Prognostic value of the expression of phosphatase and tensin homolog and CD44 in elderly patients with refractory acute myeloid leukemia

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Abstract. The leukemic stem cell marker CD44, has been reported to have prognostic significance in hematological malignancies. The present study therefore aimed to evaluate whether the expression levels of CD44 and the associated pathway components are associated with the survival rate of elderly patients with refractory acute myeloid leukemia (AML). A total of 20 elderly patients diagnosed with refractory AML were divided into two groups, following induction chemotherapy: Complete remission (CR, n=9) and non-remission (NR, n=11). Bone marrow biopsy specimens were collected, expression levels of CD44, phosphatase and tensin homolog (PTEN), mammalian target of rapamycin (mTOR) and nuclear factor-xB (NF-xB) were analyzed by immunohistochemistry and the captured images were analyzed in a blinded manner using Image Pro Plus software, version 6.0. The overall survival rates (OS) of the patients were then analyzed with log rank, and the correlation between CD44, PTEN, mTOR and NF-xB expression levels and patients survival rates were statistically analyzed using Pearson's method. Significant differences were observed between the CR and NR groups for PTEN (P=0.025) and CD44 (P=0.020) expression levels. Positive CD44 expression was significantly correlated with poor overall survival, with a hazard ratio of 6.281 (95% CI, 1.78 -22.12; P=0.0042). The expression levels of NF-xB and mTOR were slightly increased in the NR group compared with those of the CR group, although no significant differences were identified. PTEN and CD44 expression levels demonstrated trends towards negative correlation. In conclusion, the expression levels of CD44 and PTEN may be useful markers to predict the prognosis of elderly patients with refractory AML.

Introduction

Acute myeloid leukemia (AML) is a hematological malignancy with heterogeneous clinical presentations and subtypes. AML has been further classified by the French-American-British Cooperative Group (1-4) and the World Health Organization (5,6) based on the clinical features, and the biological, morphological, immunological and cytogenetic characteristics of the disease. The most common induction therapies for the treatment of adult AML have not changed significantly over the past four decades, and these are chemotherapy or stem cell transplantation (7-9).

Although efforts have been made to develop novel anti-cancer agents, the overall prognosis for AML has remained poor, particularly amongst older patients. The biological characteristics and clinical features of AML in older adults are different from younger patients, with higher rates of resistance and a poorer response to chemotherapy (10,11). Approximately 70-80% of younger adults achieve complete remission (CR), with a 5-year-survival rate of ~40-45% (12). By contrast, CR is achieved in ~40-65% elderly patients (12) and 5-year-survival rates are ≤10% (13). The most common cause of treatment failure for AML in elderly patients is refractory AML. The underlying mechanism for this resistance of AML to treatment remains unclear (14). Thus, an in-depth understanding of the molecular mechanisms associated with refractory AML is required.
Leukemic stem cells (LSCs), which are also termed leukemia-initiating cells, have been reported to be the origin of leukemic cells (15). LSCs serve key functions in the initiation and progression of leukemia and also in relapse or refractory AML, leading to resistance to induction therapies and poor survival outcomes (16). Several LSC biomarkers have been reported to be correlated with refractory or relapse AML and poor prognosis, which may provide instructive information for diagnosis, progression or treatment (17). The LSC surface marker CD44 and components of LSC-associated pathways, including phosphatase and tensin homologue (PTEN), phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and nuclear factor-κB (NF-κB), have been demonstrated to have prognostic value for adult AML patients (18). Previous studies have demonstrated that increased expression levels of CD44 in hematological malignancies is correlated with poor clinical outcomes (19,20). It has also previously been reported that patients in receipt of autologous hematopoietic stem cell transplantations exhibited lower expression levels of CD44 and improved survival outcomes (21). However, the reliability of the correlation between these LSC-associated biomarkers and the prognosis of patients with refractory AML is currently insufficient.

In the present study, the expression levels of CD44, PTEN, mTOR and NF-κB were evaluated in elderly refractory AML patients to determine whether these molecules have prognostic implications and may be potential therapeutic targets for treatment.

**Materials and methods**

**Patients and tissue samples.** Bone marrow (BM) samples were obtained from 20 elderly patients with diagnosed refractory AML (2,22), who were treated at Dongzhimen Hospital (Beijing, China) between December 2011 and April 2013, and possessed complete clinical pathological diagnosis information and the associated follow-up data. The induction chemotherapy drugs that were administered to the patients were Acla, Ara-C, cytoxan, vindesine, epirubicin and VP-16. The bone marrow samples were divided into two groups: CR (n=9) and NR (n=11), following induction chemotherapy treatment. CR was defined as patients with ≤5% leukemic blasts in the BM with signs of normal hematopoiesis and regeneration of normal peripheral-blood cell production (platelets >1x10¹¹/l without transfusions, neutrophils >1.5x10⁹/l) and an absence of leukemic cells in the peripheral blood or other locations (22). The refractory AML diagnosis for all patients in the study was confirmed histologically and cytologically. The mean age at diagnosis was 62.05±8.24 years and the range was 51-77 years, 11 patients were male and 8 patients were female. All the patients were followed up until June 2014 or death. The study was approved by the ethics committee of Dongzhimen Hospital and all samples were acquired following informed consent and ethical approval.

**Immunohistochemistry.** Immunohistochemical (IHC) analysis was performed as previously described (23), with

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CR, n (%)</th>
<th>NR, n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4 (44.4)</td>
<td>3 (27.3)</td>
<td>0.42</td>
</tr>
<tr>
<td>Male</td>
<td>5 (55.6)</td>
<td>8 (72.7)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>58</td>
<td>60</td>
<td>0.94</td>
</tr>
<tr>
<td>Range</td>
<td>55-77</td>
<td>51-73</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Demographics and baseline characteristics of elderly refractory acute myeloid leukemia patients.

<table>
<thead>
<tr>
<th>Laboratory values</th>
<th>Median (range)</th>
<th>Median (range)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell, x10⁹/l</td>
<td>3.6 (1.7-8.8)</td>
<td>4.5 (1.8-26.1)</td>
<td>0.57</td>
</tr>
<tr>
<td>Platelet count, x10⁹/l</td>
<td>109 (43-226)</td>
<td>80 (7-354)</td>
<td>0.31</td>
</tr>
<tr>
<td>Hemoglobin, g/l</td>
<td>109 (81-130)</td>
<td>82 (53-142)</td>
<td>0.13</td>
</tr>
<tr>
<td>Bone marrow blasts, %</td>
<td>0.62 (0-3)</td>
<td>30 (18-94.5)</td>
<td>0.00</td>
</tr>
<tr>
<td>Peripheral blood blasts, %</td>
<td>0.00 (0.00-2.59)</td>
<td>33 (1-80)</td>
<td>0.00</td>
</tr>
<tr>
<td>Total bilirubin, µmol/l</td>
<td>10.5 (5.5-22)</td>
<td>21.8 (8-96)</td>
<td>0.02</td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>55.7 (49.3-76)</td>
<td>64.1 (38.1-90.4)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

CR, complete remission group; NR, non-remission group.
minor modifications. Briefly, sections (4-µm thick) from the paraffin-embedded bone marrow biopsy specimens were collected, deparaffinized, hydrated, heated for antigen retrieval and treated with 1% bovine albumin serum (Sigma-Aldrich) to prevent non-specific binding. Rabbit anti-human PTEN (1:125; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-human NF-κB (1:300; Cell Signaling Technology, Inc.), rabbit anti-human mTOR (1:200; Abcam, Cambridge, MA, USA) and rabbit anti-human CD44 (1:100; Abcam) were used as the primary antibodies. Non-specific rabbit anti-human immunoglobulin G (1:200; Abcam) was used as a negative control. Following washing with phosphate-buffered saline (PBS), the sections were incubated with the primary antibodies overnight at 4˚C. Next, the sections were washed three times with PBS and incubated with goat anti-rabbit IgG secondary antibody (1:2,000; Abcam) for 30 min at 37˚C. The sections were counterstained with 5 g hematoxylin (Dako North America, Inc., Carpinteria, CA, USA) and 20 µl/ml diamino benzidine (Dako North America, Inc.) using the Cytomation Envision Plus peroxidase system (Dako North America, Inc.).

Evaluation of immunohistochemical staining. The intensity of the IHC staining was semi-quantitatively scored using a previously described method (24,25). Briefly, according to the positive area that was occupied, expression levels were scored as: - (0, negative), + (1-20%, weak), ++ (20-50%, moderate) and +++ (>50%, strong) (26,27). Images of the sections were captured using a microscope (BX51; Olympus Corp., Tokyo, Japan) and a SPOT Imaging Solutions system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and analyzed with Image-Pro Plus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). All the areas were selected from five random visual fields. The mean densitometry of the digital images (magnification, x400) were designated as representative staining intensities of PTEN, CD44, mTOR, PI3K and NF-xB, in order to determine the relative expression levels. All the slides were independently evaluated by two specialized pathologists in a blinded manner, and subjected to statistical analysis.

Statistical analysis. Values are expressed as the mean ± standard deviation. The statistical evaluations were performed with SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. Semi-quantitative results were evaluated using the χ² test. The mean density of protein expressions were measured by Image-Pro Plus and two sample t-tests were used when data were normally distributed, otherwise, non-parametric and Wilcoxon rank sum tests were used for analysis. The correlation was studied using Pearson’s coefficient. The cumulative OS analysis was plotted using Kaplan-Meier curves with log rank analysis. The variables were then analyzed by multivariate Cox regression analysis, which was performed to identify independent prognostic survival factors.

Results

Patient characteristics. A total of 20 bone marrow samples from AML patients were divided into two groups: The CR and NR groups. The median age of the patients was 59 years (range, 51-77 years). The demographics and baseline characteristics of the elderly refractory AML patients are presented in Table I. When comparing the CR and NR groups, the percentage of bone marrow blasts, peripheral blood blasts and the total bilin...
rubin levels were significantly higher in the NR group when compared with the CR group (P<0.05; Table I). These results indicated that patients in the CR and NR groups exhibited different responses to therapy. The percentage of bone marrow blasts was also significantly associated with PTEN expression (P=0.016; Table II); the percentage of bone marrow blasts was significantly higher in PTEN-negative cases when compared with PTEN-positive cases (P=0.016). It has previously been demonstrated that AML patients with lower bone marrow blast percentages following therapy may exhibit a better prognosis (28,29). Thus, the results of the present study indicate that PTEN expression  may also affect the prognosis of AML patients.

PTEN and CD44 are differentially expressed between the two groups. IHC was used to determine the expression levels of PTEN, CD44, mTOR and NF-κB in the patient samples. Representative immunohistochemical stains are presented in Fig. 1. In the CR group the mean densities were as follows: PTEN, 277.32±283.25; CD44, 408.45±303.47; mTOR, 205.72±242.53; and NF-κB, 642.66±312.21. In the NR group the mean densities were: PTEN, 109.59±60.85; CD44, 840.06±335.95; mTOR, 285.79±242.53; and NF-κB, 776.26±380.63, as presented in Fig. 2. The results demonstrated that the PTEN expression levels in the CR group were significantly increased, compared with those of the NR group (P=0.025), whereas CD44 was expressed at significantly reduced levels in the CR group compared with that of the NR group (P=0.020, Fig. 2). By contrast, slightly increased expression levels of NF-κB and mTOR were observed in the NR group compared with those of the CR group, although these differences were not statistically significant (P>0.05, Fig. 2).

Furthermore, statistical correlation analysis demonstrated that there was a negative correlation between the expression levels of PTEN and CD44 (R=-0.415, P=0.069; Fig. 3).

CD44 and PTEN expression levels are associated with patient outcomes. The present study then analyzed whether
CD44 and PTEN expression levels were associated with patient outcomes. Kaplan-Meier analysis demonstrated that the mean OS for the CR group was 10.0 months, which was significantly increased compared with that of the NR group (4.27 months; P=0.009). CD44 expression levels were significantly associated with the OS in all patients: Patients that were CD44 positive had a mean OS of 4.0 months, whereas CD44-negative patients had a mean OS of 9.27 months. The hazard ratio was 6.281 (95% CI, 1.78-22.12; P=0.0042; Fig. 4C). Multivariate Cox regression analysis also demonstrated that CD44 was an independent prognostic survival factor for patients with refractory AML (P=0.019; Table III). There was also a trend towards reduced OS in patients who were PTEN negative when compared with patients who were PTEN positive (mean OS, 4.81 months vs. 8.8 months; hazard ratio, 2.689; 95% CI, 0.89-8.08; P=0.078; Fig. 4B). The corre-
lation between PTEN positive and CD44 negative or PTEN negative and CD44 positive patients with OS, was assessed using Kaplan-Meier analysis. Patients that were PTEN positive and CD44 negative had significantly increased OS compared with those that were PTEN negative and CD44 positive. The mean OS for patients that were PTEN negative and CD44 positive was 9.86 months, whereas the mean OS for patients that were PTEN negative and CD44 positive was 2.67 months. The hazard ratio was 0.037 (95% CI, 0.006-0.222; P=0.0006; Fig. 4D).

Discussion

In the present study, IHC staining was used to detect the expression of CD44, PTEN, mTOR and NF-κB in elderly patients with refractory AML, who were divided into CR and NR groups. It has previously been reported that an increase in CD44 expression contributes to poorer AML prognosis, and CD44 may therefore serve as an adverse prognostic marker (25). The present study indicated that CD44 expression was significantly increased in the NR group compared with that of the CR group. Increased CD44 expression was associated with significantly reduced OS compared with that of CD44 negative patients, which was consistent with the results of a previous report (30). CD44 is a cell surface glycoprotein involved in diverse cellular processes in malignancy, including cell transformation (31), proliferation (31), migration (32) and anti-apoptosis (33). CD44 is also considered to be one of the particular markers that mediate efficient homing and engraftment of LSC in AML (34-36). In addition, CD44 expression is specific to LSC as it is rarely expressed on normal hematopoietic stem cells (37,38). Previous studies have demonstrated that CD44 knockout (CD44−/) mice survived significantly longer and that tumors developed more slowly compared with those of wild-type mice, when injected with murine breast carcinoma cells (30). The expression of CD44 may be associated with adverse prognosis of AML (39), which is consistent with the results obtained in the present study.

In contrast to the expression of CD44, PTEN expression was significantly increased in the CR group compared with that of the NR group. Patients that were PTEN positive tended to have an improved prognosis compared with those that were PTEN negative, although the difference was not statistically significant, possibly due to the relatively small sample size used in the present study. PTEN is a tumor suppressor gene, which is critical for cell proliferation, apoptosis and survival (40-42), and is commonly inactivated in hematological malignancies, resulting in loss of function (42,43). Loss of PTEN function has been demonstrated to be associated with tumor progression (44) and poor prognosis (45), by disturbing the balance of microenvironments, transforming normal stem cells into cancer stem cells (46-48) or promoting the generation of LSCs into unlimited self-renewal (49). The absence of PTEN expression is considered to promote leukemia development and be associated with poor patient outcomes. The results of the present study indicate that further studies with more homogeneous patient samples are required in order to draw conclusions regarding the association between expression of PTEN with the outcome of refractory AML.

It has previously been reported that knockdown of PTEN may upregulate CD44 expression in hepatoma cells and human Huh-7 cells (50). In the present study, when PTEN expression was reduced, OS was significantly improved in the CR group compared with that of the NR group. Furthermore, a significant association between CD44 and PTEN expression was identified. PTEN positive and CD44 negative cases demonstrated a strong correlation with enhanced OS. Analysis of CD44 and PTEN expression was combined and the results demonstrated that there was negative correlation between them. The results of the present study indicate that CD44 may be an adverse prognostic marker, whereas PTEN presents as a favorable biomarker in refractory AML.

PTEN has been shown to negatively regulate the PI3K/Akt/mTOR and NF-κB signaling pathways (51), and NF-κB and PI3K/Akt/mTOR signaling is known to be involved in multiple types of cancer (52), promoting carcinogenesis, cancer cell proliferation and apoptosis (27,53). It has previously been reported that the PI3K/Akt/mTOR signaling pathway may be a prognostic marker in gastric cancer. NF-κB is activated in hematological and solid tumors (54), and has also been demonstrated to be an adverse prognostic factor (55). In children with acute lymphoblastic leukemia, increased expression of NF-κB is associated with treatment failure (56). PI3K/Akt/mTOR also activates downstream signaling of Bcr-Abl, which leads to treatment failure and poor clinical outcomes in patients with imatinib (Bcr-Abl inhibitor)-resistant chronic myeloid leukemia (57). The results of the present study demonstrated that there was increased expression of NF-κB and mTOR in the NR group compared with that of the CR group. However, this increase was not statistically significant, potentially due to the small sample sizes.

In conclusion, the present study provides immunohistochemical evidence that CD44 and PTEN may be used as biomarkers for AML prognosis and for monitoring the response to therapy, which may aid the identification of additional potential strategies to treat refractory disease. The present study used an elderly patient population (>50 years of age) as AML is predominantly diagnosed in elderly patients (1). Notably, older AML patients exhibit a poor prognosis, higher resistance rate and a poorer response to chemotherapy compared with younger patients (2,3). In addition, a CR may only be achieved in 40-65% of elderly patients (4) and in patients >55 years of age, the 5-year survival rate is <10% (5). Thus, the elderly population (>50 years of age) were selected for this study with the aim of improving outcomes and the efficacy of treatments for AML. Since CD44, PTEN, mTOR and NF-κB are involved in AML progression and have been demonstrated to affect prognosis, the correlation observed between these biomarkers and prognostic implications in the elderly population may be observed in younger patients, which presents an interesting topic for further research. The present study had certain limitations due to the small sample size, therefore a study with a larger sample size is required to further validate the findings.

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