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ORIGINAL ARTICLE

Identification of MiR-125a as a Novel Plasma Diagnostic Biomarker for Chronic Lymphoblastic Leukemia

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SUMMARY

Background: **Chronic lymphocytic leukemia (CLL) is a type of malignancy in which the bone marrow makes too many lymphocytes. MicroRNAs (miRNAs) are endogenous short (~22-nucleotides) non-protein-coding regulatory RNA molecules with key roles in cellular and molecular processes linked to different cancers including CLL. Recently, some investigations have demonstrated that miR-125a downregulation is correlated with the expression of P53, NRG1 and ERBB2.**

Methods: **In this study, samples including 38 patients with CLL and 25 healthy individuals were collected. We used quantitative real-time PCR (qRT-PCR) to assess the expression of miR-125a in plasma of the CLL patients in comparison with healthy controls. Moreover, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis on miR-125a targets in the DAVID database in order to investigate the potential role of miR-125a in cancer pathways. MiR-125a exerted a variety of roles in the cancer pathway via downregulating target genes including ERBB2.**

Results: **The expression of miR-125a dramatically decreased (~2-fold) in the patients with CLL compared with the healthy controls ($p = 0.03$). Furthermore, overexpression of miR-125a was associated with different CLL staging and B symptoms (all at $p < 0.05$). The KEGG pathway enrichment analysis demonstrated the eight statistically related KEGG signaling pathways with miR-125a targetome.**

Conclusions: **The results suggested that the miR-125a expression level could be a novel potential biomarker for CLL prognosis.**

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) known as the most frequent form of leukemia in adults remains incurable while survival of patients has been longer with innovation and accomplishment of novel treatment

choices [1,2]. CLL comprises a number of features including the slow progressive course and accumulated abnormal $CD5^+$ B lymphocytes in the blood stream, lymphoid organs, and bone marrow [3]. CLL is characterized by a remarkably heterogeneous clinical course [2]. The mean age at diagnosis is about 71 years while it is infrequently encountered in population under the age 40 and is extremely rare during childhood [1]. Currently, two methods of disease classification are widely utilized to predict the prognosis of patients with CLL, namely the Rai [4] and Binet staging [5] methods. However, these methods cannot distinguish between patients with stable and progressive CLL [6].

According to research by Martin-Subero, prognostic stratification in CLL is based on IgVh mutations, ZAP-70, CD38 expression and chromosomal abnormalities. The key factor in CLL is clonal expansion and somatic hypermutation in human B lymphocytes, which are the most stated causes of IgVh mutation [7-9]. Moreover, different forms of chromosomal abnormalities such as 17p13, 17q13, 13q14, 11q23, and 6q21 have been detected in these patients [9-11]. The loss of the TP53 function due to regional abnormalities of the chromosome 17 (17p13) has been stated to be correlated with chemoresistance. In addition, deletion of 11q22.3, including the locus for *ATM*, promotes the activity of MDM2, which, in turn, reduces the p53 activity in patients with CLL [12].

MicroRNAs (miRNAs) are endogenous short (~22-nucleotides) non-protein-coding regulatory RNA molecules that control gene expression, sequence specific manner via mRNA degradation and/or translational suppression of downstream target genes [13-16]. In human acute and chronic leukemia, it has been detected that miRNAs can act either as oncogenes to cancer development or tumor suppressors to inhibit cell division or induce cell death [16-18]. Between several leukemia-associated miRNAs, microRNA-125a has been found to decrease expression levels in acute myeloid leukemia (AML) and may be associated with decreased survival in AML [19]. Moreover, miR-125a has been found to be downregulated in gastric cancer and associated with cancer progression and prognosis [20]. However, there has been no report on the exact expression pattern of miR-125a and its function in CLL.

MiR-125a has been proposed to be a negative regulator of ERBB2. Some studies have shown that miR-125a has an important effect on cell division, differentiation, and apoptosis [19]. Many investigations have stated aberrant expression of miR-125a in biological samples from malignant patients [20,21]. However, there are no relevant, exact studies on miR-125a in CLL. Given that the downregulation of miR-125a promotes AML, breast cancer, and gastric cancer support the likelihood of downregulated miR-125a in the development of CLL. In the present research, we aimed to evaluate the expression profile of miR-125a in CLL in order to find whether aberrant expression of miR-125a can be used as a prognostic biomarker. Moreover, the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis was done to explain the possible function of miR-125a in CLL pathogenesis and examine which related signaling pathways may be affected by miR-125a deregulation in CLL. Furthermore, the function and clinical significance of miR-125a were evaluated to explain whether miR-125a could assist the CLL pathogenesis and whether it might be used as a novel therapeutic target for CLL.

MATERIALS AND METHODS

Patients and the control group

We evaluated plasma samples obtained at the time of diagnosis of 38 CLL patients who had previously been diagnosed with CLL as well as 25 healthy individuals. None of the CLL patients had received prior treatment during the last six months before blood drawing. All the patients underwent molecular and phenotypic classification with available clinical data. The Binet and Rai staging systems were used for clinical staging of the patients. The patients with the Rai staging stratification were divided into the stages 0 (low risk), 1 and 2 (intermediate risk), and 3 and 4 (high risk).

Total RNA isolation and cDNA conversion were performed as previously described [22]. Briefly, RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's instructions. A total of 1 µg of total RNA was converted to cDNA using the cDNA Synthesis Kit (Takara, Japan). QRT-PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems) using the miScript® SYBR® Green PCR Kit (Qiagen) in triplicate. To normalize the expression levels of the miR-125a gene, U6 snRNA was applied as housekeeping. The relative expression of plasma miRNA in the CLL cases was analyzed with the $2^{-\Delta\Delta Ct}$ method using pooled miRNA from the healthy individuals as the reference [23].

The study was approved by the local research ethics committees, and informed consent was obtained from the contributors.

Statistical analysis

All statistical analyses were carried out using the SPSS 20.0 software package (IBM SPSS Statistics for Windows, IBM Corp; Armonk, NY, USA) and Graph Pad Prism statistical software version 5.01 (Graph Pad, San Diego, CA, USA). p-value of < 0.05 was considered statistically significant.

Systematic pathway enrichment analysis

For the first time, we used the KEGG pathway enrichment analysis to determine possible involvement of miR-125a in the cancer pathway using several tools so as to obtain predicted and validated miRNA-targets in miRecords [24] and miRTarBase [25] databases. In order to find which signaling pathway is related to miR-125a targetome and how miR-125a contributes to can-

Table 1. MiR-125a expression and clinicopathological characteristics.

Clinical features	miR-125a expression levels	
	Low (n = 18)	High (n = 20)
Age		
< 60	**6**	**10**
> 60	**9**	**13**
p	**0.634**	
Gender		
Male	**11**	**9**
Female	**8**	**10**
p	**0.579**	
Binet stage		
A	**10**	**11**
B	**8**	**9**
C		
p	**0.037**	
B-symptoms		
Yes	**7**	**8**
No	**11**	**12**
p	**0.032**	
LDH		
Normal	**12**	**7**
Elevated	**9**	**10**
p	**0.48**	
Rai stage		
0 - 2	**12**	**15**
3 - 5	**6**	**5**
p	**0.043**	

Table 2. The eight most statistically relevant KEGG signaling pathways with miR-125a-5p targetome based on TarBase 6.0.

KEGG pathway	Gene number in the pathway	p-value	Fold enrichment
HIF-1 signaling pathway	**18**	**1.2E-12**	**10.0**
Pancreatic cancer	**13**	**1.4E-9**	**10.9**
ErbB signaling pathway	**14**	**4.3E-9**	**8.8**
Central carbon metabolism in cancer	**12**	**1.6E-8 1.6E-8**	**10.2**
Epstein-Barr virus infection	**17**	**2.9E-7 2.9E-7**	**4.9**
Prostate cancer	**12**	**4.7E-7**	**7.4**
Pathways in cancer	**24**	**4.9E-7 1.6E-6**	**3.3**
Neurotrophin signaling pathway	**13**	**1.6E-6**	**5.9**

cer progression, in silico network analysis was carried out by the DAVID 6.7 database [26]. This database provides results from the KEGG pathway analysis and comprehensive set of functional annotation tools for researchers to find biological processes behind a large list of genes with miR-125a targetome [27].

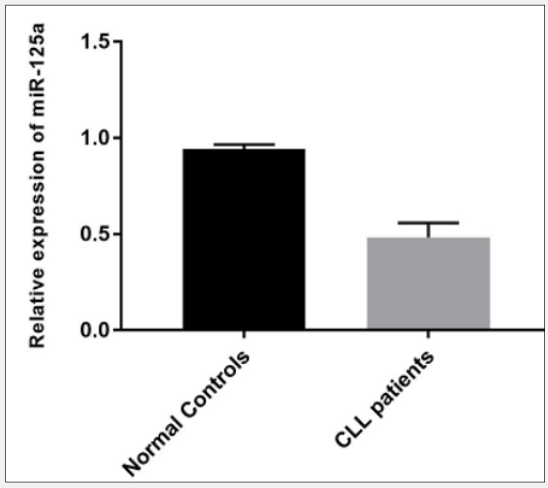

Figure 1. Comparison of CLL patients with healthy individuals; downregulation of miR-125a in the CLL patients (p = 0.03).

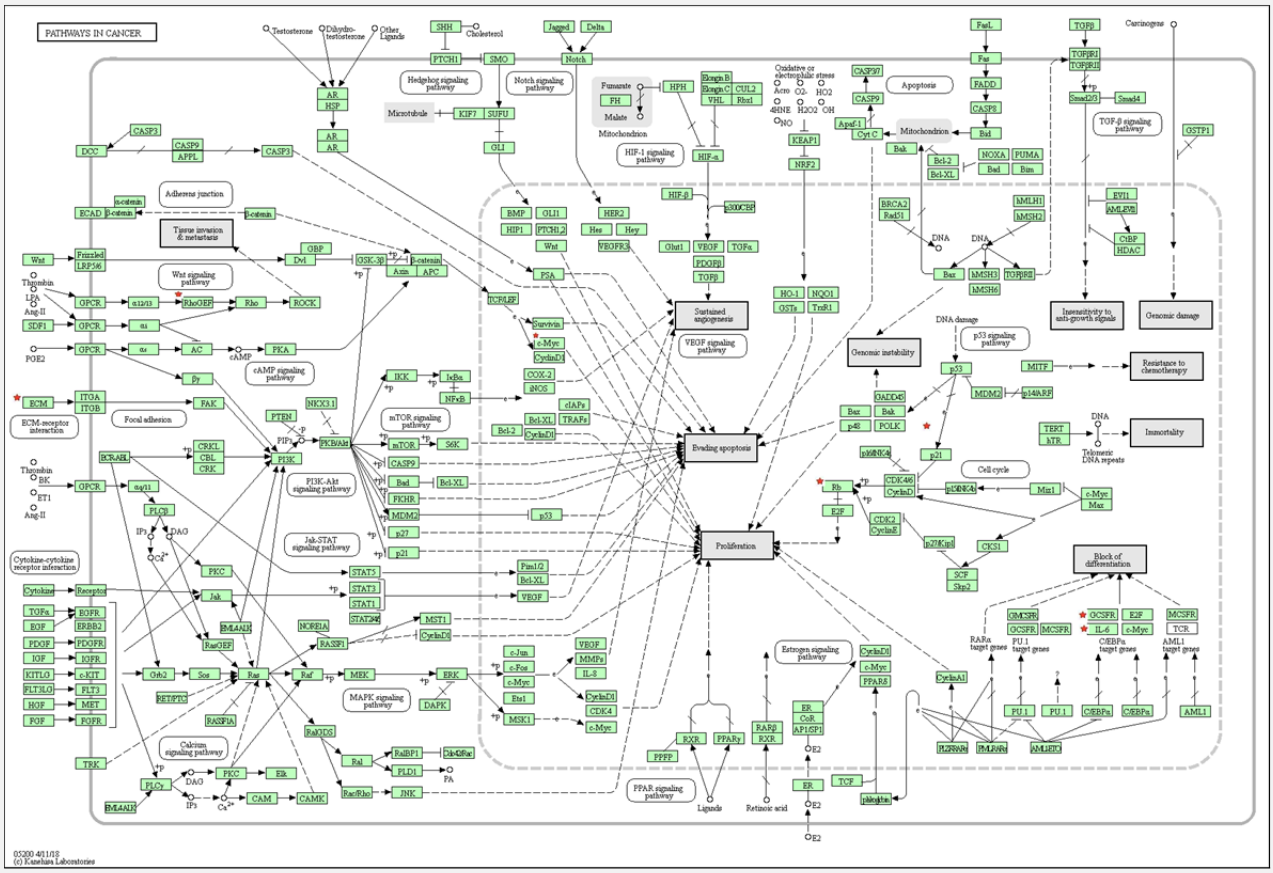

Figure 2. Involvement of miR-125a targetome in the cancer pathway; for more information, please see the KEGG database website: https://david.ncifcrf.gov/kegg.jsp?path=hsa05200.

RESULTS

MiR-125a expression in the CLL patients against normal controls

We examined the expression levels of miR-125a in plasma samples from 38 CLL patients and 25 healthy individuals. Based on the qRT-PCR analysis, the miR-125a expression level dramatically decreased in the plasma samples of the CLL cases compared with those of the healthy controls ($p = 0.03 \sim 2$-fold; Figure 1).

Correlation between the miR-125a expression level and clinicopathological characteristics

Clinical information including age, gender, LDH level, B-symptoms and different staging status was available for the 38 *CLL* patients. The cases were split into two groups of 20 and 18 cases according to the high and low level of miR-125a expression, respectively, using the mean value as a high/low cutoff. In the patients with CLL**,** the plasma miR-125a expression was associated with some known adverse prognostic factors. The presence of B symptoms and different staging of the disease were associated with increased levels of plasma miR-125a. However, the level of miR-125a expression had no correlation with LDH level, age, and gender (Table 1).

Molecular signaling pathway enrichment analysis

In order to examine and find miRNA-target interactions, two web-based miRNA-related databases including miRrecords and miRTarBase databases were used. Six and 276 mRNAs were recognized as validated and predicted miRNA-target of miR-125a, respectively (data not shown). The predicted targets were confirmed by other prediction web-based related databases. All the validated targets were obtained from the miRecords tools, which integrate the predicted targets of many miRNA target prediction tools. Functional relationships between the selected types of miR-125a targetome were investigated using the DAVID statistically significant list of attributed genes with many KEGG signaling pathways (Table 2). The KEGG analyses revealed that the enriched miRNA-targeted genes (miR125a targetome) were mostly involved in the HIF-1 signaling pathway and cancer pathway (Table 2). The potential miRNA targets for the cancer pathway are indicated in Figure 2; miR-125a target genes are demonstrated by red star marks.

DISCUSSION

To the best of our knowledge, this is the first report describing that the expression level of miR-125a can have potential diagnostic value regarding CLL as miR-125a levels are dramatically lower in the CLL patient's plasma compared to the plasma of healthy individuals. In addition, there was a significant association between miR-125a downregulation and the CLL patients' clinicopathological characteristics, including clinical staging and B symptoms. However, no significant association was found between miR-125a downregulation with serum LDH level, age and gender (Table 1, Figure 1). *CLL* is a clonal lymphocytic disorder *of mature abnormal B-lymphocytes* mainly involving bone marrow, peripheral blood, and lymphoid organs. CLL is usually considered a lymphatic disorder with a mature B-cell origin. The clonal B cells in CLL co-expression of CD5 with CD19, CD20 (dim), and CD23. The spleen and secondary lymphoid tissues are diffusely infiltrated by CLL malignant cells [28]. CLL may cause anemia and thrombocythemia can lead to excess bleeding and bruising, respectively. Sensitive, specific, and non-invasive biomarkers are needed for diagnosis of the disease. MiRNAs have emerged as attractive candidates for the diagnostic and prognostic applications of many types of malignancy [19,20,29].

One of the miR-125a targets is ERBB2; as previously shown [19], downregulation of miR-125a can induce the expression of ERBB2. ERBB2's ability to mediate resistance to therapy may involve activation of the PI3K/Akt pathway by ErbB2, which leads to increased cancer cell survival [30]; this is one of the major pathways involved in cancer progression.

Deregulation of miR-125a is associated with the expression of P53, NRG1, and ERBB2. Moreover, miR-125a expression is closely associated with gastric cancer in distinct metastasis. MiR-125a can regulate p53, which promotes ERBB2, and adjust cell growth, survival, and differentiation. According to our findings, miR-125a might have an inducing role in CLL, probably in targeting several negative regulators of CLL proliferation. According to previous investigations, the ERBB2 gene was enhanced within a patient with myelodysplastic syndrome, who developed AML [33]. Another study by Nordigarden et al. revealed that canertinib, a pan-ERBB inhibitor, is efficient in both primary samples and the murine model in patients with AML (34).

The level of miR-125a expression is observed to be lower in non-small cell lung cancer, as compared to normal tissues [19]. On the contrary, miR-125a is overexpressed in synovial sarcoma [31] and upregulated under hypoxic situations in retinoblastoma [32]. MiR-125a-5p expression, the partner of miR-125a-3p, is reduced in breast cancer [33], ovarian cancer [34], lung cancer [21], medulloblastoma [35], and AML [19], demonstrating its tumor suppressive effect in different human malignancies. A thorough investigation of the literature yielded only few studies examining the functional role for miR-125a. This is the first report indicating the clinical importance of miR-125a in CLL.

These findings suggest the significance of miR-125a as a useful biomarker in CLL patients. It can be assumed that due to ERBB2 upregulation following mir-125a downregulation, miR-125a may have a tumor suppressing effect on CLL. Moreover, miR-125a might be an ideal biomarker for early detection of CLL.

CONCLUSION

In summary, despite successes in the follow-up of CLL patients, a useful biomarker for quick and robust prognosis of the disease is still missing. In the present study, we explored the expression level of miR-125a in CLL patients in comparison with healthy individuals. We showed that the transcription level of miR-125a is downregulated in CLL patients. These findings suggest that ERBB2 could be a novel therapeutic option for miR-125a in CLL patients, which might be potentially evaluated due to the availability of canertinib. Our research showed the reduced expression in miR-125a. This miRNA might be a potential biomarker for the prognosis of CLL and also a potential therapeutic target.

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Declaration of Interest:
There is no conflict of interest.

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