Long non-coding RNA LOC644135 is a potential prognostic indicator in cytogenetically normal acute myeloid leukemia

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ABSTRACT

Objectives: Acute myeloid leukemia (AML) is a hematological malignancy with highly clinical heterogeneity resulting in poor outcomes. We aim to identify novel prognostic lncRNA in AML expecting to provide new clues for therapy in AML.

Methods: Three cohorts were enrolled in this study. Differentially expressed lncRNAs between TCGA-AML cohort and GTEx cohort was identified by DESeq2. The relationship between expression level of LOC644135 and prognosis in AML was analyzed by multiple methods.

Results: Pan-cancer analysis indicated that LOC644135 was most highly expressed in AML across 33 types of cancer. Patients with high expression of LOC644135 had poor overall prognosis in both TCGA-AML cohort and the TARGET-AML cohort. Especially, high expression of LOC644135 indicated inferior overall survival and event-free survival in CN-AML patients in the TCGA-AML cohort. Besides, CN-AML patients had higher expression of LOC644135 than normal samples. Multivariable analysis suggested that LOC644135 was an independent prognostic factor in AML. GSEA analysis showed that LOC644135 was associated with some immune-related pathways. Besides, high expression of LOC644135 was associated with less infiltration of CD8\textsuperscript{+} T cell.

Conclusion: Our findings indicated that LOC644135 was an independent prognostic factor in AML and provided a new idea in the development of therapy in AML.

1. Introduction

Acute myeloid leukemia (AML) is the most common hematological malignancy in adults with poor outcomes. Improving the prognosis of AML patients is still a challenge for clinical therapy. Developing targeted therapies, many mutations, such as FLT3-ITD, CEBPA, NPM1 and RUNX1, have been considered as the recurrent genetic mutations for AML [1]. Although landmark-targeted therapies have developed, most AML patients are destined to relapse. Therefore, there is an urgent need to identify novel prognostic biomarkers expecting to improve the outcomes of patients in AML.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules longer than 200 nucleotides. Although lncRNAs had no or limited capability of protein-coding, increasing studies have declared that lncRNAs played crucial role in diverse biological processes to function as master regulators for gene expression involving various mechanisms [2,3]. For example, lncRNAs can inhibit gene transcription by modifying RNA-binding protein in cis and preventing the association of specific transcription factors with DNA acting as molecular decoys [4,5]. Besides, lncRNAs can regulate the activity of microRNA or enhancers through sponging or binding [6–8]. Moreover, lncRNAs can also regulate gene expression post-transcriptionally through binding antisense mRNAs [9]. Recently, numerous studies have also revealed the important function of lncRNAs in tumors included AML [10]. For example, lncRNA MEG3 acted as a suppressor and promotes AML leukemogenesis not only in a p53-dependent but also in a p53-independent manner [11]. LncRNA HOXA-A52 expression was increased in NB4 promyelocytic leukemia cells, and the negative regulation mediated by HOXA-A52 contributed to the fine-tuning of apoptosis during ATRA-induced myeloid differentiation in NB4 cells [12]. In CN-AML, lncRNA AQP-1 was found to be an independent prognostic biomarker, and high AQP-1 expression predicted better prognosis [13]. However, a large number of lncRNAs have not been reported and the function of these lncRNAs remains to be investigated. Hence, in this study, we found that lncRNA LOC644135 was significantly high expression in AML and associated with poor prognosis in AML. Besides, the prognostic significance of LOC644135 was particularly outstanding in CN-AML. And then, we also preliminarily unveiled that lncRNA LOC644135 majorly participated in some immune-related signaling pathways in AML. A prognostic model based on the LOC644135-related ceRNA network indicated that the ceRNA network also played crucial role in AML, which provided clues in the improvement of outcomes in patients with AML. Our findings suggested that...
IncRNA LOC644135 could be a dependable biomarker for AML, and expecting to help solve the difficulties in AML therapy.

2. Methods

2.1. Data collection

The complete transcriptome profile, microRNA expression profile and clinical data of 151 AML patients were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov). As verification, 187 patients with pediatric Leukemia from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database were also enrolled in this study and the transcriptome profile and clinical data were available on the TARGET website (https://ocg.cancer.gov/programs/target). The transcriptome profile of 70 normal bone marrow samples was downloaded from the Genotype-Tissue Expression Project (GTEx) database (https://gtexportal.org/).

2.2. Gene expression profiling interactive analysis (GEPIA)

The GEPIA database (http://gepia.cancer-pku.cn) includes 9736 tumor and 8587 normal tissue samples from the TCGA and the Genotype-Tissue Expression (GTEx) projects (12). In the function module, we used the Ensembl Gene ID of LOC644135: ENSG00000237596 to perform the single-gene analysis and the threshold was selected as the default value.

2.3. Gene expression analysis

The log counts per million (logCPM) that transformed from raw counts by R ‘voom’ package was used to measure the expression of genes. Comparison of LOC644135 expression between TCGA-AML cohort and GTEx cohort was performed by DESeq2 [FC]≥1.5, p ≤ 0.01) [14]. The significant difference of LOC644135 expression between different European Leukemia Net (ELN) stratification groups or cytogenetic subtypes was tested by the Analysis of Variance (ANOVA) method. All statistical analyses were performed using R version 4.0.

2.4. Prognosis analysis

After rejecting fifteen M3 patients, a total of 136 patients were divided into two groups (LOC644135<sup>high</sup> and LOC644135<sup>low</sup>) based on the median expression of LOC644135. Kaplan-Meier analysis and log-rank test were performed to compare the overall survival (OS) and the event-free survival (EFS) between two groups. Multivariable analysis was conducted to demonstrate whether the expression of LOC644135 was an independent factor for prognosis in AML. A p-value less than 0.05 (typically ≤ 0.05) is statistically significant.

2.5. Differential expressed gene analysis and function enrichment analysis

Differentially expressed genes (DEGs) were identified by differentially expressed gene analysis using DESeq2 [14], the threshold value of differentially expressed gene analysis was false discovery rate (FDR)<0.05 and |FC|≥1.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses [15] were conducted using R package ‘clusterProfiler’ [16]. Gene Set Enrichment Analysis (GSEA) [17] was conducted using the gseKEGG and gsePathway function in the R package ‘clusterProfiler’ [16] with the parameters: nPerm = 1000, minGSSize = 10, maxGSSize = 1000, pvalue-Cutoff = 0.05.

2.6. Competing endogenous RNAs (ceRNA) network construction and analyses

Differentially expressed microRNAs and mRNAs selected from the DEGs were used to construct the LOC644135-related ceRNA network by GDCRNAtools [18], and the ceRNA network was visualized by R ‘igraph’ package. In the ceRNA network, three criteria are used to determine the competing endogenous interactions between IncRNA-mRNA pairs by GDCRNAtools: the IncRNA and mRNA should share a significant number of miRNAs, expression of IncRNA and mRNA must be positively correlated, and those common miRNAs should play similar roles in regulating the expression of IncRNA and mRNA. So ceRNA network was constructed satisfying the following requirements: hyperPValue>0.01, corPValue>0.01, regSim = 0. MiRcode was used to collect predicted and experimentally validated IncRNA targets. StarBase v2.0 was used to predict miRNA-mRNA interactions. Next, functional analysis based on the LOC644135-related ceRNA was performed and noting by Metascape (http://metascape.org/) [19]. LASSO Cox regression was applied to establish the prognostic model based on the LOC644135-related ceRNA network by using the R package ‘glmmnet’ [20,21]. TCGA-AML cohort was divided into the high-risk group and the low-risk group based on the median risk score. Kaplan-Meier survival analysis was used to compare the OS between two risk groups. The receiver operating characteristic (ROC) curve for the 1-year to 5-year OS was applied to further determine the reliability of the prognostic model.

2.7. Immune infiltrate analysis

The CIBERSORT tool (http://cibersort.stanford.edu) is an analytical tool to provide an estimation of the abundances of member cell types in a mixed cell population by using gene expression data. We estimated the immune cell infiltration in the TCGA-AML cohort by using the CIBERSORT. Pearson’s correlation analysis was used to evaluate the relationship between LOC644135 and immune cells.

3. Results

3.1. High expression of LOC644135 indicated poor prognosis in AML

The expression of LOC644135 was the highest in AML across 33 types of cancer in the GEPIA database (Supplementary Figure 1). Besides, comparison of the expression of LOC644135 in the Cancer Cell Line Encyclopedia (CCLE) database showed that LOC644135 was highly expressed in the AML cell lines F-36P, MONO-MAC-6, and THP-1; on the
contrary, low expression of LOC644135 was observed in the AML cell lines EOL-1, MOLM-16, and SKM-1 (Supplementary Figure 2). Notably, AML cell line MOLM-16 was cultured from the peripheral blood of a relapse AML patient after failed chemotherapy. The details of the AML cell lines were shown in Supplementary Table 1. According to the median expression of LOC644135, AML patients in the TCGA cohort were separated into LOC644135\textsuperscript{high} group and LOC644135\textsuperscript{low} group. Kaplan-Meier survival curves showed that high expression of LOC644135 was associated with inferior OS (Figure 1(a), p=0.000629) and EFS (Figure 1(b), p=0.0011) in the TCGA-AML cohort. As validation, pediatric AML patients in the TARGET cohort were divided into high- and low-expression groups based on the median expression of LOC644135. Patients in high-expression group also had shorter OS (Figure 1(c), p=0.0492) than those in low-expression group, which was consistent with the results performed in TCGA-AML cohort.

3.2. High expression of LOC644135 indicated poor prognosis in CN-AML

Sixty-two patients with cytogenetically normal AML (CN-AML) were selected from the TCGA-AML cohort. Subsequently, CN-AML patients were divided into high- and low-expression groups based on the median expression of LOC644135. Kaplan-Meier curves showed that patients with high expression of LOC644135 had significantly shorter OS (Figure 2(a), p=5.29e-08) and EFS (Figure 2(b), p=9.45e-06) than those with low LOC644135 expression.

And then, we compared the expression of LOC644135 between CN-AML patients, non-CN-AML patients and normal bone marrow samples. The significant difference was found between CN-AML patients and normal samples (Figure 2(c), p=5.6e-15).

3.3. The expression of LOC644135 was an independent prognostic factor in AML

Patients in LOC644135\textsuperscript{high} group had significantly higher white blood cells (WBC) counts than those in LOC644135\textsuperscript{low} group (Figure 3(a), p=0.002). However, patients with IDH2 mutation were significantly found in LOC644135\textsuperscript{low} group (Figure 3(a), p=0.048). Other characteristics had no statistical significance between two groups, including treatment (chemotherapy versus allo-HSCT), age (<60 years versus ≥60 years), gender (male versus female), bone marrow (BM) blast counts (<20% versus ≥20%), peripheral blood (PB) blast counts (<70% versus ≥70%).

Figure 1. Kaplan-Meier curve of overall survival (OS) and event-free survival (EFS) in different expression of LOC644135 in TCGA-AML cohort and TARGET-AML cohort. a) Kaplan-Meier curve of OS in TCGA-AML cohort. b) Kaplan-Meier curve of EFS in TCGA-AML cohort. c) Kaplan-Meier curve of OS in TARGET-AML cohort.
versus ≥70%), and frequency of other recurrent genetic mutations (FLT3-ITD, IDH1/2, etc., wild type versus mutation). The details were shown in Supplementary Table 2.

And then, some characteristics were enrolled in the multivariable analysis including IDH2 mutation, age, WBC counts, PB counts, BM count and gender. Multivariable analysis showed that the expression of LOC644135 was an independent adverse factor in AML (Figure 3(b), p=0.002). Similarly, age was an independent adverse factor in AML as well (Figure 3(b), p<0.001).

### 3.4. The biological function of LOC644135 in AML

As shown in Figure 4(a), 291 down-regulated genes and 282 up-regulated genes were identified as the DEGs and the details were showed in Supplementary Table 3. KEGG pathway analysis and GSEA analysis showed that the DEGs were enriched in some immune-related pathways, such as B cell receptor signaling pathway and Cytokine-cytokine receptor interaction (Figure 4b-f). The complete result of the GSEA analysis was shown in Supplementary Table 4. Therefore, we evaluated the correlation between LOC644135 and immune infiltrate in AML. The result indicated that LOC644135 was negatively correlated with CD8+ T cell (Supplementary Figure 3).

### 4. ceRNA network identification and analyses

The differentially expressed microRNAs and mRNAs were extracted to construct the ceRNA network (Figure 5(a)), and the LOC644135-related ceRNA network was shown in Figure 5(b). In the ceRNA network, LNC644135 was connected...
with miR-96 and miR-182 directly and associated with ten mRNAs: FASP1, FGL2, DOC1K, HDAC9, DPYD, CYBB, TMEM144, MYOF, SRC, and GIMAP8 (Supplementary Table 5). Functional enrichment analysis showed that the LOC644135-related ceRNA network was mainly enriched in the vascular endothelial growth factor receptor signaling pathway (Figure 5c). Furthermore, three genes of the LOC644135-related ceRNA network were selected to establish a prognostic model and the model had great predictive efficiency for prognosis in TCGA-AML cohort (Supplementary Figure 4).

5. Discussion

Increasing studies have demonstrated that IncRNAs were the key regulator in tumor proliferation, invasion and metastasis. Therefore, IncRNAs can be the potential biomarker or target for diagnosis and therapy. In AML, there are some well-known IncRNA such as PVT1 and MEG3 that played crucial role in proliferation and apoptosis [22], but the clinical applicability still needs to be further determined. The more we know about the function of IncRNAs in AML, the more conducive to the diagnosis and treatment of AML. Hence, in this study, pan-cancer analysis found that IncRNA LOC644135 was most highly expressed in AML. Besides, we also found that high expression of LOC644135 indicated poor prognosis in both TCGA-AML cohort. To improve the reliability of the result, we validated the result in an external independent cohort. TARGET-AML cohort contained 187 patients with pediatric AML. Although adult AML and pediatric AML are two different diseases, there are still many clinical and biological similarities between the two diseases [23,24] and the treatment for the two diseases are very familiar. Our result found that IncRNA LOC644135 was also had prognostic relevance in the TARGET-AML cohort. Notably, high expression of LOC644135 was strongly associated with poor prognosis in CN-AML patients. Multivariable analysis also indicated that LOC644135 was an independent adverse factor for prognosis in AML. Our findings illuminated that LOC644135 was a dependable prognostic biomarker for AML.

Cytogenetically normal AML (CN-AML) is the major category of AML stratified by the cytogenetics. Although the landmark targeted therapies have developed, patient outcomes in CN-AML are still widely diverse because of its clinical heterogeneity which made the prognostic stratification and treatment decision has been difficult [25]. Therefore, there is an urgent need to identify reliable prognostic biomarkers that could help to predict therapy response. In this study, we evaluated the prognostic impact of LOC644135 in patients with CN-AML in TCGA-AML cohort. We found a very strong
association between high expression of LOC644135 and poor prognosis. Additionally, the expression of LOC644135 had a significant difference between patients with CN-AML and normal samples. Therefore, our finding implied that the expression of LOC644135 can be the indicator for prognosis in CN-AML providing new thought for diagnosis and therapy in CN-AML.

To explore the biological function of LOC644135 in AML, KEGG pathway analysis and GSEA analysis were applied in differentially expressed genes. We found that DEGs were also enriched in some immune-related pathways such as B cell receptor signaling pathway and Cytokine-cytokine receptor interaction. B cell receptor signaling pathway plays a crucial role in the prevention of dysregulated activation of autoreactive B cells which can induce autoimmunity in the secondary lymphoid organs [26]. Cytokine and cytokine receptors are important components in the immune system. Cytokine therapy to activate the immune system of cancer patients has been an important treatment modality and continues to be a key contributor to current clinical cancer research [27]. Besides, Immunotherapies play anti-tumor role through activating the immune system [28]. Although immunotherapies are promising treatment for cancer patients with less toxic and side effect, immunotherapy has shown limited clinical activity in AML. Thus, classifying AML with a more precise method may be helpful to improve the clinical application of immunotherapy in AML. We found that high expression of LOC644135 might prevent the infiltration of CD8+ T cells in AML. Cancer patients with high infiltration of CD8 + T cells have better chance to benefit from immunotherapies [29]. Recent research have demonstrated that higher percentages of CD8 + T cells in bone marrow could be the predictor for the response to the checkpoint inhibitor (CPI) nivolumab in combination with hypomethylating agent in AML [30]. Thus, our findings indicated that immunotherapy might be more effective to AML with low expression of LOC644135. Taken together our findings indicated that
LOC644135 may participate in immunoregulation of AML, which provide new insight into the therapeutic strategies of AML.

To further unveil the potential mechanism of LOC644135 in AML, we constructed the LOC644135-centric ceRNA network and we found that the LOC644135-related ceRNA network mainly participated in the vascular endothelial growth factor receptor signaling pathway. Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) are the key regulators of tumor angiogenesis. Increasing evidence has shown that angiogenesis, the formation of new tumor vessels, played a pivotal role in disease progression and drug resistance in AML [31]. Therapeutic strategies targeting the VEGF/VEGFR2 pathway have been approved for clinical trials in AML [32]. Besides, our results found that low expression of LOC644135 was associated with high frequency of IDH2 mutation, which indicated that expression level of LOC644135 might predict the response of IDH inhibitors in AML. Thus, we further analyzed the efficiency of IDH inhibitors in AML cell lines and the result showed that the EOL-1 cell line that had low expression of LOC644135 were sensitive to the IDH1 targeted drug AGI-5198 and IDH2 targeted drug AGI-6780 (Supplementary Figure 5), which suggested that AML with low expression of LOC644135 might have higher sensitivity on both IDH1 and IDH2 inhibitor drugs. To further explore the potential drugs for high expression group, we searched the sensitive drugs for the AML cell line MONO-MAC-6 with high expression of LOC644135 in the Genomics of Drug Sensitivity in Cancer (GDSC) database which is the largest drug sensitivity public database [33]. We found the MONO-MAC-6 cell line was sensitive to multiple antitumor drugs (Supplementary Table 6), which provided new clues for precision treatment in AML. Furthermore, we established a prognostic model based on the LOC644135-centric ceRNA and great prognostic predictive efficiency was found in 1-year to 5-year OS. Together, these results highlighted that the LOC644135-related ceRNA network also played crucial role in AML, supporting a potential role for LOC644135-related ceRNA network in the development of diagnosis and therapy of AML.

Whereas, our study still has some limitations. For example, we have evaluated the prognostic value of LOC644135 in two
coHORTS, but the external experiments would be preferable to further verification. We preliminary unveiled the potential mechanism of LOC644135 in AML, but the role of LOC644135 and the LOC644135-related ceRNA network in AML needs to be further investigated. We will attempt to overcome these shortcomings in our following works. In conclusion, LOC644135 was highly expressed in AML and associated with poor prognosis in AML. High expression of LOC644135 also indicated inferior survival times in CN-AML patients as well. We suggested that LOC644135 might participate in some immune-related pathways and be associated with CD8+ T cells infiltration in AML. Besides, the LOC644135-related ceRNA network might also play crucial role in AML through the VEGFR pathway.

6. Conclusion
LncRNA LOC644135 is an independent poor prognostic factor for AML, especially for CN-AML, offering the insight of LOC644135 in AML and implicating new thoughts into diagnosis and treatment of AML.

Acknowledgments
We would like to acknowledge Taikang Tongji (Wuhan) hospital and the Seventh affiliated hospital of Sun Yat-Sen university for providing help during the project.

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All authors read and approved the final manuscript.

Funding
This paper was not funded.

Declaration of Interest
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures
Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Availability of data and materials
The datasets generated and/or analysed during the current study are available in the the Cancer Genome Atlas (TCGA), Therapeutically Applicable Research to Generate Effective Treatments (TARGET) and Genotype-Tissue Expression Project (GTEx) repository.

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