Original Article
Clinical significance of up-regulated ID1 expression in Chinese de novo acute myeloid leukemia

Jing-Dong Zhou1*, Lei Yang1*, Xiao-Wen Zhu2, Xiang-Mei Wen2, Jing Yang1, Hong Guo2, Qin Chen2, Dong-Ming Yao2, Ji-Chun Ma2, Jiang Lin2, Jun Qian1

1Department of Hematology, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China; 2Laboratory Center, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China. *Equal contributors.

Received February 19, 2015; Accepted April 13, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: To investigate the clinical significance of ID1 expression in Chinese de novo AML patients. Real-time quantitative PCR was carried out to detect the status of ID1 expression in 102 de novo AML patients and 28 controls. ID1 transcript level was significantly increased in AML compared to normal controls (P=0.029). The age in the patients with high ID1 expression is significantly older than in those with low ID1 expression (P=0.044). ID1 overexpression occurred with the highest frequency in the patients with poor karyotype (7/7, 100%), lower frequency in the patients with intermediate karyotype (28/60, 47%), and the lowest frequency in the patients with favorable karyotype (12/31, 39%). Both whole AML and non-M3 patients with high ID1 expression had significantly lower rate of complete remission than those with low ID1 expression (P=0.007 and 0.038). ID1 high-expressed patients showed significantly shorter overall survival (OS) than ID1 low-expressed patients in both whole AML and non-M3 according to Kaplan-Meier analysis (P=0.007 and 0.040). However, multivariate analysis indicated that ID1 overexpression was not an independent risk factor in both whole AML and non-M3 patients. However, the adverse impact of ID1 overexpression on outcome was revealed by both Kaplan-Meier analysis and multivariate analysis in the non-M3 patients less than 60 years old. Our study reveals that ID1 overexpression may be associated with higher risk karyotype classification and act as an independent risk factor in young non-M3 patients.

Keywords: ID1, expression, prognosis, acute myeloid leukemia

Introduction

Acute myeloid leukemia (AML), a clonal hematological malignancy, is a biologically, clinically, and etiologically heterogeneous disease [1, 2]. Cytogenetic alterations and molecular biological changes play crucial roles in the pathogenesis and progression of AML. Despite the advancements in the treatment of leukemia, clinical outcome of AML remains unsatisfactory. Therefore, identifying genetic and epigenetic alterations which can recognize the patients who are at the risk of poor outcome is warranted to optimize treatment strategies. Over the past years, the prognosis of AML has been evaluated mainly based on cytogenetic analysis [3, 4]. Recently, numerous genetic changes including gene mutations, deletions, amplifications and gene expression abnormalities, have been identified [5-7]. These alterations contribute to further understanding of leukemogenesis and provide more prognostic markers in AML [8, 9].

ID (inhibitors of differentiation) gene encodes for a helix-loop-helix (HLH) protein, a group of dominant inhibitors of basic HLH transcriptional factors which promote cell differentiation [10, 11]. ID1 (inhibitors of differentiation 1), a family member of ID genes, has been identified as a potential proto-oncogene for its role in inducing cell proliferation as well as invasion, and protecting cells against drug-induced apoptosis [11]. Overexpression of ID1 has been found in a variety of solid tumors [12-22]. However, few studies investigated the clinical relevance of ID1 expression in AML [23, 24]. Therefore, the current study was intended to investigate the clinical significance of ID1 expression in Chinese de novo AML patients.
**ID1 expression in AML**

### Table 1. Correlation between ID1 expression and whole AML as well as CN-AML patients parameters

<table>
<thead>
<tr>
<th>Patient’s parameters</th>
<th>Status of ID1 expression in whole AML</th>
<th>Status of ID1 expression in CN-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=51)</td>
<td>High (n=51)</td>
</tr>
<tr>
<td></td>
<td>Low (n=27)</td>
<td>High (n=21)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>29/22</td>
<td>32/19</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>51 (10-93)</td>
<td>60 (17-87)</td>
</tr>
<tr>
<td>Median WBC, ×10^9/L (range)</td>
<td>5.7 (0.3-528.0)</td>
<td>19.7 (1.1-185.4)</td>
</tr>
<tr>
<td>Median hemoglobin, g/L (range)</td>
<td>78 (32-131)</td>
<td>68 (40-138)</td>
</tr>
<tr>
<td>Median platelets, ×10^9/L (range)</td>
<td>40 (6-140)</td>
<td>42 (4-264)</td>
</tr>
<tr>
<td>BM blasts, % (range)</td>
<td>44.0 (1.0-97.5)</td>
<td>53.3 (3.0-109.0)</td>
</tr>
</tbody>
</table>

**Materials and methods**

**Patients’ samples**

Bone marrow (BM) was collected from 102 patients with de novo AML treated at the Affiliated People’s Hospital of Jiangsu University. The diagnosis and classification of AML patients were established according to the revised French-American-British (FAB) classification and the 2008 World Health Organization (WHO) criteria [25, 26]. Written informed consent was obtained from the patients before the bone marrow aspiration. The patients’ samples were collected from the patients treated at the Affiliated People’s Hospital of Jiangsu University. The diagnosis and classification of AML patients were established according to the revised French-American-British (FAB) classification and the 2008 World Health Organization (WHO) criteria [25, 26]. Written informed consent was obtained from the patients before the bone marrow aspiration.

**Table 1. Correlation between ID1 expression and whole AML as well as CN-AML patients parameters**

<table>
<thead>
<tr>
<th>Patient’s parameters</th>
<th>Status of ID1 expression in whole AML</th>
<th>Status of ID1 expression in CN-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=51)</td>
<td>High (n=51)</td>
</tr>
<tr>
<td></td>
<td>Low (n=27)</td>
<td>High (n=21)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>29/22</td>
<td>32/19</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>51 (10-93)</td>
<td>60 (17-87)</td>
</tr>
<tr>
<td>Median WBC, ×10^9/L (range)</td>
<td>5.7 (0.3-528.0)</td>
<td>19.7 (1.1-185.4)</td>
</tr>
<tr>
<td>Median hemoglobin, g/L (range)</td>
<td>78 (32-131)</td>
<td>68 (40-138)</td>
</tr>
<tr>
<td>Median platelets, ×10^9/L (range)</td>
<td>40 (6-140)</td>
<td>42 (4-264)</td>
</tr>
<tr>
<td>BM blasts, % (range)</td>
<td>44.0 (1.0-97.5)</td>
<td>53.3 (3.0-109.0)</td>
</tr>
</tbody>
</table>

**Materials and methods**

**Patients’ samples**

Bone marrow (BM) was collected from 102 patients with de novo AML treated at the Affiliated People’s Hospital of Jiangsu University. The diagnosis and classification of AML patients were established according to the revised French-American-British (FAB) classification and the 2008 World Health Organization (WHO) criteria [25, 26]. Written informed consent was obtained from the patients before the bone marrow aspiration.
obtained from all patients. The study was approved by the Institutional Review Board of the Affiliated People’s Hospital of Jiangsu University. Karyotypes were analyzed by conventional R-banding method and karyotype risk was classified according to reported previously [27]. Treatment protocol was described previously [28]. The characteristics of AML patients were summarized in Table 1. 28 healthy donors were collected as controls. Bone marrow mononuclear cells (BMMNCs) were separated by Ficoll solution and washed twice with PBS. RNA isolation, reverse transcription and RQ-PCR

Total RNA was isolated from the BMMNCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed on iCycler Thermal Cycler (Eppendorf, Hamburg, Germany). The reactions with final volume 40 μL contained 5× buffer 10 mM, dNTPs 10 mM, random hexamers 10 μM, RNAsin 80 units, and 200 units of MMLV reverse transcriptase (MBI Fermentas, Hanover, USA). The system of reverse transcription was incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C. Real-time quantitative PCR (RQ-PCR) was performed on a 7300 Thermo cycler (Applied Biosystems, CA, USA). The primer sequences of ID1 expression were 5’-CTCACGACCTCAACGG-3’ (forward) and 5’-GATCGGTCTTGTTCCCTC-3’ (reverse) with expected product of 199 bp. Reaction system with a volume of 20 μL was consisted of cDNA 20 ng, 0.4 μM of primers, 10 μM of AceQ™ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), and 0.4 μM of ROX Reference Dye 1 (Invitrogen, Carlsbad, CA, USA). RQ-PCR conditions were carried out at 95°C for 5 min, followed by 35 cycles at 95°C for 10 s, 62°C for 30 s, 72°C for 30 s, and 80°C for 30 s to collect fluorescence, finally followed by 95°C for 15 s, 60°C for 60 s, 99°C for 15 s, and 60°C for 15 s. Positive and negative controls were included in all assays. Housekeeping gene (ABL) was used to calculate the abundance of ID1 mRNA. Relative ID1 expression levels were calculated using the following equation: 

\[ N_{ID1} = \frac{(E_{ID1})^{\Delta CT}_{ID1(\text{control-sample})}}{(E_{ABL})^{\Delta CT}_{ABL(\text{control-sample})}} \]

The parameter efficiency (E) was derived from the formula E=10^{-1/[\text{slope}]} (the slope referred to CT versus cDNA concentration plot). ΔCT reflected the disparity in CT value between control and target or reference sequences. We selected the bone marrow sample from one normal control that possessed the minimal ΔCT between ID1 and ABL transcript as control and was defined as 100% expression for ID1 transcript.

Gene mutation detection

IDH1/2, DNMT3A, N/K-RAS, C-KIT, NPM1, and U2AF1 mutations were detected by high-resolution melting analysis (HRMA) as reported previously [29-32]. All positive samples were confirmed by DNA direct sequencing. FLT3-ITD and C/EBPA mutations were detected by direct DNA sequencing [33].

Statistical analysis

Statistical analyses were performed on SPSS 17.0 software package (SPSS, Chicago, IL). Mann-Whitney’s U test was used to compare the difference of continuous variables in two groups. Pearson Chi-square analysis or Fisher exact test were employed to compare the difference of categorical variables. Receiver operating characteristic curve (ROC) and area under
**ID1 expression in AML**

The ROC curve (AUC) were conducted to assess the value of ID1 expression in distinguishing AML and cytogenetically normal AML (CN-AML) patients from normal controls. Kaplan-Meier curve done by log-rank test and Cox regression backward stepwise likelihood ratio were performed to analyze the impact of ID1 expression on survival respectively. For all analyses, a two-tailed P value of 0.05 or less was determined as statistically significant.

**Results**

**ID1 expression in normal controls and AML patients**

ID1 transcript level in normal controls ranged from 0.000 to 1.000 with a median level of 0.015. The level of ID1 expression (0.000-3.536, median 0.029) was significantly increased in AML compared to normal controls (P=0.029, Figure 1). The representative electrophoresis results of RQ-PCR products were shown in Figure 2.

**Differentiating value of ID1 expression**

ROC curve was applied to evaluate the differentiating value of ID1 expression. It indicated that ID1 level might serve as a biomarker for distinguishing AML from controls (AUC=0.633, 95% CI: 0.523-0.742, P=0.032).

**Clinical and laboratory characteristics of AML patients**

The whole cohort of AML patients were divided into two groups at the median level of ID1 expression, and defined as low ID1 expression (ID1<sup>low</sup>) group (<0.029) and high ID1 expression (ID1<sup>high</sup>) group (>0.029). There were no significant differences in sex, hemoglobin (HB), platelets (PLT), and BM blasts between the ID1<sup>high</sup> and ID1<sup>low</sup> groups (P>0.05, Table 1). However, ID1<sup>high</sup> cases tended to have a higher white blood cell (WBC) than ID1<sup>low</sup> cases (P=0.062). ID1<sup>high</sup> patients had significantly older age than ID1<sup>low</sup> patients (P=0.044). No significant differences were found between the two groups in the distribution of both FAB and WHO subtypes. While, significant difference was observed in the distribution of karyotype classification between the ID1<sup>high</sup> and ID1<sup>low</sup> patients (P=0.011). ID1 overexpression occurred with the highest frequency in the patients with poor karyotype (7/7, 100%), lower frequency in the...
patients with intermediate karyotype (28/60, 47%), and the lowest frequency in the patients with favorable karyotype (12/31, 39%). No significant correlations were found between ID1 expression and ten gene mutations (P > 0.05, Table 1). In addition, among CN-AML patients, there were no significant differences in peripheral parameters, BM blasts, FAB subtypes, and gene mutations between the ID1 high and ID1 low patients (P > 0.05, Table 1).

Correlation between ID1 expression and clinical outcome

Follow-up data was obtained for 93 AML patients. After induction therapy, ID1 high patients had significantly lower rate of complete remission (CR) than ID1 low patients (33% vs. 63%, respectively, P = 0.007, Table 1). Among non-M3 patients, ID1 high cases also showed significantly lower rate of CR than ID1 low cases [30% (12/40) vs. 54% (19/35), respectively, P = 0.038]. However, there was no significant difference among CN-AML patients (52% vs. 42%, respectively, P = 0.562, Table 1). Moreover, significantly lower CR rate was observed in ID1 high groups as compared with ID1 low groups in both whole AML and non-M3 patients less than 60 years old (48% (11/23) vs. 84% (26/31) and 40% (8/20) vs. 84% (16/19); P = 0.007 and 0.008, respectively), but not in whole AML and non-M3 patients more than 60 years old (data not shown). Survival analyses were performed in 90 patients with follow-up data ranged from 1 to 92 months (median 10 months). ID1 high patients showed significantly shorter overall survival (OS) than ID1 low patients (median 5 versus 17 months, respectively, P = 0.007, Figure 3A). Significant difference was also observed in non-M3 patients. The median OS in ID1 high and ID1 low cases was 5 and 12 months, respectively (P = 0.040, Figure 3B). However, significant difference was not found among CN-AML patients (P = 0.339). Multivariate analysis including age (<60 y vs. >60 y), WBC (≥30×10⁹/L vs. <30×10⁹/L), karyotype classification (favorable vs. intermediate vs. poor), four gene mutations (mutant vs. wild-type), and ID1 expression (high vs. low) variables disclosed that ID1 overexpression was not an independent risk factor in both whole AML and non-M3 patients (Table 2). However, the adverse impact of ID1 overexpression on outcome was revealed by both Kaplan-Meier analysis and multivariate analy-
Discussion

The major biological effect of ID protein is the inhibition of differentiation and maintenance of self-renewal and multipotency of stem cells, which is coordinated with continuous cell cycling [11]. ID proteins which could be activated by oncogenic factors are essential components of oncogenic pathways [11]. De-regulation of ID proteins plays a direct role in cancer initiation, maintenance, progression, and drug resistance [11]. Additionally, ID aberration may contribute to the initiation of myeloid malignancy [34]. Thus, ID may represent a potential therapeutic target for tumors including hematopoietic malignancy.

The clinical significance of ID aberration has been widely investigated. Although ID overexpression predicts poor outcome in the majority of solid tumors [11], the impact of ID aberration remains controversial in AML patients. Tang et al revealed that high ID expression independently predicted lower CR rate and shorter disease-free survival (DFS) and OS in young (age <60 y) non-M3 or cytogenetically normal patients [23]. However, Damm et al disclosed that ID overexpression was not an independent prognostic factor in young CN-AML patients [24]. Our study further investigated the methylation status of ID in both normal controls and leukemic cell lines (data not shown). The impact of ID expression on outcome was not investigated in CN-AML patients less than 60 years due to the small size of case numbers.

Interestingly, our study further found the significant correlation between ID expression and karyotype classification and indicated that the incidence of ID overexpression was increased with the rising risk of karyotype. However, if M3 patients were excluded from analysis, we did not observe the significant association between ID expression and karyotype classification, which was in accordance with the previous investigation [23]. An early study also observed the down-regulation of ID expression in primary acute promyelocytic leukemia (APL) cells and NB4 cell lines, which could be rapidly induced upon all-trans retinoic acid (ATRA) treatment [35]. Moreover, ID overexpression inhibited proliferation and induced a G0/G1 accumulation in NB4 cells [35]. However, a later study revealed that ID overexpression enhanced the proliferation of primitive myeloid progenitor cells and immortalized bone marrow cells in vitro, and ID silencing inhibited leukemic cell line growth [34]. These results indicated that the role of ID in the process of leukemogenesis may be dependent on the context of different cytogenetics.

The association of ID expression with gene mutations has been investigated. Damm et al revealed the significantly decreased incidences of C/EBPA mut and NPM1 mut /FLT3-ITD mut in ID high patients [24]. Moreover, ID high patients showed a significantly increased frequency of FLT3-ITD mut [23, 24]. Our study did not observe the correlation between these gene mutations and ID expression, probably due to the low frequency of these gene mutations in our cases. This difference may be attributed to the differences in ethnics and in AML subtype distribution. More cases of different races are needed to further determine the association of ID expression with genetic mutations.

The underlying mechanism of regulating ID expression was poorly studied. Although a large CpG island was identified at the 5’ region of ID promoter, ID expression silencing was not associated with its promoter methylation [36, 37]. Our study further investigated the methylation status of ID in both normal controls and leukemic cell lines using bisulfite sequencing and manifested that ID promoter showed extremely low density in both normal controls and leukemic cell lines (data not shown). ID expression was shown to be regulated by histone acetylation of its promoter in leukemic cell lines [37]. Recently, two microRNAs (miR-29b and miR-381) have been demonstrated to play important roles in the regulation of ID expression in human lung adenocarcinoma [38, 39]. Garzon et al disclosed the decreased expression of miR-29b in AML [40]. Furthermore, ectopic miR-29b expression could induce apoptosis and reduce cell growth in primary AML cells and cell lines, and inhibit tumorigenicity in a Xenograft leukemia model [40]. Further studies are required to explore the role of these microRNAs in regulating ID expression in AML patients.
**ID1 expression in AML**

In conclusion, our study suggests that ID1 overexpression may correlate with higher risk karyotype classification and serve as an independent risk factor in young non-M3 patients.

**Acknowledgements**

This study was supported by National Natural Science foundation of China (81270630, 81172592), Science and Technology Special Project in Clinical Medicine of Jiangsu Province (BL2012056), 333 Project of Jiangsu Province (BRA2013136), Science and Technology Infrastructure Program of Zhenjiang (SS2012003), Medical Key Talent Project of Zhenjiang, Social Development Foundation of Zhenjiang (SH2013042, SH2013082, SH2014044, SH2014086), and Jiangsu Government Scholarship for Overseas Studies.

**Disclosure of conflict of interest**

None.

**Address correspondence to:** Dr. Jun Qian, Department of Hematology, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, People’s Republic of China; Dr. Jiang Lin, Laboratory Center, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, People’s Republic of China. Fax: +86-511-85234387; E-mail: linjiangmail@sina.com (JL); qianjun0007@hotmail.com (JQ)

**References**

ID1 expression in AML


**ID1 expression in AML**


