Identification of Heme Oxygenase-1 as a Novel Predictor of Hematopoietic Stem Cell Transplantation Outcomes in Acute Leukemia

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Key Words
Heme oxygenase-1 (HO-1) • Hematopoietic stem cell transplantation (HSCT) • Acute leukemia (AL) • Relapse • Acute graft versus host disease (aGVHD)

Abstract
Objective: The main aim of this study was to determine the correlation between clinical outcome and heme oxygenase-1 (HO-1) expression before and after hematopoietic stem cell transplantation (HSCT) in acute leukemia. Methods: HO-1 mRNA levels in 83 patients were measured using qRT-PCR. In a comparative analysis of HO-1 levels in relation to different post-transplant outcomes, the HO-1 threshold, determined via the receiver operating characteristic (ROC) curve, was effectively used to predict clinical relapse and acute graft-versus-host disease (aGVHD). The correlations among clinical relapse, aGVHD and HO-1 expression were analyzed based on this threshold. Results: Leukemia risk stratification and relative expression of HO-1 before pretreatment had significant effects on clinical relapse. Leukemia risk stratification, relative expression of HO-1 after HSCT and the interval from diagnosis to transplantation had a significant influence on aGVHD. Both relapse and aGVHD appeared to be associated with relative HO-1 expression. The relative expression rate of HO-1 was 1.131–1.186 before pretreatment, and strongly associated with post-transplantation relapse. The relative expression rate of HO-1 was 1.102–1.144 after transplantation, and closely related to aGVHD. ROC curve analysis revealed high specificity and sensitivity of HO-1 expression in predicting relapse and aGVHD after allo-HSCT. Conclusions: HO-1 expression can be effectively used as a predictor of relapse as well as a diagnostic factor of aGVHD after transplantation for allo-HSCT patients with acute leukemia.
Introduction

Allogeneic hematopoietic cell transplantation is the treatment of choice for many patients with life-threatening hematological diseases, such as those with acquired lack of marrow function, inborn errors, and hematological malignancies [1]. Owing to the rapid development of research skills and techniques, hematopoietic stem cell transplantation (HSCT) has become one of the most widely used techniques for treating benign blood diseases, solid tumors, autoimmune, genetic, heart and neurological diseases, along with other disorders [2]. However, graft-versus-host disease (GVHD) in allogeneic bone marrow transplantation (allo-HSCT) is a major complication with an incidence rate as high as 40%–60% and mortality rate of ~50%. Current measures to prevent and treat GVHD include cytotoxic drugs, immunosuppressive agents or renovating of T cells in grafts before and after transplantation [3]. Cytotoxic drugs and immunosuppressive agents can decrease recipient immune function and induce secondary infection as well as occurrence of other tumors. Removal of T cells in grafts not only reduces the incidence of GVHD, but also weakens graft-versus-leukemia or graft-versus-tumor effect, leading to leukemia or cancer recurrence and graft failure [4].

Previous studies have determined the effect of exogenous hemin/Heme oxygenase (HO)-1 on cellularity and the hemopoietic clonal potential of cells. Hemin may produce mobilization of hemopoietic cells and committed precursors from adherent mesenchymal stem cells into suspension [5]. The degradation of heme is now considered critical in cellular defense. Heme levels are maintained and regulated by either the synthesis of heme or the degradation of heme by HO [6]. HO-1 is a stress-inducible enzyme that catalyzes the degradation of heme proteins into free iron, carbon monoxide (CO) and biliverdin, which is then rapidly converted into bilirubin. These catabolic end products exert antioxidant, antiapoptotic, and immune-modulating effects, leading to an overall cytoprotective function of HO-1 [7, 8]. The increased production of bilirubin and CO is regarded as beneficial and critical to cellular defense mechanisms. Iron, which can stimulate free radical formation, is immediately bound by ferritin. During immune-mediated diseases, such as MS, type 1 diabetes, collagen induced arthritis (CIA) and organ transplant rejection, several immunoregulatory functions have been attributed to CO, which is an HO-1 product. Thus, CO and bilirubin are seminal to the protection that occurs from elevated levels of HO-1 protein and HO activity [6, 9].

HO plays an important role in attenuating overall production of reactive oxygen species (ROS) through its ability to degrade heme, produce CO and biliverdin/bilirubin and release free iron, which possess potent antioxidant and antiapoptotic properties [10]. Ursu et al. found that HO-1 induces a paracrine signalling in macrophages via reactive oxygen species production, mediating apoptosis of heart muscle cells at later stages of myocarditis [11]. Recent studies have shown that induction of HO-1 results in suppression of diabetes-induced glomerular injury and apoptosis in association with decreased NF-κB-induced inflammation and oxidative stress [12]. Induction of HO-1 also increased NO levels and reduced aortic superoxide production and NF-κB expression [13]. Mesenchymal stem cells (MSCs) have the capacity to repair renal injury, accelerate tubular proliferation and improve renal function, and upregulate HO-1 expression and activity. Abraham et al. has reported that transplantation of BMSC using the IBM-BMT strategy in conjunction with HO-1 induction offers a novel approach for the treatment of type 2 diabetes and would restore insulin sensitivity and glucose tolerance [14]. Wu et al. provided evidence that HO-1/MSCs improved allogeneic LTx outcomes by attenuating inflammatory responses and acute cellular rejection, as well as enhanced immunomodulatory effects compared with BMMSCs [15]. These processes are essential for MSC growth and differentiation into the osteoblast lineage, consistent with the reported role of HO-1 in hematopoietic stem cell differentiation [16]. Induction of HO-1 by cobalt-protoporphyrin IX in recipient mice before conditioning and bone marrow transplantation has been shown to reduce GVHD and improve survival [10]. Another previous report suggests that HO-1 in dendritic cells function as an inhibitor of the alloimmune response mediated by CD4+ T cells. In view of its potent immune-modulating
capacity, HO-1 maybe a promising target in design of therapies to prolong allograft function [17].

In this study, 83 acute leukemia patients subjected to HSCT in our ward were analyzed to determine the effects of HO-1 expression on relapse and aGVHD post-transplantation.

Materials and Methods

Patients and specimens

All patient samples were obtained using institutional review board-approved protocols from the Biomedical Ethics Committee of Hospital Affiliated to Guizhou Medical University, with written informed consent. Clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. HO-1 expression was evaluated in 83 acute leukemia (21 relapsed and 62 non-relapsed) patients who received HSCT from June 2010 to June 2014 in the Affiliated Hospital of Guizhou Medical University. Out of the 83 patients, 48 were male and the remaining 35 were female. The median age of patients was 24 (10 to 53) years. Within our patient group, 48 were diagnosed as acute myeloid leukemia (AML) and 35 as acute lymphoblastic leukemia (ALL). All patients underwent myeloablative treatment. The number of human leukocyte antigen (HLA)-matched sibling donor hematopoietic stem cell transplantation (MS-HSCT) cases was 38 and haplo-identical HSCT (Haplo-HSCT) cases were 45. The median follow-up time was 32 (1 to 53) months after transplantation. HO-1 expression in each sample was detected at least three times. Characteristics of patients are presented in Table 1. Diagnosis of all selected patients was performed according to the guidelines edited by Zhang [18]. All patients had indications for transplantation.

Transplantation process

All patients were subjected to a modified busulfan/cyclophosphamide (BU/CY) regimen. Patients for haploidentical transplantation were additionally treated with rabbit anti-human immunoglobulin (ATG). Patient conditions were evaluated at +1, +2, +3, +6, +9, +12, +18 and +24 months via assessment of bone marrow morphology, cytogenetics, gene fusion, and immunophenotype, among other analyses. The appearance of one of the following conditions was considered a relapse after patients had achieved complete response (CR) post-transplant: 1) bone marrow myeloblasts type I + type II (monoblasts + naive mononuclear cells or lymphoblasts + naive lymphocytes) >20%; 2) leukemia cell infiltration into the extramedullary region. Patients were administered a regimen of cyclosporine (CsA), methotrexate (MTX) and mycophenolate mofetil (MMF) for GVHD prevention. Diagnosis of acute GVHD (aGVHD) was based on criteria specified by the Fred Hutchinson Cancer Research Institute, United States of America [19].

Samples and HO-1 detection

Bone marrow (BM) sample (2–5 mL) from patients was collected in EDTA anti-coagulated tubes before pretreatment and periodically after successful transplantation. Peripheral blood hematopoietic stem cells (2–3mL) from donors were collected in EDTA anti-coagulated tubes before mobilization. PBS was added to BM samples at a 1:1 dilution. Bone marrow mononuclear cells (BMMNCs) were isolated using Ficoll lymphocyte separation liquid, followed by mixing with 2mLTRIzol (Invitrogen, Carlsbad, CA, USA). The mixture was stored at -20°C for total RNA extraction using TRIzol. The RNA concentration was determined based on the A260/A280 value on an ultraviolet spectrophotometer. RNA was reverse-transcribed into cDNA using an Easy Script First-Strand cDNA Synthesis Super Mix kit (Beyotime, Shanghai, China). Specifically, 0.5μg total RNA, 1μL primer, 1μL 2×ES Reaction Mix, 1μL RT Enzyme Mix, and distilled water were mixed to a final volume of 20μL and reacted at 37°C for 60 min. After incubation at 70°C for a further 10 min, the reaction was terminated. Finally, qPCR was performed on an Eppendorf thermocycler using a Trans Start SYBR Green qPCR SuperMix kit (TransGen Biotech, Beijing, China). Specifically, 2μL cDNA, 12.5μL 2×Trans Start SYBR Green qPCR Super Mix, 0.5μL Passive Reference Dye, 0.5μL forward primer, 0.5μL reverse primer, and 9μL distilled water were mixed into a final volume of 25μL. The reaction conditions for amplification were as follows: 3 min at 95°C, followed by 45 cycles at 95°C for 30 s and 60°C for 20 s, 72°C for 30 s. The following primers were used: GAPDH: 5’-CAGCCTCAAGATCATCAGCA-3’ (hGAPDH-F-528, forward), 5’-TGTTGTTCTAGAGTCCTTCCA-3’ (hGAPDH-R-633, reverse); HO-1: 5’-CGGTCCTCAGGCAAGGTGATAGAAGGAG-3’ (hHO-1-F, forward), 5’-CTGAGTTAAAGCCATCGGAGACGC-3’ (hHO-1-R, reverse). cDNA was amplified in a 96-well plate,
with GAPDH as the internal control. Amplification using each primer pair was performed twice, and data presented as means±SD. Relative expression of HO-1 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

**Statistical analysis**

All data were analyzed using SPSS 19.0 software package. Data were presented in terms of median values (25 to 75% quartile). The $\chi^2$ test was used to determine the association between two categorical variables. Variable HO-1 mRNA expression was compared using the Kruskal–Wallis H test among groups. The relationships among HO-1 expression, post-transplant relapse and aGVHD were analyzed via two-variable correlation analysis. The Cox proportional hazards model was applied for multivariate analysis. The threshold of the relative expression rate of HO-1 was analyzed using receiver operating characteristic (ROC) curve before pretreatment and after transplantation. Data were considered significant at $P< 0.05$.

**Results**

**Reconstruction of hematopoiesis**

Hematopoiesis was successfully reconstructed in all 83 patients (100%) after transplantation (neutrophils ≥0.5×10$^9$/L and platelet (PLT) ≥20×10$^9$/L). The median times were 18d (11–38d) and 20d (13–39d), respectively. Sex chromosome and DNA fingerprint analyses confirmed that all 83 patients were complete chimera. Blood types of patients were converted to donor blood types, even if they had different blood types. Complete donor implantation was achieved for all 83 patients.

**Relapse and aGVHD after transplantation**

Overall, 59 of 83 patients had aGVHD with a median time of 34d (11–96d). Among these patients, 37 were classified as grades I–II and 22 as III–IV. Thirty-nine patients were diagnosed as skin type, 16 as gastrointestinal type and 4 as mixed type. Relapse was observed in 21 patients at the end of the follow-up period. Eight patients died (six due to relapse and two of severe aGVHD), leading to a mortality rate of 9.6%. Disease-free survival (DFS) was 73.5% and overall survival rate (OS) was 82.8%.

**Analysis of factors affecting prognosis of allo-HSCT**

COX regression analysis was performed on the potential factors affecting prognosis of allo-HSCT, including age, gender, risk stratification, HLA-matched degree, origin of donor, intervals from diagnosis to transplantation, relative HO-1 expression, relapse rate and aGVHD post-transplant. Post-transplant relapse was significantly affected by both leukemia risk stratification and relative HO-1 expression before pretreatment while aGVHD was markedly influenced by leukemia risk stratification, relative expression rate of HO-1 after transplantation and the interval from diagnosis to transplantation (Table 2).

**Comparison of HO-1 expression patterns among patients with different outcomes after transplantation**

We analyzed the relative HO-1 expression rates of BMMNCs in 83 acute leukemia patients before pretreatment and after transplantation. Notably, HO-1 expression

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Relapse</td>
</tr>
<tr>
<td>Non-relapse</td>
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</table>
before pretreatment was significantly higher in relapsed than non-relapsed patients while expression in aGVHD patients was markedly lower than that of non-aGVHD cases ($P<0.05$) (Tables 3, 4).

**Correlations among HO-1 expression, relapse and aGVHD**

The correlations between relative HO-1 expression before pretreatment and post-transplant relapse as well as relative HO-1 expression and post-transplant aGVHD were assessed by application of the two-variable correlation method. The relative HO-1 expression rate before pretreatment ranged from 1.131 to 1.186, which was highly correlated with relapse after transplantation. The relevance ratio was 0.823. Our data indicate that expression of HO-1 before pretreatment could be effectively applied to predict relapse after transplantation. The relative expression rate of HO-1 after transplantation was 1.102–1.144, and highly correlated with aGVHD. The relevance ratio was -0.806, signifying that lower expression of HO-1 is indicative of aGVHD after transplantation (Table 5).

### Table 2. Multivariate factors affecting allo-HSCT prognosis.

* $P<0.05$, ** $P<0.01$

<table>
<thead>
<tr>
<th>Item</th>
<th>Relapse rate</th>
<th>aGVHD rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.144</td>
<td>0.053</td>
</tr>
<tr>
<td>Age</td>
<td>0.360</td>
<td>0.192</td>
</tr>
<tr>
<td>Type of disease</td>
<td>0.624</td>
<td>0.124</td>
</tr>
<tr>
<td>Risk stratification</td>
<td>0.003**</td>
<td>0.028*</td>
</tr>
<tr>
<td>HLA-matched degree</td>
<td>0.743</td>
<td>0.051</td>
</tr>
<tr>
<td>Origin of donor</td>
<td>0.283</td>
<td>0.182</td>
</tr>
<tr>
<td>HO-1 relative expression rate</td>
<td>0.018*</td>
<td>0.130</td>
</tr>
<tr>
<td>(before pre-treatment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-1 relative expression rate</td>
<td>0.505</td>
<td>0.046*</td>
</tr>
<tr>
<td>(post-transplant)</td>
<td></td>
<td></td>
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<tr>
<td>interval from diagnosis to transplantation</td>
<td>0.128</td>
<td>0.048*</td>
</tr>
</tbody>
</table>

### Table 3. The correlation between HO-1 expression and relapse post-transplant.

$R_{relapse/n-relapse}=$quartile of HO-1 relative expression rate at relapse/quartile of HO-1 relative expression rate non-relapse, ** $P<0.01$

<table>
<thead>
<tr>
<th>HO-1 expression</th>
<th>mRNA level ($R_{relapse/non-relapse}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>expression</td>
<td>median 25% quartile 75% quartile</td>
<td></td>
</tr>
<tr>
<td>Before pre-treatment</td>
<td>1.204</td>
<td>1.165</td>
</tr>
<tr>
<td>Post-transplant</td>
<td>1.072</td>
<td>1.074</td>
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</tbody>
</table>

### Table 4. The correlation between HO-1 expression and aGVHD post-transplant. $R_{aGVHD/n-GVHD}=$quartile of HO-1 relative expression rate at aGVHD/quartile of HO-1 relative expression rate non-GVHD, *** $P<0.001$

<table>
<thead>
<tr>
<th>HO-1 expression</th>
<th>mRNA level ($R_{aGVHD/n-GVHD}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>expression</td>
<td>median 25% tantile 75% tantile</td>
<td></td>
</tr>
<tr>
<td>Before pre-treatment</td>
<td>1.165</td>
<td>1.148</td>
</tr>
<tr>
<td>Post-transplant</td>
<td>0.849</td>
<td>0.860</td>
</tr>
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</table>
(59) experiencing aGVHD post-transplant were classified as positive and the non-aGVHD group (24) as negative. The HO-1 threshold determined on the basis of the ROC curve was specific and sensitive in predicting relapse and aGVHD after allo-HSCT (Table 6).

**Discussion**

Allo-HSCT is currently a widely used treatment method for malignant leukemia. However, relapse and aGVHD are critical factors that often lead to failure of treatment and long-term survival after allo-HSCT. Relapse in most leukemia patients is caused by residual leukemic cells that are not completely removed by pretreatment and graft versus leukemia (GVL), and a few cases are the result of malignant transformation of donor cells [20]. Several factors influence the prognosis of allo-HSCT, including disease status at the time of transplantation, HLA-matched degree, age of receiver, and schemes of pretreatment, among others [21, 22]. In the current study, we focused on the relationship between HO-1 expression and prognosis of patients with acute leukemia after allo-HSCT.

HO-1 is a key enzyme in heme catabolism associated with proliferation, apoptosis and drug resistance in a variety of tumor cells. The protein is proposed to be an important molecular factor promoting the proliferation and anti-apoptosis of leukemic cells [23-25]. Ewing et al. additionally demonstrated that GVHD in allo-HSCT mice is reduced when HO-1 expression is increased, along with prolonged survival time [26]. The 83 patients in our study were grouped according to median values. Single factor analysis disclosed relevant associations among HO-1 expression, relapse and aGVHD.

High expression of HO-1 before pretreatment was closely related to relapse after transplantation. Moreover, expression of HO-1 and aGVHD post-transplant were highly correlated, indicating that HO-1 expression at different time-points has different (even contrasting).

The relative HO-1 expression rate of patients ranged from 1.131 to 1.186 prior to pretreatment, which was highly correlated with post-transplant relapse. After allo-HSCT, the relative HO-1 expression rate was between 1.102 and 1.144, and closely associated with aGVHD. Application of the threshold relative HO-1 expression value based on the ROC curve was specific and sensitive in predicting relapse and aGVHD after allo-HSCT. However, the current investigation was limited to only 83 patients, and further studies on larger patient populations are warranted to confirm these findings.

In conclusion, HO-1 is a survival-related factor of leukemic cells, and high expression is critical for suppression of post-transplant aGVHD. Measurement of HO-1 expression before and after transplantation may therefore present an effective method to predict relapse and post-transplant aGVHD, providing a new direction in clinical research on prognostic factors of allo-HSCT.
Acknowledgments

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Disclosure Statement

The authors have declared that no competing interests exist.

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