regulatory molecules with more than 200 nucleotides that participate in various cellular processes. Methylation at the promoter regions of these regulatory molecules has been reported to vary between ALL patients and healthy controls. This study aimed to evaluate methylation status at promoter regions of lncRNAs between these two groups.

Methods: In the current study, 80 ALL patients and 80 healthy controls were enrolled. The intravenous blood samples were obtained from all patients and controls. The extracted DNA from blood samples underwent sodium bisulfite treatment. Thereafter, methylation levels in the promoter regions of lncRNAs RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10 were evaluated using methylation specific-high resolution melting (MS-HRM). Moreover, the receiver operating characteristic curve (ROC) analysis was performed to examine the sensitivity and specificity of the tests.

Results: The methylation levels of all studied lncRNAs including RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10 were significantly increased ($p<0.05$). ROC curve analysis also showed that all lncRNAs could be used as diagnostic markers.

Conclusion: This study showed that methylation alterations of lncRNAs could be considered as novel biomarkers for early detection of ALL. Furthermore, owing to the possible role of studied lncRNAs as tumor suppressors, they could be reliable treatment targets for methylation modifications. Further research is still required to elucidate the role of these lncRNAs in ALL etiology.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous hematological disorder in which hematopoietic stem cells are hindered at early stages of differentiation. These malignant cells may clonally expand in the bone marrow, peripheral cells, or other organs.[1] The ALL incidence was 1.57 per 100000 people in the United States in 2014. In 2018, 5690 new ALL cases and 1470 deaths were reported in the US.[2,3] The age range in the majority of ALL cases is between 1-4 years and its incidence significantly decreases during adulthood.[2] Both lineages of lymphocytes, T and B cells, are involved in ALL pathogenesis; however, in the majority of pediatric cases, B precursor type is involved.[4] Despite the vast conducted research, there are still many ambiguities about ALL etiology. However, there is an agreement that both genetic and environmental factors contribute to its occurrence.Given the role of non-coding RNAs (ncRNAs) in regulating physiological processes, they are considered responsible agents for connecting the genetic and environmental factors.[5]

NcRNAs are a group of regulatory molecules that are not translated to proteins. They consist of different families including small nuclear RNAs (snRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and long non-coding RNAs (lncRNAs).[6] lncRNAs are one of the recently identified molecules with a length of more than 200 nucleotides. They have some similarities with mRNAs including a cap at 5' end, containing more than one exon, being transcribed by RNA polymerase II (RNA pol II), and being located in the cytoplasm or the nucleus. However, this class of ncRNAs has some differences with mRNAs

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including poor conservation among species, inability to transcribe to a protein, lower expression level, and tissue and stage specific expression.[7] Up/down regulation of lncRNAs in cancerous cells compared to healthy ones indicates these molecules may act either as an oncogene or a tumor suppressor.[8] Despite extensive study of lncRNAs in a variety of cancers, there is still little information about their participation in ALL pathophisiology.[9] Previous studies identified many lncRNAs with aberrant expression levels in acute leukemia that participated in tumor suppression, chromosomal translocations, myeloid differentiation, autophagy, chromatin remodeling, apoptosis, DNA methylation, and cell migration.[7]

DNA methylation, which mainly occurs in the context of CpG dinucleotides, is a cellular regulatory mechanism that participates in numerous processes including genome imprinting, X-inactivation, and gene expression regulation.[10] Hematopoietic stem cells undergo extensive DNA methylation changes during differentiation. Basically, these alterations are determinants of cell fate rather than a simple consequence of changes during hematopoietic development.[11,12] It has been reported methylation changes participate in the development of hematologic malignancies which result in transcriptional iregularities.[13] Interestingly, DNA methylation alteration can be used as a novel biomarker for prognosis, diagnosis, and treatment of hematologic malignancies.[14] Milani *et al.*[15] identified methylation changes in 40 genes that could be used to discriminate between ALL subtypes and also between ALL and healthy controls. In a recent study, pediatric T-ALL cases were classified based on their DNA methylation profiles. Moreover, the methylation status of T-ALL was altered compared to normal thymocytes.[16] Genes with aberrant methylation levels are involved in main cellular processes related to cancer progression including cell cycle regulation, apoptosis, gene expression, and DNA-damage response.[17]

In the current study, methylation changes of five lncRNAs were studied. These lncRNAs have been previously reported to correlate with cancer and ALL. RP11-137H2-4 was upregulated in pre-B ALL, as well as participating in cancer specific features such as cell proliferation, migration, apoptosis, and response to treatment. Moreover, suppressing this lncRNA in a glucocorticoid (GC) resistant pre-B ALL cell line resulted in a normal glucocorticoid (GC) response and adjusted the expression of members of both the NRAS/BRAF/NF-κB MAPK cascade and cell cycle pathway.[18] Gioia *et al.*[19] reported that RP11-624C23.1, RP11-203E8, and RP11-446E9 participated in apoptosis, cell proliferation, migration, and DNA damage response (DDR) in cancer cells. Furthermore, both RP11-624C23.1 and RP11-203E8 showed an equivalent impact on DDR, as well as higher apoptosis levels in response to genotoxic stress. Yet silencing of these two lncRNAs by the DDR pathway could provide a selective advantage to leukemic cells by elevating resistance to the DNA damages. RP11-68I18.10 which had an elevated expression in the cardiac and skeletal muscles, along with RP11-137H2.4, RP11-624C23.1, RP11-203E8, and RP11-446E9 showed significantly decreased expression levels in children with ALL.[20,21]

Regarding previous studies, methylation alterations in the promotor regions of five lncRNAs including RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10 were studied. The results of this study may help to have a better understanding of epigenetic alterations in ALL. The results might also be used as a novel biomarker for the classification and diagnosis of this fatal disease.

Materials and Methods

Participants and sample collection

In the present study, a total of 160 subjects, comprised of 80 ALL patients and 80 healthy controls were enrolled. The patients were recruited from "Tabriz Pediatric Hospital" during the years 2018-2019. The children between the ages 1 to 14 who were pathologically diagnosed with lymphoblastic malignancy were included. Cases with a history of acute viral infection, autoimmune diseases, and endocrine disorders were excluded from the study. Healthy control samples were taken from people whose medical examinations and routine laboratory tests were normal and who had not used any specific medication. Blood samples were obtained from the ALL patients before undergoing the chemotherapy process. The blood samples were also collected from the healthy volunteers without a history of cancer or other severe illnesses. Afterwards, the samples were stored in a -80°C freezer. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1398.732). Written consent forms were also signed by the parents or legal guardians of the children. The samples were obtained according to the medical and ethical guidelines of Tabriz University of Medical Sciences to ensure participants' safety.

DNA extraction and sodium bisulfite treatment

In brief, total Genomic DNA was extracted from blood samples using a FavorPrep Blood Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's guidelines. Afterwards, the concentration and quality of extracted DNA was measured using a NanoDrop spectrophotometer. To remove possible RNA contamination, DNA samples were treated with RNAase I (Takara, Japan). The samples with DNA concentration lower than 100 ng/μL or the A260/A280 ratio outside the range of 1.7-1.9 were excluded. DNA samples then underwent sodium bisulfite conversion using an EZ-96 DNA Methylation-Gold™ (Zymo Research Corp. Irvine, CA, USA), according to the company instructions. In all steps, DNA samples were stored at -20°C immediately.

Methylation specific-high resolution melting

To evaluate methylation alterations between cases and controls, the methylation specific-high resolution melting (MS-HRM) method was applied in this study. According

to the principles of primer design for HRM, specific primer pairs were designed (www.ncbi.nlm.nih.gov/BLAST) (Accession number: 596-4067-4790-4067-2553) for GC-rich segments of promotors using Gene Runner (version 3.05) (Table 1). Each PCR reaction contained 2 µL of the bisulfite-treated DNA template, 10 µL of master mix (SYBR Premix Ex Taq™ II), 2 µL of specific primer pairs, and 6 µL of double-distilled water. The 2-step Real-time PCR (Applied Biosystems, StepOnePlus) was performed as "initial denaturation at 95˚C for 5 minutes; 40 cycles of denaturation at 95˚C for 10 seconds, annealing and extension temperature at 57 °C for 20 sec, and elongation at 72˚C for 25 sec". Then, HRM analysis was performed at a temperature range of 55˚C-95˚C with a ramp rate of 0.3˚C/15 seconds. The control DNA (HUM Diagnostic Biotechnology, Iran) with known methylation ratios of 0%, 25%, 50%, 75%, and 100% was used in each assay to interpret the methylation levels of unknown samples. The resulting melting curves were analyzed using HRM software v.2.2 (ABI Applied Biosystems, USA). The melting curves were normalized relative to 2 normalization regions before and after major fluorescence reductions. The resulting plots from the samples with unknown methylation statues were displayed relative to the standard curves.

Statistical analysis

Nonparametric tests were used when data were abnormally distributed. Mann-Whitney U and t-tests were also employed for statistical analyses. Moreover, the receiver operating characteristic curve (ROC) analysis was carried out to examine the sensitivity and specificity of the tests.
Each real-time PCR reaction was done in triplicate. P-value<0.05 was considered as statistically significant. All statistical analyses were performed with SPSS software version 22 (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics and clinical features

In the current study, 80 patients with ALL were enrolled. The age of the patients ranged from 2 to 14 years old. The obtained immunophenotyping data indicated pre-B cell ALL and mixed-ALL were the most and the least types among the patients, respectively. In detail, among 80 patients, 33 patients with pre-B cell ALL (45.21%), 9 patients with pro-B cell ALL (11.25%), 9 patients with B cell ALL (11.25%), 5 patients with mixed-ALL (5%), and 24 patients with T cell ALL (11.25%) were diagnosed (Table 2).

Table 2. Clinical data of the patients.

Disease phenotype	Number of patients	Percentage	Female	Male
Pre-B cell	3	21/45	12	21
Pro-B cell	9	25/11	4	5
B cell	9	25/11	6	3
Med-x ALL	5	25/6	2	3
T cell	24	30	11	13

Quantification of DNA methylation by MS-HRM assay

The MS-HRM method was used to evaluate the methylation alterations in lncRNAs (RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10) in patients with ALL and in healthy volunteers as controls. In this method, the samples with unknown methylation levels were compared to the control samples with adjusted methylation levels (0, 25, 50, 75, and 100%). The normalized melting curves along with ALL cases and healthy controls in each lncRNA are depicted in Figures 1 and 2. In each lncRNA, only one location in their promotor region was studied. Statistical analyses showed that in analyzed lncRNAs, the methylation alterations were significant between cases and controls ($P \leq 0.05$). As summarized in Table 3, the methylation levels of RP11-624c23.1, RP11-203E8, RP11-446E9, RP11-137H2.4, and RP11-68118.10 were 14.27% (P=0.000003), 6.828% (P=0.000092), 1.551% (P=0.02447), 3.184% (P = 0.001837), and 25.88% (P=0.000086), higher in cases with ALL compared to the healthy controls, respectively(n=80). The bar chart is as Figure 3.
ROC curves analysis was done to study the capability of methylation alterations in lncRNAs as therapeutic biomarkers. As depicted in Table 4 and Figure 4, alterations in all lncRNAs could be used as diagnostic biomarkers. Among these lncRNAs, RP11-624c23.1 with a 0.8962 area under curve (AUC) had the highest diagnostic capability.

Discussion

Hematopoietic stem cells (HSCs) undergo various stages of specification, differentiation, and expansion to produce mature blood cells. This complex process, the so-called haematopoiesis, requires strict regulation of the involved mechanisms such as gene expression and signal transduction between HSCs and bone marrow.[22] It has been reported that epigenetic factors like DNA methylation has a fundamental role in regulating hematopoiesis.[23,24] Different quantitative and qualitative methods have been utilized to detect DNA methylation alterations.[25] Methylation sensitive-high resolution melting (MS-HRM) is a simple

Table 1. Primers used for MS-HRM.

Primer sequence (reverse)	Primer sequence (forward)	Gene name
5′- ACGCCTAATTTCCTATACGTACGT-3′	5′- CGTTTATTGAGTTATTGTCGTTCG-3′	RP11-624C23.1
5′- AACGAAAACGAAAAATAAAATCG-3′	5′-GAAGTTAGAGTTTCGTAGGGGTC-3′	RP11-446E9
5′-AATCGCACACTGAAACGCAC-3′	5′-CACGGTCCGGAATATCCACC-3′	RP11-137H24
5′- TCCCAACTACTTCCTAATACTCGAA-3′	5′-TTTAGGTTTTTTTGGAGATTTTTTC -3′	RP11-203E8
5′- TAAAAACTACGAACGACCATATACG-3′	5′-AAGTTTAAAATTTTAAAGATTTCGT-3′	RP11-68I18.10

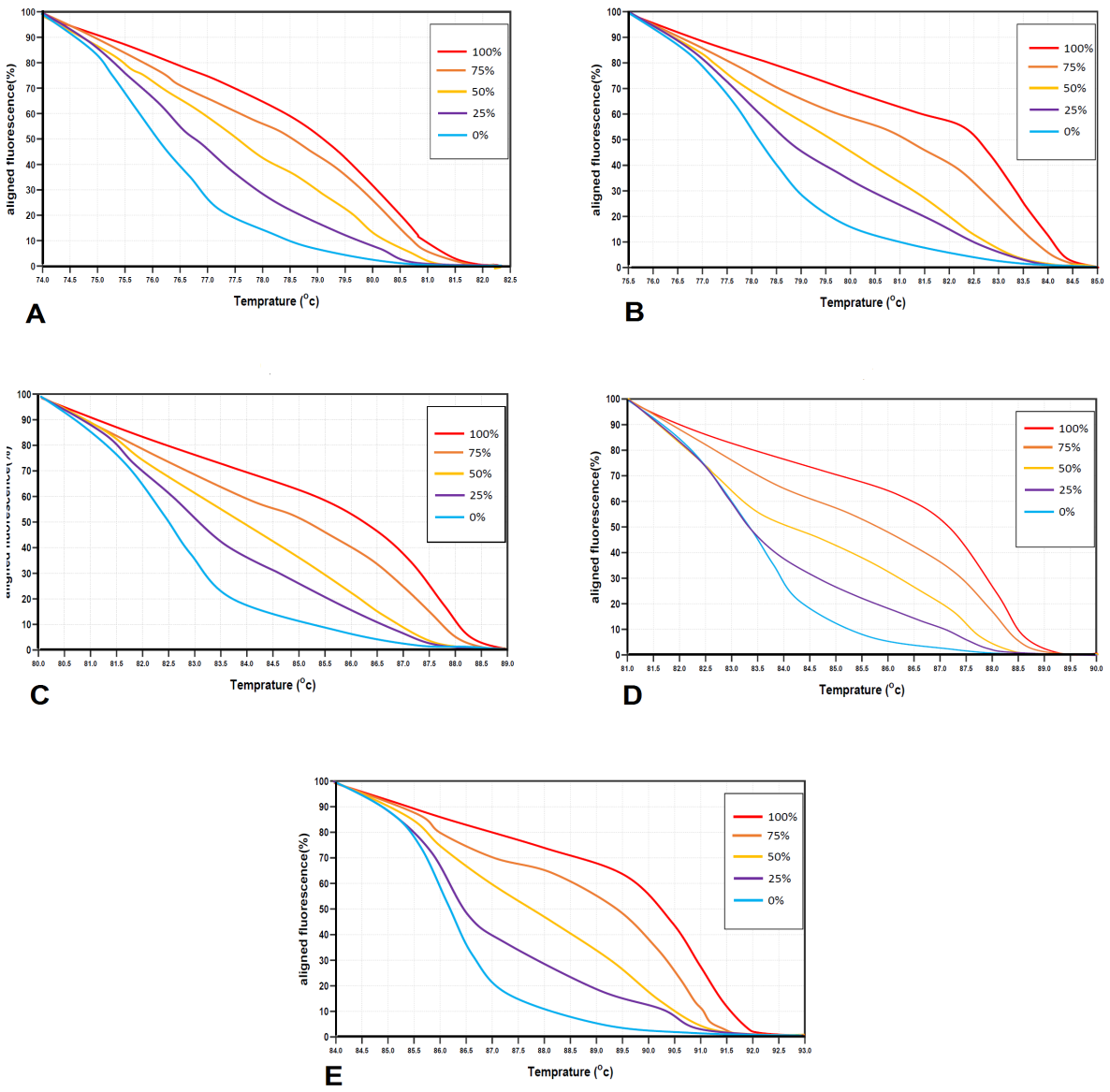

Figure 1. HRM control melting curves for each studied lncRNA. A) RP11-446E9, B) RP11-203E8, C) RP11-68118.10, D) RP11-624c23.1, E) RP11-137H2.4.

Table 3. Mean±SE methylation level in cases and controls (n=80).

LncRNA	Methylation level in ALL cases (%)	Methylation level in controls (%)	Difference	*P*-value
RP11-624c23.1	26.8±1.952	12.53±2.111	14.27	0.000003
RP11-203E8	23.67±1.041	16.85±1.92	6.828	0.00009
RP11-446E9	15.68±1.828	14.13±1.848	1.551	0.02447
RP11-137H2.4	17.63±1.041	14.45±1.088	3.184	0.00184
RP11-68118.10	42.82±4.435	16.94±1.43	25.88	0.00009

Table 4. Diagnostic performance of studied lncRNAs.

LncRNA	Area under curve (95 CI%)	Cutoff (%)	Sensitivity (%)	Specificity (%)	*P*-value
RP11-624c23-1	0.8962 (0.5274 to 0.9044)	> 22.07	0.7647	0.8824	0.0001
RP11-203E8	0.846 (0.5897 to 0.9381)	> 19.78	0.8235	0.8235	0.0006
RP11-446E9	0.6107 (0.8157 to 1.000)	> 10.65	1	0.2353	0.0370
RP11-137H2.4	0.7993 (0.5897 to 0.9381)	> 15.75	0.8235	0.7647	0.0029
RP11-68118.10	0.8339 (0.5897 to 0.9381)	> 27.79	0.8235	0.8235	0.0009

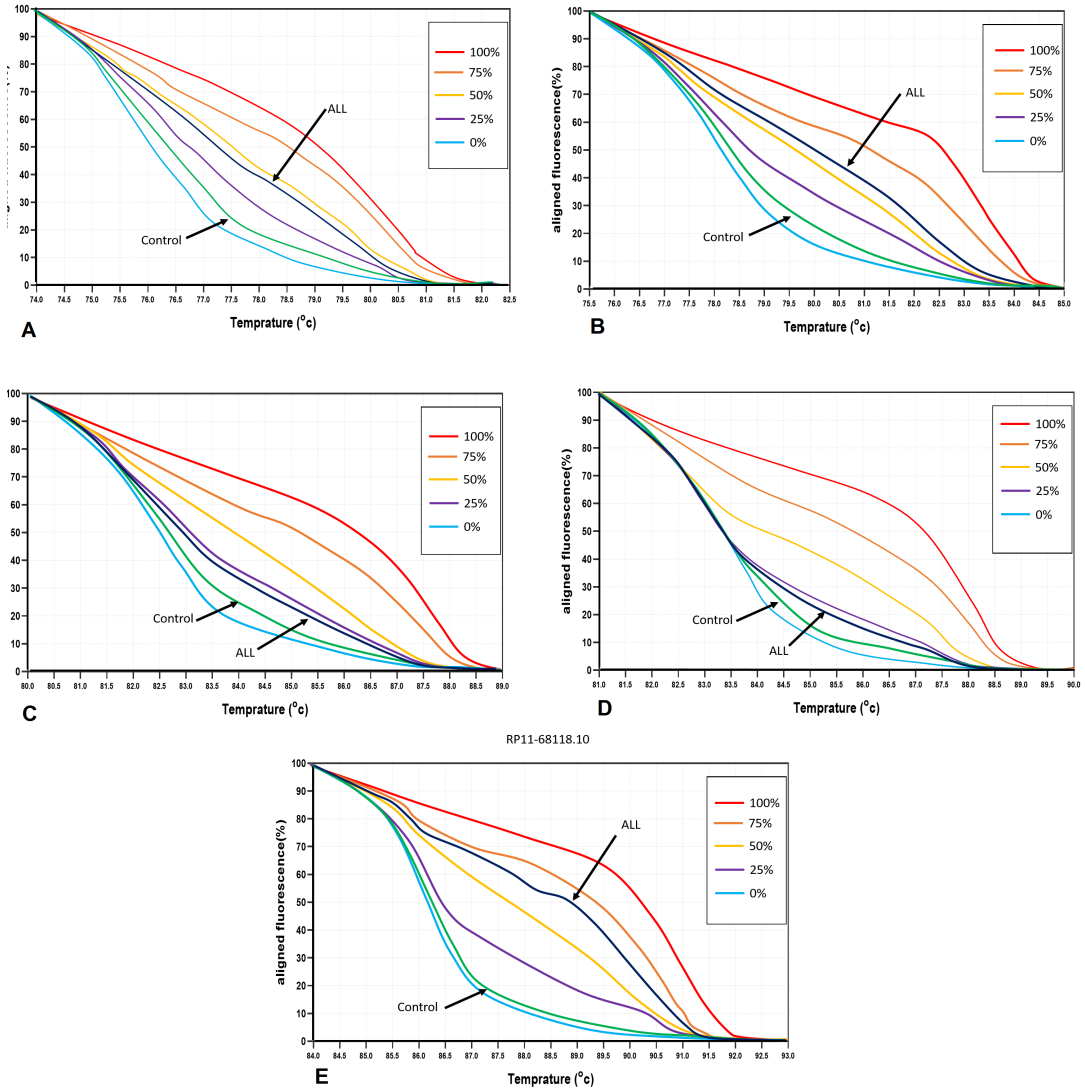

Figure 2. HRM aligned melting curves for each studied lncRNA. A) RP11-446E9, B) RP11-203E8, C) RP11-68118.10, D) RP11-624c23.1, E) RP11-137H2.4.

and reproducible technique that detects the methylation alteration of bisulfite converted DNA semi-quantitively.[26] Resent study reported that the increased expression of lncRNA reduced the rate of migration and proliferation of leukemia cells, indicating the important role of lncRNA s in signaling pathways, which are dependent on the migration and proliferation.[21] Another study demonstrated that an increased expression of lncRNAs results in augmented apoptosis while facing genotoxic stress, which displays the contribution of lncRNAs in apoptosis-dependent

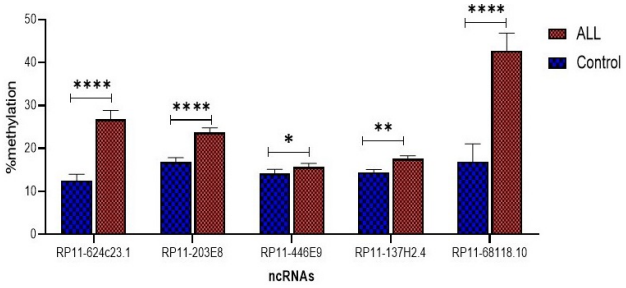

Figure 3. Bar charts of methylation in patients with ALL and controls. Data is presented as mean±SE.
(*p<0.05, **p<0.005, ***p<0.005, ****p<0.0005)

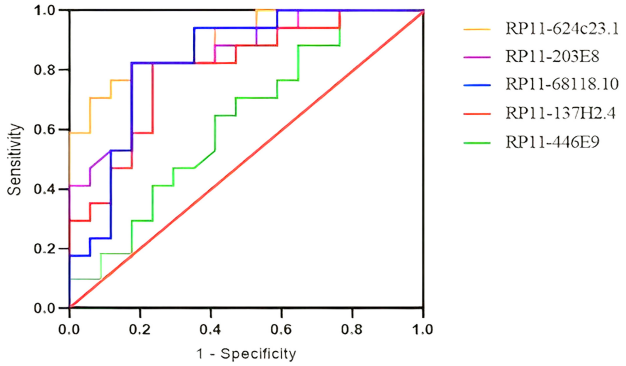

Figure 4. ROC curves of studied lncRNAs.

signaling pathways. Moreover, it was shown that lncRNAs play important roles in regulating DNA damage response (DDR). LncRNA plays a role in activating cell death initiated by DNA damage, In addition, the increased expression of lncRNA results in a decreased proliferation and cell migration.[21]

The studies of Ouimet *et al.*[18] 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 resulting in *RP11-137H2.4* silencing. They revealed precise roles of lncRNAs in cell cycle pathways and NRAS/BRAF/NF-κB MAPK cascade and significantly increases apoptosis in the cells.

In the current study, the methylation levels of lncRNAs (RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10) were studied using the MS-HRM method in children with ALL. Using high-throughput or low-throughput methods, many genes with methylation alterations in ALL patients have been identified that participate in vast types of cellular functions including Wnt signaling, tight junction, transcriptional repression, estrogen receptor action, cell adhesion, cell cycle control, apoptosis, calcium metabolism, and various types of proteolytic activities.[27] To the best of our knowledge, this is the first case-control study that analyzed methylation alterations of the mentioned lncRNAs in ALL patients compared to the healthy controls. Our results indicated methylation levels of the promoter regions in all studied lncRNAs were significantly increased ($P \leq 0.05$). Moreover, ROC curve analysis showed all lncRNAs could be used as reliable diagnostic factors. Specifically, RP11-624c23.1 among other studied lncRNAs had higher AUC value. Majority of the patients under this study suffered from pre-B and T cell ALL while fewer numbers had pro-B cell, B cell, and mixed-ALL (Table 2). In a previous study conducted by our team, the expression levels of the same lncRNAs decreased in ALL patients.[21] Higher methylation levels which may result in expression suppression is a classic phenomenon in tumor suppressors and regulatory molecules in cancer cells. Hence, the lncRNAs analyzed in this study might have a tumor suppressor role. Furthermore, methylation suppression in ALL could worsen the clinical status of patients including their prognosis.[27] Contrary to our results, in the study by Ouimet *et al.*[18], RP11-137H2.4 was upregulated in patients with pre-B ALL, and suppressing the lncRNA resulted in better cell functionality. However, it has been reported that suppressing RP11-624C23.1 and RP11-203E8 increased the cell ability to deal with DNA dameges.[19] Interestingly, ALL symptoms were worsened if the methylation alteration would be seen in more than one gene. In the analysis of 3 genes of p15, p73, and p57, ALL patients who had higher methylation levels in more than one gene had a significantly worse survival rate.[28]

Diagnosis and treatment of ALL remains a major challenge for both scientists and physicians due to the vast and different participation of cellular and molecular mechanisms and pathways.[29] DNA methylation is a promising novel biomarker for the diagnosis, prognosis, and treatment of ALL. However, we are still unable to use it as a definite biomarker due to the lack of a specific standard method for methylation evaluation, inability to detect allelic differences in methylation, and inability to reproduce the results.[24] Current study showed that methylation alterations in lncRNAs might be used as novel diagnostic markers with reliable sensitivity and specificity. However, further cohort studies with larger sample sizes are suggested for confirming these results. Moreover, epigenetic markers have a reversible nature that can be used to suppress proto-oncogenes or elevate tumor suppressor level.[25] Therefore, given the possible tumor suppressor role of studied lncRNAs, further studies about methylation modifications may shed light on novel treatments for ALL.

Conclusion

In general, the methylation levels of RP11-137H2.4, RP11-624c23.1, RP11-203E8, RP11-446E9, and RP11-68118.10 were significantly increased in ALL patients. This alteration could be considered as a novel diagnostic marker in future studies. The results of this study help to have a better understanding of ALL and the factors involved. Studies with a larger sample size may further elucidate the role of lncRNAs in ALL etiology.

Ethical Issues

The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1398.732). Written consent forms were also signed by the parents or legal guardians of the children. The samples were obtained according to the medical and ethical guidelines of Tabriz University of Medical Sciences to ensure participants' safety.

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Author Contributions

ZM and SG designed and directed the project and developed the theoretical formalism, performed the analytic calculations. ZM and AR supervised the project. ZM performed the experiments and wrote the article. LR and BJ developed the theoretical framework. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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