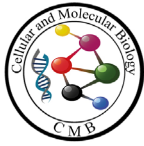

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Expression of CD123 related long non-coding RNA in Acute Myeloid Leukemia bone marrow mononuclear cells and its clinical significance

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ABSTRACT

Acute myelogenous leukemia (AML) is a very common hematopoietic malignancy. Hematopoietic stem cell transplantation can improve the therapeutic effect of AML, but the 5-year survival rate is very low. CD123 imbalance, abnormal gene expression, and epigenetics play an important role in the pathogenesis of AML. This research was to explore the differential expression of CD123-related long non-coding RNA (lncRNA) in AML bone marrow mononuclear cells and provide a theoretical basis for targeted therapy of AML. High-throughput sequencing was performed to screen differentially expressed lncRNA in bone marrow mononuclear immunophenotypes of CD123+ and CD123- from patients with primary AML, and real-time quantitative PCR was adopted for screening and validation. There were 933 differentially expressed lncRNAs in the CD123+ group and the CD123- group, 407 lncRNAs were up-regulated and 463 lncRNAs were down-regulated in the CD123+ group. 14 lncRNAs with more than 2 times of difference were screened for identification, and it was found that compared with CD123- group, there was no substantial difference in the expression of JHDM1D-AS1, LINC01355, CASC15, FAM13A-AS1, HSPC324, LOC339803, LINC00877, and MAG12-AS3 in CD123+ group ($P>0.05$). The expressions of LOC101929698, BaALC-AS2, BOLA3-AS1, and FBX19-AS1 were considerably up-regulated ($P<0.05$), while the expressions of LOC100132249 and LINC02085 were considerably down-regulated ($P<0.05$). In summary, differentially expressed lncRNAs in bone marrow samples of CD123+ and CD123- group of newly diagnosed AML patients may be involved in the process of AML and seriously affect the prognosis of patients.

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Introduction

Leukemia is a kind of hematopoietic stem cell clonal disease, which is characterized by high heterogeneity and abnormal cell proliferation, accompanied by cell differentiation and maturation disorders (1). Acute myelogenous leukemia (AML) ranks first in the incidence of adult leukemia (2). With the continuous development of hematopoietic stem cell transplantation technology and the development of new therapeutic drugs, the therapeutic effect of AML has been considerably improved (3). However, there is evidence that clinically, 30% of AML patients still fail to meet the criteria of complete remission, and the 5-year survival rate is only 40% - 50% (4). With the rapid development of molecular biology techniques, the diagnosis of leukemia has gradually shifted from manual to instrumental. Flow cytometry-based on fluorescence antibody detection has been widely applied in the recognition of molecular markers of leukemia cells and the diagnosis of diseases (5).

CD molecule is very important in lymphocyte immune typing and is involved in lymphocyte immunological function (6). The abnormal functions of leukemia stem cells (LSCs), such as self-renewal, differentiation, and proliferation, are involved in the occurrence, development, recurrence, and refractory of AML (7). LSCs have a variety of cell phenotypes, such as CD123, CD44, and CD47. Of which CD123 is the Interleukin-3 receptor alpha chain (IL-3Rα). As one of the members of the hematopoietic growth factor receptor family, it is currently recognized as the most targeted immunophenotype expressed on LSCs (8,9). One study indicated that the positive expression of CD123/IL-3Rα is closely related to the poor prognosis of elderly patients with leukemia (10). Other studies confirmed that high expression of CD123/IL-3Rα is associated with low clinical remission rate, high recurrence rate, short disease-free survival, and poor prognosis of AML (11,12). Therefore, it is of great significance to explore CD123-related gene molecules in AML stem cells to improve the therapeutic effect of AML.

Long non-coding RNA (lncRNA) is a class of RNA molecules that do not encode proteins and are more than 200 nucleotides long. LncRNAs are involved in the regulation of post-transcriptional activation/inhibition, post-transcriptional mediation, miRNA sponge adsorption, chromatin remodeling, expression genetics, and other processes (13). Many studies confirmed that abnormal expression of lncRNA is involved in and regulates tumor occurrence and development (14-16). Studies indicated that lncRNA is involved in the occurrence, development, recurrence, metastasis, and drug resistance of leukemia and other hematologic diseases. Cheng et al. (2020) constructed a

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prognostic circRNA-lncRNA-miRNA-mRNA network of AML and screened a prognostic ceRNA network containing 32 lncRNAs (17). Tao et al. (2021) showed that lncRNA CD27-AS1 showed a tendency of high expression in AML patients and seriously affected the survival of patients (18). Therefore, the screening of differentially expressed lncRNAs in AML is of great significance for early diagnosis, preoperative typing, prognosis prediction, treatment selection, and survival extension of AML.

Therefore, in this study, differentially expressed lncRNAs in bone marrow mononuclear cells of CD123+ group and CD123- group of AML patients were detected based on high-throughput sequencing technology, and real-time quantitative PCR was used to screen and verify lncRNAs. It was hoped to provide a basis for further exploring the molecular mechanism of lncRNA regulating CD123/IL-3Rα expression in AML patients and provide new biomarkers and targets for the diagnosis and treatment of AML.

Materials and Methods

Experimental materials

Human peripheral blood lymphocyte isolation fluid (Ficoll) and Trizol reagent were purchased from Invitrogen Company in the United States. A reverse transcription kit (Hifair®II 1st Strand cDNA Synthesis SuperMix) and fluorescent quantitative PCR kit (Hieff® qPCR SYBR® Green Master Mix) were purchased from Shanghai Yisheng Biotechnology Co., LTD.Fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridnine chlorophyll protein (perCP) fluorescent agents, as well as cell membrane surface antigen monoclonal antibodies were purchased from Becton Dickinson Company in the United States. Phosphate buffer (PBS) was purchased from Seville Biotechnology LTD.

Immunotyping of leukemia

Patients with leukemia (non-M3) who visited *Hematology Department of Affiliated Hospital of Yan'an University* from December 2020 to December 2021 were recruited as the research subjects. The patient met the diagnostic criteria for leukemia in The Diagnostic Criteria for Hematological Diseases, and the patient and his family members had signed informed consent. 5-10 mL of fresh bone marrow was extracted by bone marrow puncture in the lateral decubitus position. Heparin was treated with anticoagulation and diluted to (0.5-1.0) $\times 10^6$ cells /mL using PBA. 20 μL of different combinations of antibodies was added, mixed well, and incubated for 20 min under dark conditions. The red blood cells were fully lysed and washed and then tested on the machine. FITC, PE, and perCP fluorescent were adopted to label non-correlated CD123, TdT, CD38, CD34, and HLA-DR; myeloid related CD13, CD33, CD14, CD15, CD11b, and CD117; T cell-related CD2, CD3, CD4, CD5, CD7, and CD8; B cell-related CD10, CD19, CD20, CD22, CyCD79a, and other cell membrane surface antigens. CD34≥10% and MPO≥10% were considered as a positive reaction, and the positive rate of cell population surface antigen in other gates ≥20% was considered as a positive reaction. Finally, the CD123+ group and CD123- group were labeled.

Flow cytometry of leukemia stem cell CD123

4-10 mL of bone marrow fluid of CD123+ group and CD123- group were taken, treated with heparin anticoagulant, and PBS solution was added to prepare 1×106 single-cell suspension at a concentration of 1×106 cells /mL. The single cell suspension was added into four flow cytometry test tubes and numbered. Mouse anti-human CD34-PE, CD38-FITC, and CD123-Percp monoclonal antibody reagents were added, respectively, and the homotype controls were IGG1-PE, IGG1-FITC, and IGG1-percP, respectively, and the negative control was set. After the mixture was fully mixed, the samples were stained for 20 min at 4°C, centrifuged at 1,000 rpm for 10 min, washed twice, and diluted with PBS to 500 μL. Cells were sorted by flow cytometry and analyzed by CellQuest. The cell population collected on the left and right was set, the polarization plate was changed, and the parameters were adjusted so that the polarization plate was in the best position. The left and right separation channel was opened so that the left and right two liquids flew separately, and then the sorting was started. The cells were centrifuged and collected, and the measurements were repeated ten times. Finally, the CD123+ group and CD123- group were obtained.

Differentially expressed lncRNAs screened by high-throughput sequencing

Trizol assay was adopted to extract total RNA from three pairs of primary AMLs CD123+ and CD123- mononuclear cells, and the RNA quality was detected by multi-function ultraviolet (UV) spectrophotometer and 1% agarose gel electrophoresis. LncRNA sequencing process included rRNA removal, RNA fragmentation, reverse transcription, connecting street, fragment selection, PCR amplification, and machine sequencing. Subsequently, bioinformatics analysis techniques were employed to analyze the expression differences of lncRNAs, predict new lncRNAs, analyze the co-expression of lncRNA-mRNA and predict the function of lncRNAs.

Extraction of bone marrow mononuclear cells

3-4 mL bone marrow solution of AML patients were taken and treated with heparin anticoagulant, and the same volume of lymphocyte separation solution was added, mixed, and centrifuged at 2,000 rpm for 20 min. The middle white film layer was taken, and the cells were diluted with three times the volume of PBS solution, centrifuged at 1,000 rpm for 10 min, and washed with PBS solution. Finally, the supernatant was discarded, and bone marrow mononuclear cells were obtained.

Differential expression of lncRNA verification by real-time fluorescence quantitative PCR

Trizol assay was used to detect total RNA in CD123+ and CD123- groups of AML patients and RNA quality was detected by multi-function UV spectrophotometer and 1% agarose gel electrophoresis. CDNA of RNA was extracted according to the instructions of Hifair® II 1st Strand cDNA Synthesis SuperMix. Reverse transcription was implemented according to the instructions of the Hieff® qPCR SYBR® Green Master Mix kit, and real-time quantitative PCR reaction procedures and reaction system were set. Fourteen quantitative detection primers with differential expression of lncRNA were designed and synthesized, and the specific information is shown in Table 1. Using β-actin as inteRNAl reference gene, the relative expression level of target lncRNA was detected by $2^{-\Delta\Delta Ct}$ method,

$\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$, $\Delta\Delta Ct = \Delta Ct_{experimental\ group} - \Delta Ct_{control\ group}$.

Statistical methods

SPSS 19.0 was used for experimental data processing and classification analysis, and mean± standard deviation (mean ±SD) was used to represent the experimental data. Non-parametric rank-sum test (Mann-Whitney U test) was used to analyze the data that did not conform to the normal distribution, and an independent sample *T*-tes`t was used to analyze the data that did conform to the normal distribution. When $P<0.05$, the difference between groups was considered statistically significant. $P<0.05$ was considered statistically significant, $P<0.01$ was considered highly significant, and $P<0.001$ was considered extremely significant.

Results

Differential expression detection of lncRNA in CD123+ and CD123- groups of AML patients

High-throughput sequencing results showed that a total of 933 differentially expressed lncRNAs were found in CD123+ and CD123- groups of AML patients. A total of 470 lncRNAs were up-regulated and 463 were down-regulated. The volcanic diagram of overall lncRNA expression distribution is shown in Figure 1. Subsequently, heat maps of lncRNA expression differences in different groups were

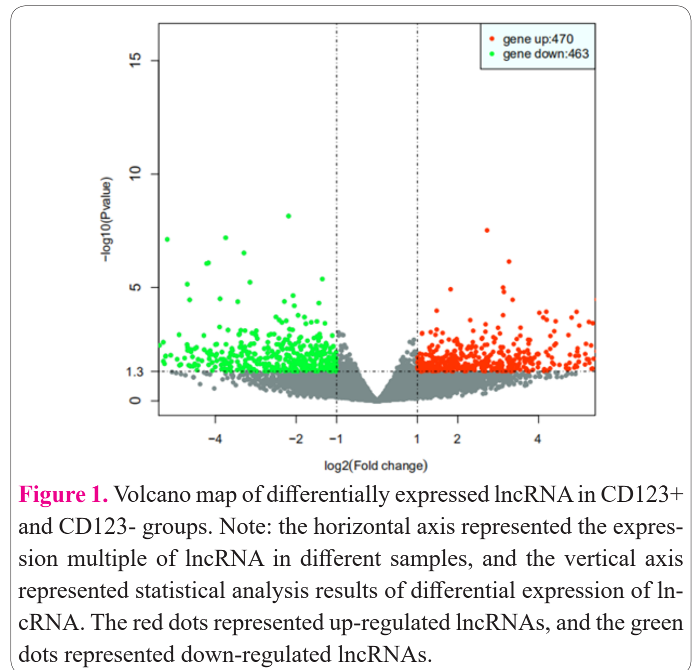

Figure 1. Volcano map of differentially expressed lncRNA in CD123+ and CD123- groups. Note: the horizontal axis represented the expression multiple of lncRNA in different samples, and the vertical axis represented statistical analysis results of differential expression of lncRNA. The red dots represented up-regulated lncRNAs, and the green dots represented down-regulated lncRNAs.

Table 1. Real-time fluorescence quantitative primer information.

Gene		Primer sequences (5'→3')
BAALC-AS2	F	CAGCCTTCGGGGTGAACTTA
	R	GCTTGCCCCCACTCTTTTTG
BOLA3-AS1	F	TGAGAAAGATCGCTCCGCTC
	R	TTTGCGGACAGTTCTACCCC
LOC101929698	F	AGCAAAGCCTACGTCACACA
	R	GGTCTCCTTGACAACCCAG
JHDMID-AS1	F	GGAAAACGTCCCACCCGAAT
	R	AGCGGACGACAGTGATGAAT
LINC01355	F	GCTGAAATGGAAGCCAACCA
	R	TGGTGGAGCAGACACGATTA
CASC15	F	TGACCTCCTTCATTCTGCGT
	R	AGTCTCCTACCGGAACATGC
FAM13A-AS1	F	GCCATAGGAGTGCGGTCTTC
	R	CACTCGGCAACACTGATTCTA
LOC100132249	F	CACAGGAGCGAGAACTGAGAA
	R	ATCGGGGCCGTTGGTATG
LINC02085	F	AGGCAGACGAAAAATGGAGC
	R	TCTTGAATGAATGCCCAGCA
HSPC324	F	AGTGCCAGCTTTGCCCTATC
	R	TGATGAGGGTTCTCTGCGTC
FBX19-AS1	F	GTCGAGACAATGGAAGGGGAT
	R	GTCCTCTCCCTCATTTCCGC
LOC339803	F	TGCCAGTACAACAGCCACAA
	R	CAACCAAATCGGGAAAGCCG
LINC00877	F	CCCCAGACGAAAGGATACGC
	R	TTCCTTGTTCATCACCGGCT
MAGI2-AS3	F	TTTCTTCAGCCTCTGTGCGC
	R	CTCTTGGATGCAAACGGCAG
β-actin	F	CTCCATCCTGGCCTCGCTGT
	R	GCTGTCACCTTCACCGTTCC

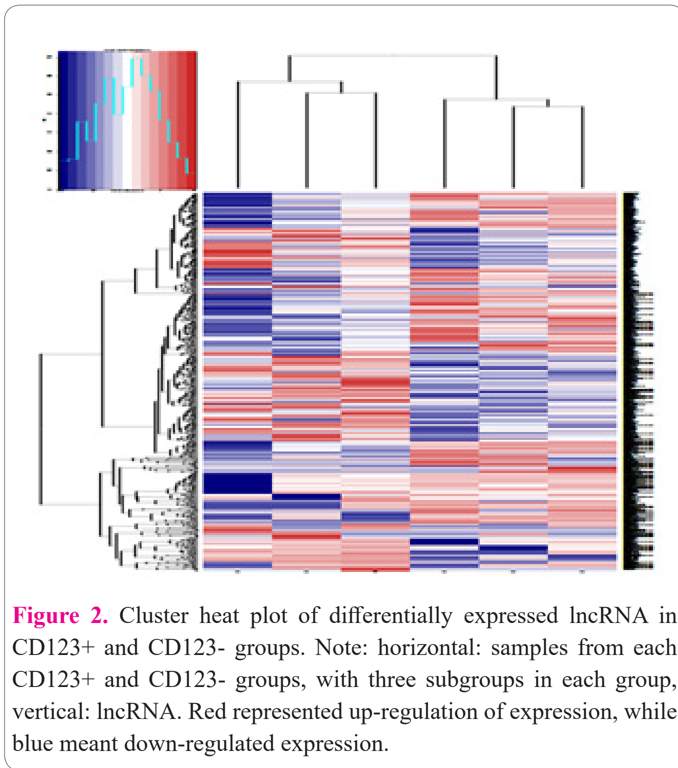

Figure 2. Cluster heat plot of differentially expressed lncRNA in CD123+ and CD123- groups. Note: horizontal: samples from each CD123+ and CD123- groups, with three subgroups in each group, vertical: lncRNA. Red represented up-regulation of expression, while blue meant down-regulated expression.

analyzed and drawn in Figure 2. There were substantial differences in lncRNA expression trends between CD123+ and CD123- groups.

Screening of differentially expressed lncRNA in CD123+ and CD123- groups of AML patients

According to the differential expression data atlas and CD123-related lncRNAs, 14 differentially expressed lncRNAs with high differential expression abundance and differential multiple ≥2 times were selected. They were BAALC-AS2, BOLA3-AS1, LOC101929698, JHDM1D-AS1, LINC01355, CASC15, FAM13A-AS1, LOC100132249, LINC02085, HSPC324, FBX19-AS1 and LOC339803, LINC00877, and MAG12-AS3 Information of differentially expressed lncRNAs was shown in Table 2.

Analysis of differentially expressed lncRNA target genes in CD123+ and CD123- groups of AML patients

Biological techniques were adopted to predict the differentially expressed lncRNA target genes in CD123+ and CD123- groups of AML patients, and it was divided into cis-regulation and trans regulation according to the regulation mode of lncRNA. Then, X target genes were predicted. Subsequently, GO functional annotation of predicted target genes and KEGG pathway enrichment analysis were performed, and the results are shown in Figures 3 and 4. The target genes of lncRNA were identified as organic substance metabolic process, primary metabolic process, cellular metabolic process, and single-organism cellular process. They were mainly enriched in metabolic pathways, pathways in cancer, and the PI3K-Akt signaling pathway.

Verification of differential expression of lncRNA in CD123+ and CD123- groups of AML patients

Real-time fluorescence quantitative PCR was performed to detect the differential expression of lncRNA in CD123+ and CD123- groups of AML patients, including BAALC-AS2, BOLA3-AS1, LOC101929698, JHDM1D-AS1, LINC01355, CASC15, FAM13A-AS1, LOC100132249, LINC02085, HSPC324, FBX19-AS1, LOC339803,

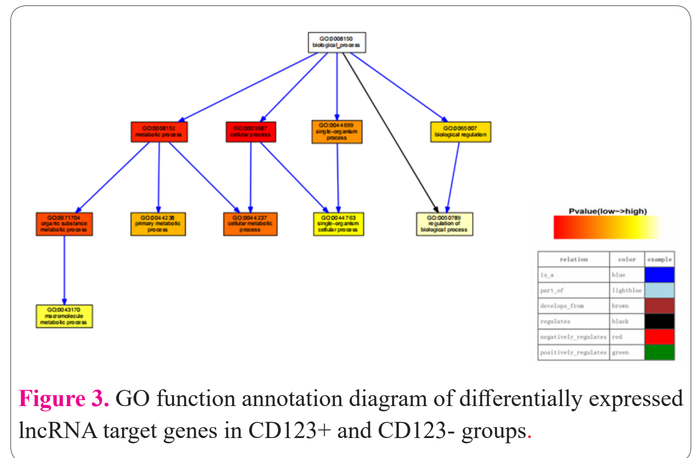

Figure 3. GO function annotation diagram of differentially expressed lncRNA target genes in CD123+ and CD123- groups.

Table 2. High-throughput sequencing results of differentially expressed lncRNAs.

Gene	CD123-			CD123+			*P*
	B2	B3	B4	A1	A3	A5	
BAALC-AS2	187	3	254	18	3	25	0.000
BOLA3-AS1	17	29	37	104	154	60	0.003
LOC101929698	3	5	5	113	8	12	0.000
JHDM1D-AS1	187	20	69	272	331	130	0.008
LINC01355	356	137	219	120	8	111	0.008
CASC15	5	1	9	107	51	49	0.006
FAM13A-AS1	157	139	270	98	25	152	0.048
LOC100132249	132	23	1	105	68	367	0.040
LINC02085	191	256	21	93	10	6	0.020
HSPC324	94	31	714	53	21	18	0.001
FBX19-AS1	857	149	259	152	63	192	0.013
LOC339803	84	41	60	131	71	144	0.037
LINC00877	402	384	18	184	17	56	0.040
MAG12-AS3	18	0	11	524	9	76	0.001

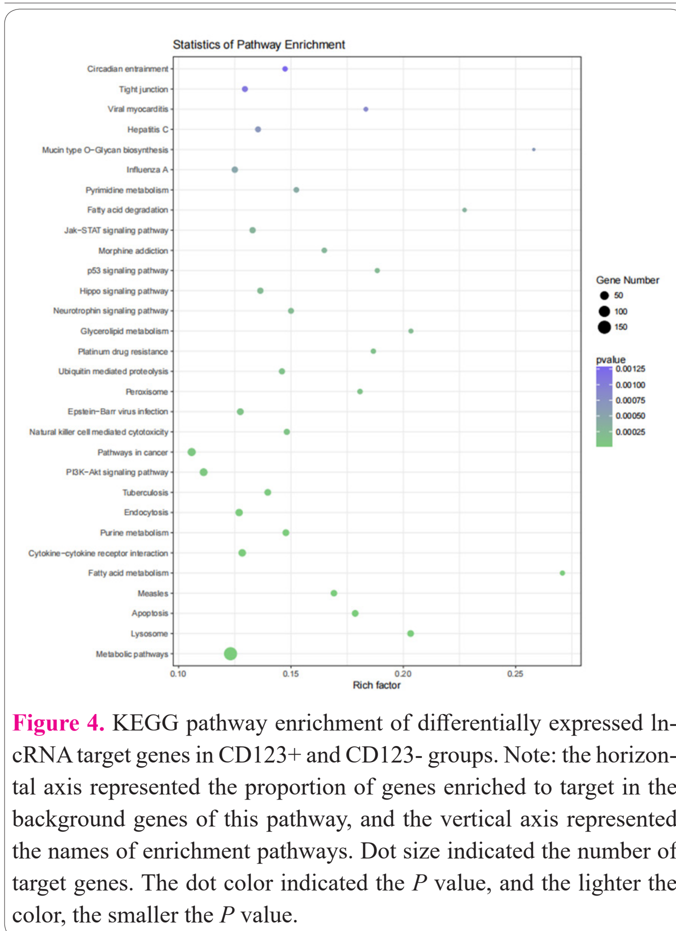

Figure 4. KEGG pathway enrichment of differentially expressed lncRNA target genes in CD123+ and CD123- groups. Note: the horizontal axis represented the proportion of genes enriched to target in the background genes of this pathway, and the vertical axis represented the names of enrichment pathways. Dot size indicated the number of target genes. The dot color indicated the *P* value, and the lighter the color, the smaller the *P* value.

LINC00877, and MAG12-AS3. From Figure 5, there was no substantial difference in the expression of JHDM1D-AS1, LINC01355, CASC15, FAM13A-AS1, HSPC324, LOC339803, LINC00877, and MAG12-AS3 in CD123+ and CD123- groups of AML patients (*P*>0.05). The expression of LOC101929698 in the CD123+ group was considerably higher than that in CD123- group (*P*<0.05). BAALC-AS2, BOLA3-AS1, and FBX19-AS1 in the CD123+ group of AML patients were considerably higher than those in CD123- group (*P*<0.001). LOC100132249 in the CD123+ group was considerably lower than that in the CD123- group (*P*<0.001). LINC02085 in the CD123+ group was considerably higher than that in CD123- group (*P*<0.01).

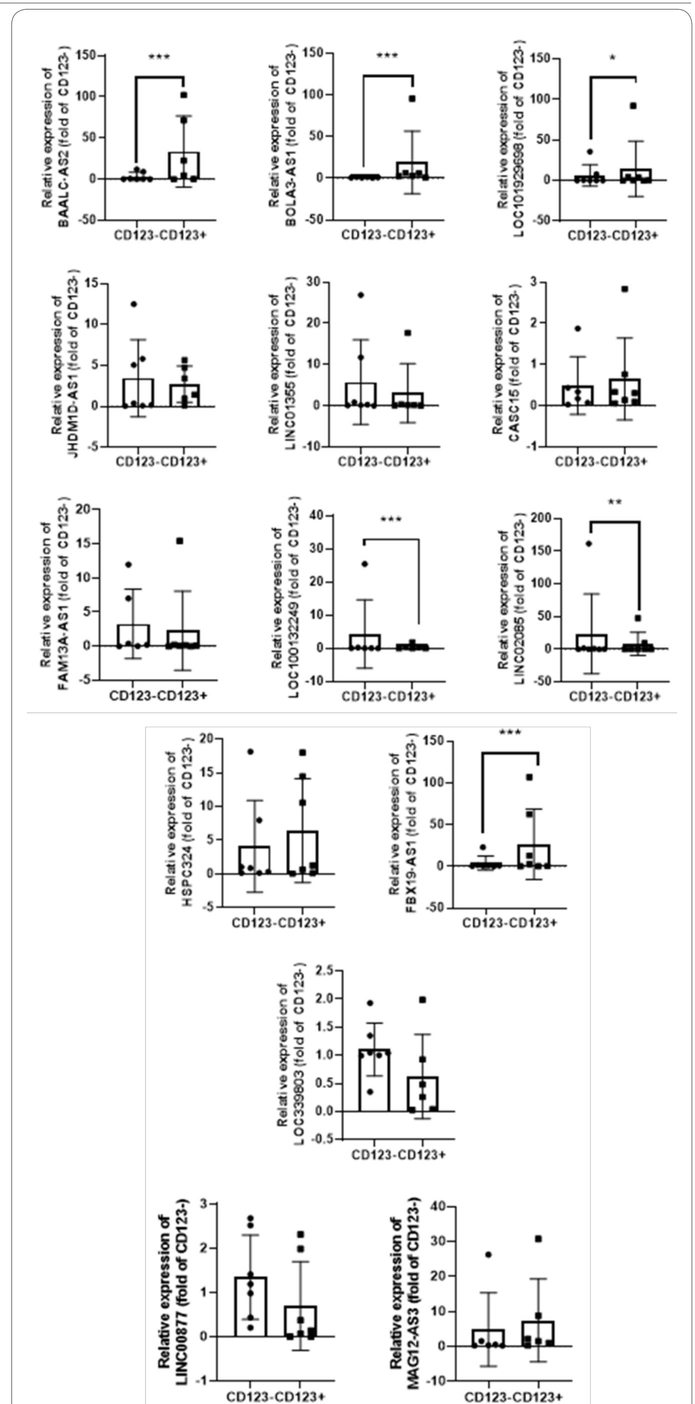

Figure 5. Relative expression levels of differentially expressed lncRNA. Note: * represented substantial difference between groups, *P*<0.05; ** indicated the highly substantial difference between groups, *P*<0.01; *** indicated the extremely substantial difference between groups, *P*<0.001.

Discussion

AML is a kind of malignant hematologic tumor disease. The clonal proliferation and differentiation disorders of bone marrow podocytes are the main pathological features of AML, and the survival rate of patients is low (19). Studies confirmed that relapse and drug resistance of AML might be related to LSCs, which have many and specific cell phenotypes, among which CD123 is the most highly targeted immunophenotype of LSCs recognized at present (20,21). CD123 is a soluble and pleiotropic cytokine that regulates hematopoietic and immune cell formation and function. In addition, studies revealed that CD123 is closely related to clinical remission rate, recurrence rate, disease-free survival, and prognosis of AML patients (22). Loff et al. (2020) used chimeric antigen receptor T cells targeting CD123 to effectively eradicate CD123+ leukemia in vivo and in vitro (23). Bras et al. (2019) showed that AML patients showed high expression of CD123, which was mainly distributed in NPM1 and PLT3-ITD mutant leukemia, and CD123 expression was positively correlated with disease recurrence (24). Aref et al. (2020) confirmed the expression of CD25 and CD123 in bone marrow samples of AML patients by flow cytometry and found that low-risk/medium-risk AML was associated with CD25+/CD123+, and the overall survival of AML patients with CD25+/CD123+ expression was considerably shortened (25). Therefore, the prediction of CD123 expression is of great significance for the treatment of AML, as well as prolonging the survival period and improving the prognosis of patients.

Many studies confirmed that lncRNAs are involved in regulating the process of various diseases, especially

tumor diseases (26,27). Song et al. (2020) showed that lncRNA-MALAT1 was up-regulated in relapsed acute lymphoblastic leukemia and affected the proliferation and apoptosis of leukemia cells by binding miRNA (28). Yang et al. (2020) showed that the expression level of lncRNA SNHG16 in peripheral blood/mononuclear cells of normal subjects was lower than that in peripheral blood/leukemia cell lines of AML patients, and silencing lncRNA SNHG16 expression promoted apoptosis of leukemia cells and inhibited proliferation (29). Therefore, lncRNA plays an important role in the treatment and diagnosis of AML. In this study, differentially expressed lncRNAs in CD123+ and CD123- mononuclear cells of AML patients were analyzed by high-throughput sequencing technology. The results revealed that a total of 933 differentially expressed lncRNAs were screened, and there were 470 up-regulated lncRNAs and 463 down-regulated lncRNAs in the CD123+ group compared with the CD123- group.

Target genes with differential expression of lncRNA were predicted, and GO functional annotation of target genes and KEGG pathway enrichment analysis was performed. It turned out that the target genes of lncRNA were identified as organic substance metabolic process, primary metabolic process, cellular metabolic process, and single-organism cellular process, which were mainly enriched in metabolic pathways, pathways in cancer, and PI3K-Akt signaling pathway. Metabolic disorders of exosomes in AML patients are mainly involved in glycolysis/gluconeogenesis, and targeted dysmetabolic pathways can be used as therapeutic targets for AML (30). Dysregulation of the PI3K-Akt pathway is common in a variety of diseases, including cancer, diabetes, and cardiovascular diseases. In cancer diseases, two mutations can be seen that enhance the kinase activity within PI3K. The PI3K-Akt pathway is one of the abnormally up-regulated cellular pathways in AML and other diseases and is closely related to the prognosis of AML patients (31). Combined with the GO functional annotation of lncRNA target genes and KEGG pathway enrichment results in this study, differential expression of lncRNA may be involved in the regulation of AML disease process through regulating metabolic pathway or PI3K-Akt pathway and affect the survival and prognosis of patients.

Subsequently, 14 differentially expressed lncRNAs were selected and identified in this study. The results showed that LOC101929698, BAALC-AS2, BOLA3-AS1, and FBX19-AS1 were considerably up-regulated in the CD123+ group compared with CD123- group, while LOC100132249 and LINC02085 were considerably down-regulated. BOLA3-AS1 is a divergent transcription of BOLA3, located on human chromosome 2p13.1. Wei et al. (2021) showed that BOLA3-AS1 is up-regulated in gastric cancer cells (32). Jiang et al. (2021) comparatively analyzed the expression profile of glycoly-associated lncRNA in endometrial cancer and found abnormal expression of BOLA3-AS1 (33). LOC101929698 is located on human chromosome 20q11.21. BAALC-AS2 is the antisense RNA2 of BAALC and is located on human chromosome 8q22.3. FBX19-AS1 is an F-box and fuleucine repeat protein 19 antisense RNA located on human chromosome 16p11.2. LOC100132249 is located on human chromosome 9q13. LINC02085 is located on human chromosome 3q12.3. At present, there are few studies on LOC101929698, BAALC-AS2, FBX19-AS1, LOC100132249, and LINC02085. However, the results of this study showed that LOC101929698, BAALC-AS2, FBX19-AS1, LOC100132249, and LINC02085 were differentially expressed in bone marrow CD123+ and CD123- in AML patients, indicating that these lncRNAs may be involved in the process of AML.

To investigate the expression difference of CD123 related lncRNA in AML bone marrow mononuclear cells, CD123+ and CD123- stem cells were selected from AML patients, differentially expressed lncRNAs were screened by high-throughput sequencing, and their expression was identified by real-time quantitative PCR. The results showed that compared with CD123- group, LOC101929698, BAALC-AS2, BOLA3-AS1, and FBX19-AS1 expressions were considerably up-regulated in the CD123+ group, while LOC100132249 and LINC02085 expressions were considerably down-regulated. Moreover, differentially expressed lncRNA can regulate the AML process by regulating the metabolic pathways and PI3K-Akt signaling pathway. In this study, however, only real-time fluorescence quantitative PCR was adopted to identify differentially expressed lncRNAs. Subsequently, cell models should be used to analyze the effects of overexpression/silencing of some lncRNAs on cell biological functions and to analyze the expression differences of lncRNAs in AML patients with different types, clinical symptoms, survival, and prognosis. In conclusion, this study may provide new targets for the treatment of AML.

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