AMLS Archives of Medical Laboratory Sciences

Original Article

The Expression Level of CCDC26 and FOXCUT Genes in Acute Lymphoblastic Leukemia

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Received: January 3, 2021, 2021; Accepted: February 27, 2021

Abstract

Background and Aim: Acute lymphoblastic leukemia (ALL) is a malignant disease of lymphoid progenitor cells affecting both children and adults. Long non-coding RNAs (lncRNAs) are one kind of non-coding RNAs (ncRNAs), reported modulating the initiation or progression of diverse cancers. However, the role of CCDC26 and FOXCUT long non-coding RNAs in ALL has been unknown. In this study, we explored the expression of FOXCUT and CCDC26 lncRNAs in acute lymphoblastic leukemia cell lines.

Methods: Acute T lymphoblastic leukemia cell lines, RPMI 8402, Jurkat, B lymphoblastic leukemia, Daudi, and Ramos cell lines were used. After culturing the cells, RNA extraction and cDNA synthesis were performed. The real-time PCR technique was then used to study the expression of CCDC26, FOXCUT, C-kit, and FOXC1 genes.

Result: We found a significant increase of CCDC26 expression in RPMI 8402 ($p < 0.0001$) and Ramos ($p < 0.05$) cell lines compared to the control, while decreased expression of these genes was observed in Jurkat and Daudi cell lines. Furthermore, FOXCUT gene had a significant increase in expression in all cell lines compared to the control ($p < 0.01$ in Daudi and RPMI 8402 cell lines) ($p < 0.001$ in Jurkat and Ramos cell lines).

Conclusion: Our results demonstrated that CCDC26 and FOXCUT genes can play a regulatory role in acute lymphoblastic leukemia and may serve as a potential diagnostic biomarker and therapeutic target of acute lymphoblastic leukemia.

Keywords: Acute Lymphoblastic Leukemia; Long Non-Coding RNA; CCDC26; C-Kit; FOXCUT.

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Please cite this article as: Barati S, Mousavi SH, Motallebzadeh-Khanmiri J, Khani-Eshratabdi M. The Expression Level of CCDC26 and FOXCUT Genes in Acute Lymphoblastic Leukemia. Arch Med Lab Sci. 2021;7:1-9 (e16). https://doi.org/10.22037/amls.v7.33294

Introduction

Acute lymphoblastic leukemia (ALL) is caused by a multistage somatic mutation in a single B or T lymphoid progenitor cell, occurring in one of the stages of development. The proliferation and accumulation of clonal blast cells in the bone marrow inhibits hematopoiesis and finally leads to anemia, neutropenia, and thrombocytopenia (1, 2). ALL is one of the most common and severe-to-treat childhood malignant neoplasia, accounting for 25% of all cancers in children under the age of 15 and also includes 20% of acute leukemia in adults (3, 4).

In the United States, more than 5900 patients are diagnosed with ALL, 25% of whom die annually (5). Despite clinical advancement in ALL diagnosis and treatment, patients with ALL, especially those with refractory or relapsed forms, still suffer from poor prognosis, severe adverse events, and low survival rate (6). It is thus essential to understand the underlying signaling pathways of ALL pathogenesis, development, metastasis, and death, to identify a new biomarker for ALL diagnosis and novel molecular targets for ALL treatment (7, 8).

Numerous agents are involved in the development, prognosis, and treatment of Patients with ALL. Among the cases that have been recently studied in some cancers are long non-coding RNAs

(lncRNAs). Some studies have shown that lncRNAs have a significant effect on cell death and cell growth by controlling apoptosis in solid tumors, leukemias, and gliomas. Long non-coding RNAs (lncRNAs) have recently been studied in some cancers (9, 10). Some studies have shown that lncRNAs have a significant effect on cell death and cell growth by controlling apoptosis in solid tumors, leukemias, and gliomas(11). lncRNAs are mRNA-like transcripts, ranging in size from 200 nucleotides to 100 kb. All but a few are locking the ability to synthesize proteins(12). Some studies on the role of lncRNAs indicate that their control mechanisms are complex in regulating the pattern of gene expression, involved in regulating gene expression at the transcriptional and post-transcriptional levels (13). For example, transcriptional regulation involves the epigenetic inactivation of some genes, but post-transcriptional regulation by lncRNAs involves several factors(14). FOXCUT is located on chromosome 6 and is transcribed from upstream of the FOXC1 promoter, which is why it is called FOXC1 upstream promoter transcript (15). FOXC1 is a member of the Forkhead family, which is an important transcription factor in regulating a variety of biological processes, including tumorigenesis and cell migration. FOXC1 is also associated with invasive phenotypes and increased cell proliferation in lung cancer cells. An increase of FOXC1 has been reported in approximately 20% of AML samples, which is a barrier to monocyte/macrophage differentiation and increases the clonogenic potential of AML cells. Silencing of FOXCUT reduces the expression of FOXC1. Silencing both FOXCUT and FOXC1 genes reduces cell proliferation and cell migration. FOXC1 is involved in four important biological processes (proliferation, tumorigenesis, metastasis, and angiogenesis) (16, 17).

CCDC26 is located on chromosome 8 (24q8), and its protein contains 109 amino acids. Despite the ambiguous nature of CCDC26 function as an oncogene or anti-oncogene, there is considerable evidence of CCDC26 association with specific tumors, including glioma and AML, in which an increase in all or part of the CCDC26 genetic locus has been observed in children with acute leukemia (18). CCDC26 also appears to control growing myeloid leukemia cells by regulating kit gene expression. However, the role of lncRNAs in the ALL cell line has not been investigated(19).

There is currently no extensive evidence on the function of CCDC26, and it is yet to be known. CCDC26 is located on chromosome 8 (24q8), and its protein contains 109 amino acids; however, its mRNA is encoded, and this protein has no resemblance to known proteins. Moreover, some studies have suggested an association between CCDC26 and cancers, including acute myeloblastic leukemia (AML) and glioma(19). The C-kit or CD117 is a cytokine receptor located on chromosome 4 (4q13) which is expressed on the surface of hematopoietic stem cells (HSCs) as well as other cells. Changed forms of this receptor may be related to cancer types. The binding of the C-Kit to its receptor on HSCs activates the C-Kit, resulting in autophosphorylation and initiation of message transmission, which as a result, leads to cell survival, migration, and cell proliferation. With the CCDC26 gene silencing, an increased expression is seen in the tyrosine kinase receptor. Kit tyrosine kinase receptors cause the survival of cancer cells in HSCs. CCDC26, therefore, appears to be a tumor suppressor non-coding RNA because its expression suppresses the expression of the C-Kit gene. According to studies on the K562 cell line, inhibiting the expression of the CCDC26 gene appears to inhibit cell death and cause increased cell survival. Also, in cell lines in which CCDC26 is silenced, kit gene overexpression is seen. Kit Protein is important for the self-renewal and maintenance of HSCs. In leukemia, CSCs remain at this stage and cause malignancy. Therefore, it is possible to suppress the tumor via inhibiting or reducing the expression of the kit gene by CCDC26 (17, 19).

Glyceraldehyde 3-phosphate dehydrogenase is a glycolytic enzyme located on chromosome 12 that is involved in DNA repair, membrane fusion, and cell death. GAPDH is overexpressed in many human cancers, such as melanoma, and is directly related to tumor progression. Its glycolytic and anti-apoptotic function helps to proliferate and protect

tumor cells, but under oxidative stress, GAPDH expression causes aging and cell death(20).

Considering the importance of C-Kit in adjusting hematopoietic stem cell signaling and also elucidating the role of CCDC26 in regulating the expression of this gene, the main purpose of this study is to determine the expression LncRNAs status of FOXCUT and CCDC26 in acute lymphoblastic leukemia cell line and further studies will be performed on samples from patients with ALL. If the results of in-vivo studies are similar, these two genes could be used to develop diagnostic, prognostic, and therapeutic methods for patients with ALL. In this experimental study, we investigated the expression of CCDC26, C-Kit, FOXCUT, and FOXC1 genes in ALL cell lines, and peripheral blood mononuclear cells (PBMCs) were used as the control group.

Methods

Cell culture: Acute T lymphocytic leukemia cell lines of RPMI 8402, Jurkat, B lymphoblastic leukemia, Daudi, and Ramos cell lines were obtained from Pasteur Institute of Iran. Cell lines were cultured according to ATCC instructions in 25 ml flasks (T25) in RPMI-1640 medium enriched with 10% fetal bovine serum (FBS, Gibico, USA). During the culture period, the flask containing the cells was incubated in an incubator at 37 ° C containing 5% CO2, and the culture medium was changed every two days. After enough growth of the cells, some cells were frozen at -80 ° C and then stored in a nitrogen tank, and another flask was used for cell counting and RNA extraction. During culture, cell survival was assessed by slide hemocytometer and trypan blue.

Isolation of PBMCs

Peripheral blood lymphocyte cells of average humans were also used as a control sample. Initially, samples were obtained from a consenting healthy person without any particular disease, consumption. 10 ml of peripheral blood was collected in tubes containing EDTA, and PBMCs were isolated using Faicol gradient centrifugation according to instructions. RNA was extracted immediately after isolation.

Extraction of RNA: YTZOL pure RNA commercial kit (Yekta Tajhez Azma, Iran) was used to extract RNA. First, 1 ml Trizol Reagent was added to the cell precipitate obtained from culture and immediately transferred to a freezing temperature of -30°C. After one hour, the sample was defrosted, 200 µl chloroform was added to the sample and it was centrifuged at 120000 g for 15 minutes at 4°C.

The clear supernatant was added to a microtube containing 500 µl of cold isopropanol and placed at -30°C for 10 minutes. After full shaking, the RNA solution and isopropanol were centrifuged and the supernatant was discarded.

The RNA precipitate was washed twice with 70% ethanol (Adding 1 ml of 70% ethanol in every step and centrifugation at 10000 g for 7 minutes at 4°C). Finally, the RNA precipitate was solved in 20 µl of DEPC water and transferred to a -70°C until the time of the cDNA synthesis.

cDNA synthesis: For cDNA synthesis in this study, a cDNA synthesis kit (Yekta Tajhez Azma, Iran) was used. RNase-free equipment and solutions were used in every step of the procedure. For each RNA sample, a 0.5 ml tube was selected and 6.2 µl template RNA, 1 µl Oligo (dt) primer, 8.9 µl DEPC water was added, with the final total volume of 13.4 µl.

After shaking and centrifuging for a short time, they were kept at 70° C for 5 minutes and then kept on ice. Then, 4 µL First Strand butter 5X, 1 dNTPs, 0.5 RNasin, 1µL M-MLV were added to the microtube, which was kept 60 minutes at 42°C for reverse transcription, 5 minutes at 70°C, and one week at -20° C for storage.

Quantitative Real-time PCR: To perform the reaction, first, the primer was designed (Confirmed on Primer blast website), and the efficiency of the primers (Table 1) was evaluated using five consecutive dilutions (1. 1.5, 1.25, 1.125, and 1.625).

Table 1. The sequence of primers designed in this study

Gene Symbol	Primer	Sequence (5`to 3`)	Product Size	Tm
C-KIT	Forward	GGGCCACCGTTTGGAAAG	80	60
C-KIT	Reverse	TTACATTCAACCGTGCCATTG		60
FOXC1	Forward	GGCGAGCAGAGCTACTACC	86	59
FOXC1	Reverse	TGCGAGTACACGCTCATGG		59
FOXCUT	Forward	CTAAGGACGGGGCTGAATTGG	84	62
FOXCUT	Reverse	GGCAGGCAACGTTAGTCATC		62
CCDC26	Forward	AGGCCTGAGGAGAGAAGACAC	90	60
CCDC26	Reverse	AGAGCAGCCTGAAAAATGGA		60
GAPDH	Forward	TGCACCACCAACTGCTTAGC	87	58
GAPDH	Reverse	GGCATGGACTGTGGTCATGAG		58

The mentioned dilutions were added to Master Mix according to the protocol and placed in the Real-Time device. Finally, the Ct of each concentration was placed in the REST2009 program, and the reaction efficiency of each primer was calculated. The Master mix used was a Taq DNA Polymerase 2x Master MixRed-Amplicon.

Ingredients for the reaction were 6.5 µL Master Mix Real-time (2x), 1 µL Primer (Forward and Reverse), 1 µL cDNA, and 7.5 µL DEPC Water. The PCR reaction was performed by a thermocycler with 35 cycles. All tests in this study were performed in triplicates.

Statistical analysis: In this study, one-way ANOVA statistical analysis was performed using GraphPad Prism V6.07 software. Data are displayed as mean ± SD. The confidence interval was 95% in all experiments, and P values less than 0.05 were considered significant. In graphs, P values less than/equal to 0.05, 0.01, 0.001 and 0.0001 are shown with one star (*), two stars (**), three stars (***) and four stars (***), respectively.

Results

In this study, the quantitative expression of FOXCUT, FOXC1, CCDC26, and C-kit genes were evaluated by Real-Time PCR reaction by line-gene k with Takara master mix. The expression of the named LncRNAs was normalized using GAPDH as the internal control. The results of melting and amplification curves (linear) of FOXCUT, FOXC1, CCDC26, C-kit, and GAPDH (as internal control) transcripts were analyzed using Line Gene software. The standard curves of FOXCUT, FOXC1, CCDC26, C-kit, and GAPDH genes, show efficiency, R2 (correlation coefficient), line slope and intercept. The data obtained from the sample Ct were first analyzed using $2^{-\Delta\Delta CT}$ methods.

Increased Expression of FOXCUT and FOXC1 Genes in Cell Lines Compared to the Control Group

Comparing the expression of FOXCUT and FOXC1 genes in the Daudi cell line shows that FOXC1 expression (P>0.317) and FOXCUT expression (P<0.01) had increased expression compared to control, while FOXCUT expression was increased compared to that of FOXC1 (P<0.05). In the Jurkat cell line, both FOXC1 (P<0.9958) and FOXCUT (P<0.001) genes had increased expressions compared to the control group, and FOXCUT showed an increased expression compared to FOXC1 (P<0.001).

In Ramos cell line, the expression of FOXCUT (P<0.001) and FOXC1 (P.V: 0.8362) genes increased compared to the control group, and FOXCUT expression was increased compared to FOXC1 (p <0.001). Finally, in RPMI 8402 cell line, FOXC1 (p = 0.999) and FOXCUT (P <0.01) genes had increased expressions compared to the control group, and FOXCUT expression was increased compared to FOXC1 (P<0.01). The results are summarized in Figure1.

3.2. Different Expression of CCDC26 and C Kit Genes in Cell Lines Compared to the Control Group

Comparison of CCDC26 and C-kit gene expression in the Daudi cell line shows that CCDC26 expression was decreased compared to control (p >0.999), while C-kit expression was increased (p <0.05) and expression of C-kit was increased compared to CCDC26 (p <0.05). Besides, in the Jurkat cell line, CCDC26 gene expression was

decreased compared to control (p = 0.9974), and C-kit gene expression was increased compared to control (p <0.05) and in general, C-kit gene expression was increased compared to that of CCDC26 gene (p <0.05).

In RPMI 8402 cell line, CCDC26 gene expression was observed to increase compared to control (p<0.0001), and C-kit gene had a decreased expression compared to control (p >0.999), and in total, CCDC26 gene expression was increased compared to that of C-kit. (p <0.0001). Similarly, in the Ramos cell line, CCDC26 expression was increased compared to control (p <0.486), and C-kit expression was decreased compared to control (p <0.05), and CCDC26 expression was increased compared to C-kit expression (p <0.05). The results are summarized in Figure 2.

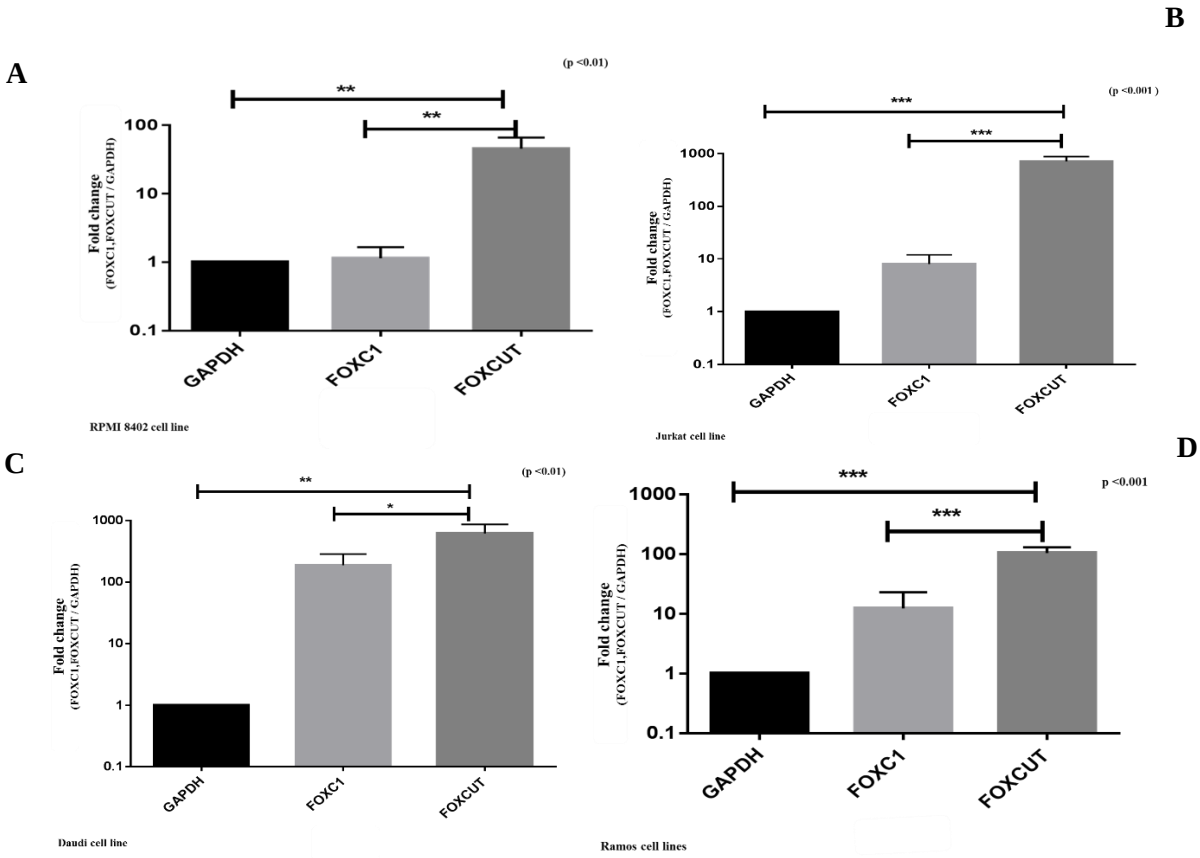

Figure1: Expression of FOXCUT and FOXC1 genes in cell lines compared to control samples. A: FOXC1 and FOXCUT genes had increased expressions compared to the control, and FOXCUT expression was increased compared to FOXC1. B: both FOXC1 and FOXCUT genes had increased expressions compared to the control, and FOXCUT showed an increased expression compared to FOXC1. C: FOXC1 expression and FOXCUT expression had increased expression compared to control, while FOXCUT expression was increased compared to that of FOXC1 . D: FOXCUT and FOXC1 genes increased compared to the control, and FOXCUT expression was increased compared to FOXC1.

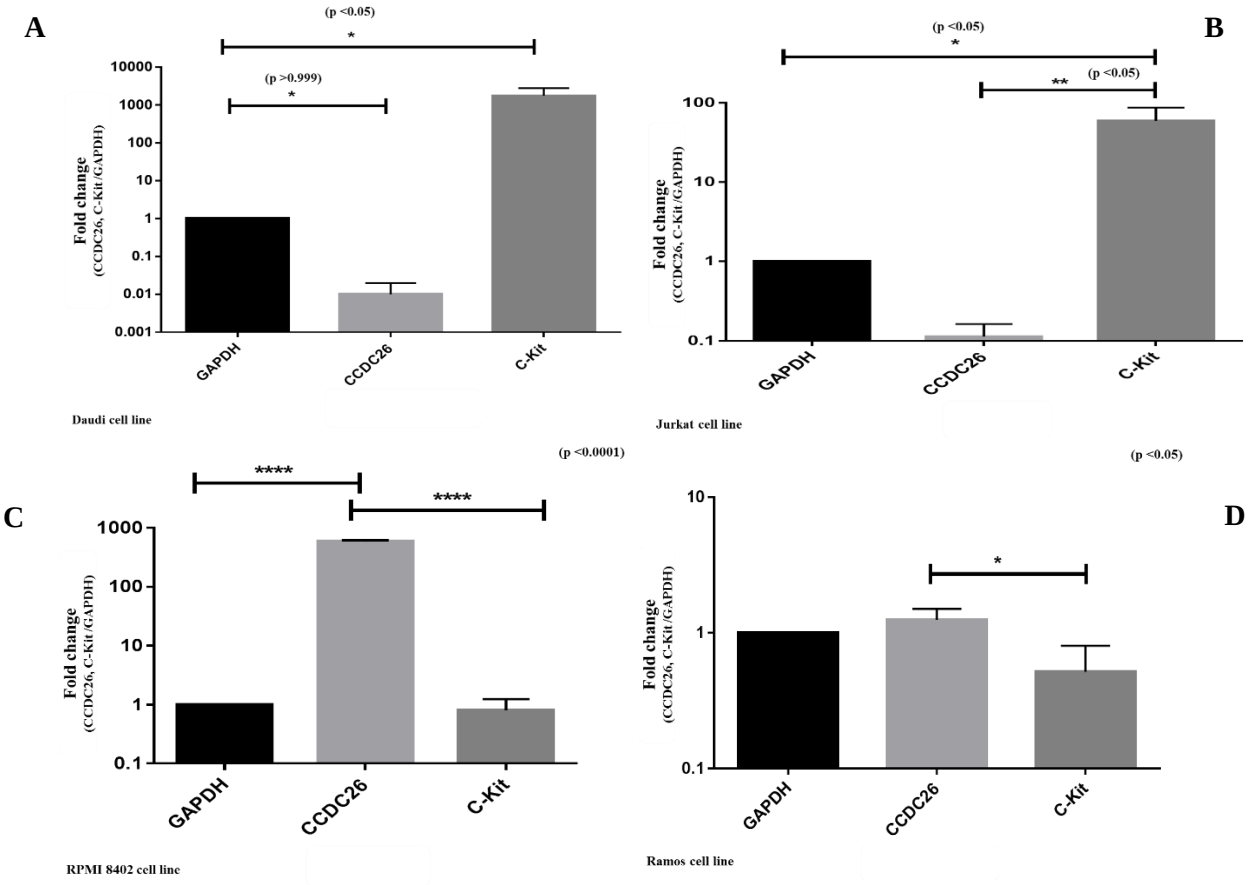

Figure2: Expression of CCDC26 and C-kit genes in cell lines compared to control samples. A: CCDC26 expression was decreased compared to control, while C-kit expression was increased and expression of C-kit was increased compared to CCDC26. B: CCDC26 gene expression was decreased compared to the control, and C-kit gene expression was increased compared to control, and in general, C-kit gene expression was increased compared to that of the CCDC26 gene. C: CCDC26 expression was increased compared to the control and C-kit expression was decreased compared to the control and CCDC26 expression was increased compared to C-kit expression. D: CCDC26 gene expression was observed to increase compared to control and C-kit gene had a decreased expression compared to control, and in total, CCDC26 gene expression was increased compared to that of C-kit.

Discussion

Acute lymphoblastic leukemia is neoplasia that results from multistage somatic mutations in a single lymphoid progenitor cell (B or T) at one of several stages of development. However, our knowledge of ALL genomics is still incomplete. This study was performed to answer the question of whether the expression of FOXCUT and CCDC26 lncRNAs are altered in acute lymphoblastic leukemia. Studies on the role of lncRNAs in regulating the pattern of gene expression indicate the complexity of their control mechanisms, with these molecules at different levels of regulation of gene expression, including regulation at the transcriptional and post-transcriptional levels. Transcriptional regulation involves the role of lncRNAs in the epigenetic inactivation of specific genes. Nevertheless, post-transcriptional regulatory levels are affected by the action of lncRNAs. Research conducted by Pen et al. indicated that FOXCUT is a lncRNA (long non-coding RNA) on chromosome 6, transcribed upstream of the FOXC1 promoter(21). FOXC1 is a crucial transcription factor in regulating a variety of biological processes, including tumorigenesis, epithelial transmission, proliferation, and cell migration (9). On the other hand, Somerville TD and colleagues showed that FOXC1 expression increased in at least 20% of patients with acute myeloid leukemia and

inhibited monocyte/macrophage differentiation (22). In 2014, Pan et al. showed that FOXCUT non-coding RNA (upstream of the FOXC1 promoter) played an important role in carcinogenesis and esophageal progression of squamous cells to cancer. Experiments were performed on 82 cancer-infected tissues compared to non-cancerous tissues (21). It was found that a significant increase in expression was seen in cancer tissues in FOXC1 and FOXCUT (21, 23). In 2016, M. Swaminathan showed that FOXC1 plays an important role in AML disease onset by regulating HSC by blocking myeloid cell differentiation and increasing clonogenic potential. In this study, the prognostic value of FOXC1 and a factor for disease recurrence or non-response to chemotherapy was investigated(24). In 2017, B Han et al. showed that FOXC1 plays an important role in tumour progression and metastasis in tissue with basal cell carcinoma and that FOXC1 expression was increased in this type of cancer compared to other types of breast cancer (25). Similarly, according to the results of the present study, the expression of FOXCUT and FOXC1 in all cell lines, including Daudi, Ramos, Jurkat, RPMI 8402, had a significant increase compared to the control sample. Therefore, it seems that a significant increase in these two genes in cancer cells can increase cell proliferation, cell migration, inhibit cell differentiation and enhance tumorigenesis and expression of several factors leading to cell metastasis. The results of the present study somewhat confirm the results of other studies that have shown the effect of FOXCUT expression on the FOXC1 gene, which ultimately leads to increased tumorigenesis in various diseases. It seems that these two genes, in addition to the malignancies that have been studied in the past, can also play an essential role in the development or progression of ALL disease. In addition to their role in other malignancies, according to the present study, these two genes can also proliferate and cancer cells in ALL by increasing the expression of FOXC1 gene. Therefore, these two genes can be considered as therapeutic targets for the treatment of this malignancy.

CCDC26 on chromosome 8 (8q24) is thought to be a long non-coding RNA. Despite the ambiguous nature of CCDC26, extensive genome analysis has shown some evidence of CCDC26 association with specific tumors, including glioma and AML. An increase in all or part of the CCDC26 genetic locus has been observed in children with acute leukemia. CCDC26 also appears to control growing myeloid leukemia cells by regulating kit expression. In 2015, Tetsuo Hirano showed that some non-coding RNAs, including CCDC26, increased expression in acute myeloid leukemia in childhood and showed the role of CCDC26 as a tumor suppressor, and also showed that decreasing CCDC26 expression increased Ckit gene expression. Also, they found that the kit gene plays an important role in tumor survival and spread and suggests increasing CCDC26 expression, which reduces C-kit expression, for treatment in these patients (19). According to the data obtained from statistical analysis in the study, CCDC26 expression in Daudi and Jurkat cell lines has decreased compared to C kit as a factor in cancer spread, and CCDC26 expression has increased compared to Ckit expression in Ramos and RPMI 8402. It is possible that the expression of CCDC26 could be a factor in suppressing the C-kit gene and inhibiting its function. Inhibition of the Ckit gene in these cell lines reduces cell proliferation, increases apoptosis, and decreases drug resistance. This probably indicates a link between the two genes, as well as the controlling effect that CCDC26 has on the C-kit gene. The only study on the effect of CCDC26 gene expression on the C-kit gene in the AML cell line confirms the findings of the present study, which somehow demonstrates the inhibition of C-kit function by CCDC26.

Conclusion

On the whole, the present study revealed a novel lncRNAs network in ALL cells and a new insight into ALL treatment. The expression patterns of CCDC26 and FOXCUT in Daudi, Ramos, Jurkat, RPMI 8402 cell lines could indicate the potential role of these non-coding RNAs in regulating the biology of this type of leukemia, as CCDC26 shows an inhibitory role in cancer cells' growth and proliferation, but FOXCUT can play a positive role in the proliferation and spread of ALL cells.

Therefore, this study provides the first data on the expression of these genes in acute lymphoblastic leukemia, which is hoped to be the first step in further studying these genes in cancers, especially leukemias, and will be a starting point for obtaining sufficient information to treat patients.

Conflict of Interest

The authors declared that they have no conflict of interest.

Acknowledgments

The authors acknowledge the financial support from Tehran University of Medical Sciences, Department of Hematology, and Blood Transfusion Sciences.

Funding/Support

This work was supported by the Tehran University of Medical Sciences Foundation, grant number: 95-01-31-31461.

Ethics

The present study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1395.330).

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