

Overexpression of long non-coding RNA ANRIL in B-acute lymphoblastic leukemia

Mehran Bahraini MSc¹, Mohammad Faranoush MD², Sepideh Naseri Mobaraki PhD³, Mostafa Paridar MD PhD⁴, Ali Amini PhD¹, Rima Manafi Shabestari PhD¹, Majid Safa PhD^{1,*}

1. Department of Hematology and Blood Banking, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran

2. Pediatric Growth and Development Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences, Tehran, Iran

3. Department of Hematology, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran

4. Ministry of Health and Medical Education, Deputy of Management and Resources Development, Tehran, Iran

*Corresponding author: Dr Majid Safa, Department of Hematology and Blood Banking, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran. Email: safa.m@iums.ac.ir. ORCID ID: 0000-0003-0070-6620

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Abstract

Background: Dysregulation of LncRNA antisense non-coding RNA in the INK4 locus (ANRIL) expression is implicated in pathogenesis and disease progression of a variety of cancer types. However, the expression level of ANRIL in pediatric patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has not been elucidated, yet. The present study is an attempt to evaluate the expression level of ANRIL at different clinical stages in pediatric patients with BCP-ALL.

Materials and Methods: This case-control study was conducted in Tehran, Iran on peripheral blood samples obtained at diagnosis, complete remission, and relapse phases from a total of 50 pediatric BCP-ALL patients who were admitted to Mahak Hospital and Rehabilitation Complex, and Rasul Akram Hospital. The ANRIL expression analysis was performed by the quantitative real-time polymerase chain reaction (qRT-PCR) method. To test the statistical significance, a nonparametric Mann-Whitney U test was used.

Results: The mean fold-changes of ANRIL gene expression in newly diagnosed patients were [31.51 (18.28 to 44.75)] compared to the control group [1.06 (0.73 to 1.38)] indicating significant overexpression ($P < 0.001$). ANRIL fold-changes significantly declined following achievement of complete remission [1.24 (0.80 to 1.69)] compared to the newly diagnosed patients ($p < 0.001$) and increased as the patients experienced relapse [285.4 (269.70 to 301)] ($P < 0.001$).

Conclusions: LncRNA ANRIL may contribute to BCP-ALL pathogenesis and disease progression; casting new light on the application of ANRIL as a potential biomarker or therapeutic target in BCP-ALL.

Keywords: ANRIL long non-coding RNA, Gene expression, Precursor B-cell lymphoblastic leukemia

Introduction

Acute lymphoblastic leukemia (ALL) (1) is the most common malignancy of early B or less commonly T-lymphoid precursors in the bone marrow, peripheral blood, and extramedullary organs (1). It constitutes up to 26% of all neoplasms diagnosed in children aged between 0 and 14 years old (2). The age-specific incidence of ALL demonstrates a bimodal distribution, with the highest risk of development in children younger than 5 years of age. The risk of developing ALL then progressively decreases until the late twenties and begins

to rise again from 30 years onwards (3). The outcome for children (1 to 14 years old) with ALL has significantly improved over the past decades; however, the prognosis is not favorable for infants less than 12 months of age (4). The majority of pediatric B-cell precursor ALL (BCP-ALL) patients respond well to the therapeutic approaches based on risk stratification. These approaches include chemotherapy with prognosis-adjusted toxicity, immunotargeted therapy, and hematopoietic stem cell transplantation. Nonetheless, a minority of patients display

resistance which is associated with poor outcomes (5). Although it is widely recognized that genetic alterations may have a major role in the pathogenesis of ALL, these modifications alone are not able to develop leukemia (6). Currently, it is well-documented that the accumulation of genetic and epigenetic changes is responsible for the development of the special features of ALL (7).

Over the past decades, it is comprehensively identified that non-coding ribonucleic acids (ncRNAs), especially long-non-coding ribonucleic acids (LncRNAs), contribute to the tumorigenesis via epigenetic remodeling (8, 9). Recent advances in genomics and transcriptomics have declared that a great part of the human genome is transcribed to RNAs; however only less than 2% of the human transcriptome is protein-coding RNAs, and the remaining 98% is ncRNAs (10). LncRNAs (>200 nucleotides in length) mirror the features of translatable RNA species, such as polyadenylation, 5' capping, transcription by RNA polymerase II, and splicing (11-13). It was initially considered that LncRNAs lack biological functions, however, recent studies have demonstrated that LncRNAs are associated with a spectrum of imperative biological processes, such as cell growth, maintenance of genome integrity, apoptosis, cell differentiation, and transformation (14-17). LncRNAs are considered to be predominantly involved in epigenetic regulation of gene expression primarily in transcriptional rather than post-transcriptional level. Functionally, LncRNAs usually interact with histone modifiers, typically polycomb repressive complex (PRC) -1 and 2 (18). Based on the orientation, this epigenetic regulation could occur either by cis or trans-acting LncRNAs encoded at the proximity of their target genes or geographically distant locations of the genome, respectively (19). The expression of LncRNAs is developmentally regulated and tissue-specific, while their expression quantity is

lower than protein-coding genes (1). By the advent of high-throughput sequencing methods for transcriptomics studies like RNA-sequencing and tiling array, LncRNAs appeared to be one of the missing players in cancer development and have recently attracted considerable attention in the field of cancer biology (18). B-ALL-associated long RNA-2 (BALR-2) is overexpressed in BCP-ALL patients and its overexpression is associated with poor overall survival and diminished response to treatment with prednisone (20). Likewise, BALR-6 up-regulation is associated with elevated cell proliferation and diminished apoptosis in BCP-ALL cells (21). High levels of LncRNA HOXA cluster antisense RNA2 (HOXA-AS2) induce glucocorticoid resistance by increasing cell proliferation and suppressing cell apoptosis in BCP-ALL (22). Aberrant up-regulation of LncRNA ZEB1-AS1 predicted poor prognosis of childhood B-ALL (23). Moreover, overexpression of RP11-137H2.4 LncRNA has been implicated in loss of cell cycle arrest, apoptosis, and cell migration in pediatric B-ALL (24). Hence, knowledge of the expression pattern of LncRNAs during tumorigenesis provides a future direction toward targeted therapies in cancer.

Antisense non-coding RNA in the INK4 locus (ANRIL), also known as cyclin-dependent kinase inhibitor 2B antisense RNA 1 (CDKN2B-AS1), is a LncRNA located within the 9p21.3 locus encompassing CDKN2B/CDKN2A gene cluster. This gene cluster contains three protein-coding genes and ANRIL LncRNA is located in the antisense position to them. The protein-coding genes include MTAP, CDKN2A, and CDKN2B which encode methylthioadenosine phosphorylase, p^{16INK4A}, and p^{15INK4B} respectively. P^{14ARF} is another splice variant of CDKN2A gene (25, 26). P^{15INK4B} and p^{16INK4A} function as tumor suppressors and exert a prominent role in cell proliferation, senescence, and apoptosis. Functionally, these proteins are

cyclin-dependent kinase inhibitors that suppress CDK4/6 to inhibit the phosphorylation of retinoblastoma (27, 28). ANRIL preliminary identified by evaluating the germ-line deletion of this gene cluster in inheritable melanoma-neural system tumors (29). ANRIL gene consists of 21 exons with various isoforms, which are expressed on a tissue-specific basis (30, 31). It is reported that ANRIL exerts its epigenetic activity by interaction with PRCs and microRNA sponging. Several studies indicate that ANRIL recruits the histone modifiers of PRC-1 and -2 to the neighboring genes *CDKN2A/B* and suppresses their expression via the chromatin modification mechanism (32, 33).

The exact mechanism of action of ANRIL LncRNA is not yet fully understood. However, its aberrant expression or dysregulation is frequently reported in a broad spectrum of cancers (18). By investigating the expression pattern of ANRIL LncRNA, this study took the initial step to approximate its role in pediatric BCP-ALL etiology. In this regard, relative quantification of the transcriptional level of ANRIL LncRNA in distinct phases of BCP-ALL has been carried out.

Materials and Methods

Patients

In this case-control study, a total of 50 peripheral blood specimens were collected following written informed consent obtained from patients at different clinical stages of pediatric BCP-ALL, including 20 newly diagnosed (ND) patients, 20 complete remission (CR) samples as well as 10 relapsed cases who presented to Mahak Hospital and Rehabilitation Complex, and Rasul Akram Hospital (Tehran, Iran). The details of the samples were listed in Table I. In addition, 20 peripheral blood specimens from age and sex-matched healthy children displaying no signs of any disease were taken as controls from Massoud Clinical

Laboratory (Tehran, Iran). The diagnosis of BCP-ALL was made mainly according to the World Health Organization (WHO) criteria (based on cytomorphology, immunophenotyping, and cytogenetic analysis) (34). ND patients with BCP-ALL were treated based on Berlin-Frankfurt-Munster (BFM) protocol (35) and complete remission was defined as the presence of less than 5% blasts in the bone marrow, the absence of leukemic blasts in cerebrospinal fluid (CSF) and peripheral blood, and no evidence of extramedullary disease (36). Relapse was considered as the recurrence of $\geq 25\%$ lymphoblasts in the bone marrow and/or localized leukemic infiltrates at any site(37).

Ethical Consideration

The Medical Ethics Committee of Iran University of Medical Sciences (agreement number IR.IUMS.REC.1397.1008) permitted this study.

Mononuclear cells separation, RNA extraction, and cDNA synthesis

Mononuclear cells were isolated from patients' peripheral blood using the density-gradient centrifugation method by Pancoll human (PAN-Biotech GmbH, Aidenbach, Germany). Total RNA was extracted from mononuclear cells using TriPure isolation reagent (Roche Applied Science) according to the manufacturer's recommendation and following the PrimeScript™ RT reagent Kit protocol (Takara, Tokyo, Japan), 1µg of RNA was used for complementary DNA (cDNA) synthesis.

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using specific gene primers and Real Q Plus 2X Master Mix Green without ROX (Ampliqon, Odense, Denmark) according to the instructions of the manufacturer in a Light Cycler 96 Real-time PCR system (Roche Diagnostics, Lewes, UK). Following conditions were employed for

PCR amplification: 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s for the annealing/elongation step. All samples were done in triplicate, and the fold-changes were calculated based on the $2^{-\Delta\Delta C_t}$ relative expression formula. ACTB gene was used as an internal control. Primer sequences are represented in Table II.

Statistical analysis

All data were presented as median with interquartile range. Mann-Whitney U test was used to analyze two independent samples. Spearman rank correlation test was used to analyze the association between ANRIL gene expression and patients' laboratory parameters. Statistically, differences were defined as significant at a p-value less than 0.05. Statistical analysis was performed using Prism 6.01 software (GraphPad, La Jolla, California, USA).

Results

ANRIL is up-regulated in ALL patients' samples at diagnosis and down-regulated after complete remission.

This case-control study included 50 BCP-ALL patients. In ND, there were 15 males (75 %) and 5 (25 %) female patients; in the CR group, 12 were males (60%) and 8 were females (40%); in relapses, there were 7 males (70%) and 3 females (30%) The mean age of the patients in ND, CR, and relapse phases were 6.3, 8.3, and 10.8 years, respectively. In the control group, there were 13 males (65%) and 7 females (35%). The mean age of the healthy individuals was 7.9 years (Table I). The ANRIL mRNA expression levels in ND, CR, and relapse phases were 31.51 (18.28 to 44.75), 1.24 (0.80 to 1.69), and 285.4 (269.70 to 301), respectively (Figure 1). Current findings showed that ANRIL expression was remarkably higher in ND patients samples in comparison to the control group ($P < 0.001$). Intriguingly, the transcriptional expression level of ANRIL was significantly down-regulated in the

CR phase after induction therapy compared with ND patients. Interestingly, this study demonstrated that overexpression of ANRIL levels might be associated with the recurrence of BCP-ALL. ANRIL expression was markedly overexpressed in relapsed patients by more than 200 fold-changes compared with the CR group. Moreover, the expression level of ANRIL mRNA was significantly higher in relapsed patients compared to ND patients ($P < 0.001$). In addition, the correlation analysis between mRNA expression of the ANRIL and laboratory findings mentioned in Table I have been conducted through Spearman rank correlation. Current results demonstrated that there was no statistically significant correlation of ANRIL gene expression levels with patients' white blood cell (WBC) count, peripheral blood blast percentage, hemoglobin concentration, and platelet count (Table III).

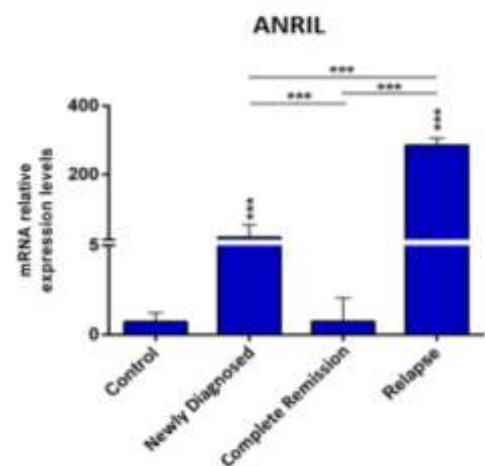


Figure 1. ANRIL expression level was higher in newly diagnosed and relapsed patients compared to patient's samples after complete remission. ANRIL expression levels in peripheral blood samples from newly diagnosed (n=20), complete remission (n=20), relapsed patients (n=10), and healthy volunteers (n=20) were determined by

quantitative real-time polymerase chain reaction. (***)P-value<0.001). ANRIL: antisense non-coding RNA in the INK4 locus.

Table I: Demographic characteristics and laboratory variables of the patients.

Variables	New case (N=20)	Complete remission (N=20)	Relapse (N=10)	Control (N=20)
Sex (%)	15 males (75%) 5 females (25%)	12 males (60%) 8 females (40%)	7 males (70%) 3 females (30%)	13 males (65%) 7 females (35%)
Age (years)	1 to 15 (mean: 6.3)	1 to 16 (mean: 8.3)	2.5 to 15 (mean: 10.8)	2 to 14 (mean: 7.9)
WBC count ($\times 10^9/L$)	2.2 to 92 (mean: 20.2)	4.4 to 18.7 (mean: 8.61)	6 to 118 (mean: 36.4)	3.1 to 8.5 (mean: 6.31)
Blast (%)	21 to 94 (mean: 54)	0	28 to 90 (mean: 61)	0
Hemoglobin (g/dl)	2.8 to 13.7 (mean: 8.9)	8.8 to 16.7 (mean: 13)	7.2 to 11.6 (mean: 8.5)	13.5 to 16.7 (mean: 14.8)
Platelet ($\times 10^9/L$)	17 to 392 (mean: 120)	57 to 564 (mean: 221.68)	35 to 189 (mean: 142)	189 to 312 (mean: 246)

Name	Primer	Sequence	Size (bp)
ACTB	Forward	5'-GGAAATCGTGCGTGACATTAAG-3'	181
	Reverse	5'-GAAGGAAGGCTGGAAGAGTG-3'	
ANRIL	Forward	5'-TCTGATTCAACAGCAGAGATCAAAG-3'	166
	Reverse	5'-CTGACTCGGGAAAGGATTCCAG-3'	

PCR: polymerase chain reaction; ACTB: Actin beta; ANRIL: Antisense non-coding RNA in the INK4 locus.

Table II: The forward and reverse primer sequences used for real-time PCR.

Variables	New case		Complete remission		Relapse	
	r	P	r	P	r	P
WBC count ($\times 10^9/L$)	+0.534	0.061 (ns)	+0.502	0.092 (ns)	+0.302	0.101 (ns)
Blast (%)	+0.614	0.052 (ns)	+0.603	0.058 (ns)	+0.598	0.091 (ns)
Hemoglobin (g/dl)	-0.275	0.122 (ns)	-0.266	0.159 (ns)	-0.259	0.200 (ns)
Platelet ($\times 10^9/L$)	-0.391	0.089 (ns)	-0.401	0.078 (ns)	-0.382	0.099 (ns)

Ns = non-significant; - r value = correlation coefficient

Table III: Correlation between ANRIL gene expression and laboratory characteristics

Discussion

Since the expression of genes involved in cell proliferation, differentiation, apoptosis, and metastasis directly or indirectly regulates by lncRNAs, it has been proposed that these molecules could act as tumor suppressors or oncogenes (38, 39). Moreover, it has been stated that lncRNAs could be considered as a

potential diagnostic or prognostic biomarker in many cancers (40). Less well appreciated is the alteration of ANRIL expression levels and its presumptive role in BCP-ALL. In the present study, it was observed that the expression level of ANRIL is drastically increased in ND patients. Current results suggest that ANRIL might have a tumor promoter role in pediatric BCP-ALL. Present data were

in harmony with other studies in which the oncogenic role of ANRIL has been indicated. In previous studies, it has been shown that ANRIL was up-regulated in acute myeloid leukemia (AML) patients and several solid tumors such as liver (41, 42), bladder (43), stomach (44), lung (45-47), nasopharyngeal (48, 49), esophageal (50), ovary (51), thyroid (52), and breast cancers (53). In these cancers, one of the major consequences of ANRIL overexpression was silencing of *CDKN2A/B* tumor suppressor genes which results in high cellular proliferation and low apoptosis rate. High levels of ANRIL expression in the tissue in all these malignancies has been correlated with invasive clinicopathological findings such as advanced tumor-node-metastasis stage, high histological tumor grade and size, and poor overall survival. Additional studies reported that ANRIL is up-regulated in adult T-cell leukemia/lymphoma (ATLL) patients and cell lines infected by HTLV-1 through E2F1-mediated strengthening of ANRIL promoter. Furthermore, ANRIL knockdown in ATLL cells suppressed proliferation and induced apoptosis (54). In a further study on 108 patients with multiple myeloma (37) who received high-dose melphalan (as the initial line of therapy) and then undergone autologous stem cell transplantation (ASCT), it was observed that patients with TT genotype of rs2151280 in the ANRIL gene have more redundant expression of ANRIL and inferior expression of p14 gene which indicates the reduced p53 activity. These findings may explain the correlation of impaired p53-dependent response to the melphalan treatment and high risk of relapse after ASCT in MM patients (40). In colorectal cancer, ANRIL also causes chemoresistance by induction of ATP-binding cassette subfamily C member 1 (ABCC1) via binding Let-7a miRNA (55). Zhu et al. indicated that CTCF, a transcriptional regulator protein and a putative positive regulator of ANRIL promoter (56), was significantly

overexpressed in ND pediatric BCP-ALL patients and its expression downregulated to the normal level after complete induction therapy and rebounded as patients experiencing relapse (57).

Conclusion

LncRNAs have recently been recognized as the novel player in the multiple aspects of cell biology and dysregulation of their expression has been reported in the vast majority of human diseases including cancers. This study showed the ANRIL gene is markedly overexpressed in ND and relapsed BCP-ALL samples suggesting that it might play an oncogenic role in the leukemic cells. Accordingly, it could be considered as a potential diagnostic and prognostic biomarker or therapeutic target in BCP-ALL. However, the precise mechanism of function attributed to ANRIL LncRNA in pediatric BCP-ALL remains ambiguous.

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Conflict of interest

The authors declare no conflict of interest.

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