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HO-1, RET and PML as possible markers for risk stratification of acute myelocytic leukemia and prognostic evaluation

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Abstract. Heme oxygenase-1 (HO-1) is an inducible isoform of HO that is activated in response to oxidative stress and has anti-apoptotic and pro-proliferative effects on leukemia cells. RET, a tyrosine kinase receptor; its expression levels are associated with the differentiation degree of acute myelocytic leukemia (AML) cells. The promyelocytic leukemia (PML) gene inhibits cell proliferation and tumor growth, participates in the differentiation of hematopoietic progenitor cells and induces cell apoptosis. However, the association between the expression levels of HO-1, RET and PML genes and the risk stratification of AML and prognosis have not previously been reported. In the present study, HO-1 was expressed in the human AML Kasumi-1, HL-60 and THP-1 cell lines, and HO-1 expression was regulated by Hemin (20 μmol/l) and ZnPPIX (10 μmol/l). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis demonstrated that expression of RET and PML were positively and negatively correlated with HO-1 expression, respectively. Bone marrow samples (18 favorable, 55 intermediate, 15 adverse and 2 unknown karyotype AML cases and 20 healthy donors) were collected from 90 randomly selected AML patients upon their first visit. The mRNA and protein expression of HO-1, RET and PML in samples was detected by RT-qPCR and western blot analysis. At the mRNA level, the adverse group expressed significantly higher levels of HO-1 and RET compared with the levels in the favorable and normal groups. The PML mRNA expression levels in adverse patient samples was lower compared with those of the intermediate group and favorable group. Western blot analysis demonstrated that the expression levels of HO-1, RET and PML proteins in all risk groups exhibited the same pattern of expression as was observed for the mRNA levels. The overall survival and relapse-free survival rates were shortest in AML patients with high HO-1 expression (Kaplan-Meier; log-rank, P<0.01). The results of the present study therefore indicate that HO-1, RET and PML may be critical in the risk-stratification and prognosis of AML. However, additional samples and clinical data should be collected and analyzed in order to provide stronger evidence for this hypothesis.

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Introduction

Acute myelocytic leukemia (AML) is a malignant, clonal disorder of the highly heterogeneous myeloid progenitor cells in the hematopoietic system (1). Treating patients with different prognoses upon first visit by various protocols may dramatically prolong survival and improve the quality of life. Risk stratification based on cytogenetic characteristics has been proposed to divide AML patients into 3 subgroups based on their cytogenetics: Favorable, intermediate and unfavorable risk, with the 5-year overall survival (OS) rates of 36, 22 and 5%, respectively (2). In the last few decades, markers for determining prognosis have been well studied, but performing accurate risk stratification upon diagnosis remains difficult (3). Meanwhile, molecular mechanisms for the etiology and progression of AML remain unclear, and AML genomes are expected to identify additional prognostic markers (4).

AML risk stratification and prognosis are affected by abnormal gene expression and gene mutation. Heme oxygenase-1 (HO-1) is an inducible isoform of HO-1 that is activated in response to oxidative stress, disease or other physiological stresses. The activation of HO-1 may result in a reduction of cell apoptosis and promotion of cell proliferation. In a previous study of 43 AML patients, 33 expressed high levels of HO-1 (77%) (5). In addition, higher levels of HO-1 are expressed in chronic lymphocytic leukemia (CLL) cells compared with normal lymphocytes (6). In a previous study, inducing high expression of HO-1 gene increased the expression of multidrug-resistance gene, accompanied by elevated IC_{50} values in a number of types of tumor cells (7). In another previous study, silencing the HO-1 gene facilitated

the apoptosis of leukemia cells or tumor cells by reducing the antioxidant capacity (8). Moreover, inducing high HO-1 expression inhibited the expression of anti-oncogene P53 and increased the cell proliferation capability 1-fold compared with that of the control group (9). RET is a tyrosine kinase receptor that has been associated with papillary thyroid carcinoma (10), multiple endocrine neoplasia type 2 and gastric cancer (11); its expression level is associated with the degree of differentiation of AML cells (12). The expression of promyelocytic leukemia (PML) protein was first observed in patients with acute PML. The PML protein shares similar characteristics with numerous tumor inhibitors by suppressing cell proliferation and tumor growth, by participating in the differentiation of hematopoietic progenitor cells and by inducing cell apoptosis (13). Although PML is expressed in the majority of cell lines, it is only expressed in myeloid cells of human bone marrow (14). It has previously been reported that PML protein expression was weakened or even vanished when colon and breast cancer cells locally invaded and metastasized (15). Dynamic expression of PML gene is indicative of tumor progression, and has been used in the diagnosis and treatment of acute promyelocytic leukemia; therefore, it may be employed as a diagnostic marker in AML (16,17). To the best of our knowledge, the association between the expression levels of HO-1 and RET and AML and the risk stratification and prognosis of AML have not previously been reported. The present study aimed to endogenously regulate the expressions levels of HO-1 in Kasumi-1, HL-60 and THP-1 cells, and to investigate whether RET and PML genes were associated with HO-1 expression using RT-qPCR. In addition, bone marrow samples were collected from 90 randomly selected AML patients to analyze the association between abnormal expression levels of HO-1, RET and PML and the AML risk stratification and prognosis by using RT-qPCR and western blot analysis.

Materials and methods

Cell lines and cell culture. Human AML cell lines Kasumi-1, HL-60 and THP-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). HO-1 is highly expressed in U937 cells but expressed at lower levels in HL-60 and Kasumi-1 cells. Hemin (20 μmol/l; Sigma-Aldrich, St. Louis, MO, USA) and ZnPPIX (10 μmol/l; Sigma-Aldrich) were used to upregulate and downregulate HO-1 expression, respectively. The cell lines were cultured in RPMI 1640 media with 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) with 100 U/ml penicillin and 100 mg/ml streptomycin (Solarbio Science & Technology, Beijing, China) in a 37˚C humidified atmosphere containing 5% CO_2/95% air.

Patients and samples. The 90 AML patients (mean age, 36 years; range, 13-75 years) comprised 55 males and 35 females. A total of 20 healthy volunteers were also selected (mean age, 25 years; range, 18-56 years), consisting of 12 males and 8 females: They were either hospitalized patients or bone marrow donors who visited Hospital Affiliated to Guiyang Medical College from February 2011 to August 2014. According to the Southwest Oncology Group classification system (18), the patients were divided into 1 case of M1, 45 cases of M2, 24 cases of M4 and 20 cases of M5. The patients were divided into a low-risk group (18 cases), an intermediate-risk group (55 cases) and a high-risk group (15 cases) based on the guidelines of National Comprehensive Cancer Network (19,20). In addition, chromosome karyotype was unknown in 2 cases. Bone marrow (BM) samples were collected from clinically examined patients and normal donors. All of the patients and volunteers signed written informed consent forms for their samples to be stored and used for research purposes in advance. All data were collected and analyzed anonymously. The present study was approved by the Ethics Committee of the Hospital Affiliated to Guiyang Medical College. The clinical features of AML patients are listed in Table I.

Total RNA extraction and cDNA synthesis. TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was applied to extract total RNA from Kasumi-1, HL-60, THP-1 cells and patient samples. Ultraviolet spectroscopy was performed using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) to determine the RNA concentration and purity. Total RNA (2 μg) was reverse-transcribed into first-strand cDNA using the first-strand PrimeScript™ RT Reagent kit with gDNA Eraser (Takara Bio, Inc., Tokyo, Japan) according to the manufacturer's instructions. The final reaction volume was 20 μl.

Quantitative polymerase chain reaction (qPCR). cDNA was subjected to qPCR using SYBR-Green PCR Master Mix (TianGen Biotech, Beijing, China) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems Life Technologies, Foster City, CA, USA). Melting curve analysis was applied to guarantee the specificity of amplification. β-Actin was used as the internal control. The primer sequences were as follows: HO-1, sense, 5'-ACC CATGACACCAAGGACCAGA-3' and antisense, 5'-GTG TAAGGACCCATCGGAGAAGC-3', with a PCR product of 157 bp; RET, sense, 5'-TCGAGGGATGCTTACTGGGA-3' and antisense, 5'-GTTGTTCTCATGCAGCCGTG-3', with a PCR product of 160 bp; PML, sense, 5'-CAGGAGCCC CGTCATAGGAA-3' and antisense, 5'-GAGGGCTGGAAG AGACCGA-3', with a PCR product of 143 bp; β-actin, sense, 5'-TCACCCACACTGTGCCCATCTACGA-3' and antisense, 5'-CAGCGGAACCGCTCATTGCCAATGG-3', with a PCR product of 195 bp. Briefly, qPCR (total volume, 20 μl) was performed with 0.5 μl cDNA, 0.75 μl forward primer, 0.75 μl reverse primer, 9 μl SYBR-Green PCR Master Mix and 9 μl dd H_2O. The fluorescence was automatically measured using the Mastercycler® ep realplex (Eppendorf). The thermal cycling conditions were: 1 min at 94˚C followed with 40 cycles at 94˚C for 10 sec, and 60˚C for 15 sec, then 72˚C for 5 min and finally paused at 4˚C. The relative gene expression was calculated relative to β-actin according to the following equations: i) ΔCt = Ct (Target gene) - Ct (β-actin); ii) $\Delta\Delta Ct$ = ΔCt (Treatment) - ΔCt (Control); and iii) Gene expression = $2^{-\Delta Ct}$ or Gene expression = $2^{-\Delta\Delta Ct}$.

Western blot analysis. BM cells were harvested from the clinically examined patients and normal donors. After washing twice in ice-cold PBS, the cells were lysed by sonication in RIPA buffer containing 1 mM Phenylmethanesulfonyl fluoride

Table I. Clinical features of 90 AML pretreatment patients.

Characteristic	No. patients
Gender	
Male	55
Female	35
Age	
<60	78
≥60	12
FAB subtype	
M1	1
M2	45
M4	24
M5	20
Chromosome karyotype	
Favorable	18
Intermediate	55
Adverse	15
Not obtained	2
Type of AML	
De novo	90
Secondary	0
PB blasts, %[a]	46 (0-97)
BM blasts, %[a]	60 (22-95)
WBC count, g/l[a]	43.4 (0.4-420)
Hb concentration, g/l[a]	80 (56-156)
PLT count, g/l[a]	65 (30-119)
Induction	
DA	25
IA	45
TA	14
Mortality before induction	6
CR following induction therapy, (%)	60 (73.1)
Consolidation	
MDAC/HDAC	80
Mortality before CR/lost before consolidation	10
HSCT	
Auto-HSCT	6
Allo-HSCT	28

[a]Median (Range).PB, peripheral blood; BM, bone marrow; WBC, white blood cells; Hb, hemaglobin; PLT, platelet; DA, daunorubicin and cytarabine; IA, idarubicin and cytarabine; TA, pirarubicin and cytarabine; CR, complete remission; MDAC, medium dose cytarabine; HDAC, high dose cytarabine; HSCT, hematopoietic stem cell transplantation.

(Solarbio Science & Technology) for 20 min. The lysate was then centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant was collected and mixed with loading buffer. The final solution was boiled for 10 min. An equal amount of protein (100-150 μg) was loaded and run on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore Corporation, Milford, MA, USA), which were then blocked in 5% non-fat milk in TBST buffer at 4˚C overnight. The membranes were treated with corresponding primary antibodies at a dilution of 1:1,000 dilution (monoclonal mouse anti-human HO-1 32.8 kDa, cat no. ab12220; monoclonal rabbit anti-human Ret 124 kDa, cat no. ab134100; polyclonal rabbit anti-human PML 69 kDa, cat no. ab53773; monoclonal mouse anti-human β-actin 42 kDa, cat no. ab6276; Abcam, Cambridge, UK) for 2 h while shaking at room temperature. Subsequently, the membranes were washed in TBST 3 times (10 min each) and incubated with the corresponding conjugated secondary antibody at a dilution of 1:1,000 [horseradish peroxidase-labeled (HRP)goat anti-rabbit IgG (H+L), cat no. A0208; HRP-labeled goat anti-mouse IgG (H+L), cat no. A0216; Beyotime Institute of Biotechnology, Shanghai, China) for 2 h at room temperature. After washing in TBST three times (10 min each), protein bands were visualized on film by enhanced chemiluminescence (7sea Biotech, Shanghai, China) following the manufacturer's instructions. The expression levels of the target genes were normalized to that of β-actin.

Statistical analysis. Data were statistically analyzed using SPSS software, version 11.5 (SPSS Inc, Chicago, IL, USA). All data are presented as the mean ± standard deviation. Statistical significance among groups was determined by one-way analysis of variance and *q*-text. Optical density from western blotting was quantified with Quantity One software (version 4.5.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference. Kaplan-Meier survival curves were plotted and analyzed with the log-rank test.

Results

Association between mRNA expression levels of RET and PML with HO-1. The association between the mRNA expression levels of RET and PML with HO-1 were quantitatively assessed using qPCR and the SYBR® Green I technique in human AML cell lines U937, HL60 and Kasumi-1. $2^{-\Delta\Delta Ct} \geq 1$ was considered to indicate gene upregulation, and $2^{-\Delta\Delta Ct} < 1$ was considered to indicate downregulation. Under the control of Hemin (20 μmol/l), RET and HO-1 was upregulated, and PML was downregulated. Under the control of ZnPPIX (10 μmol/l), PML was upregulated, and RET and HO-1 was downregulated in all 3 cell lines. Thus, regulating the expression of HO-1 was positively associated with RET and negatively associated with PML (Fig. 1).

mRNA expression levels of HO-1, RET and PML in favorable, intermediate and adverse AML patient groups. The adverse group (3.2241±0.3262) expressed significantly higher levels of HO-1 compared with the intermediate group (2.4356±0.6064)), intermediate group expressed significantly higher expression levels of HO-1 compared with the favorable group (0.8954±0.0200), and the favorable group expressed significantly higher levels of HO-1 compared with the normal group (0.7812) (Fig. 2A). RET mRNA was expressed at significantly higher levels in intermediate patients compared with those in the favorable group (1.1025±0.011, $P<0.0001$), RET mRNA was expressed at significantly higher levels in favorable patients compared with the normal group

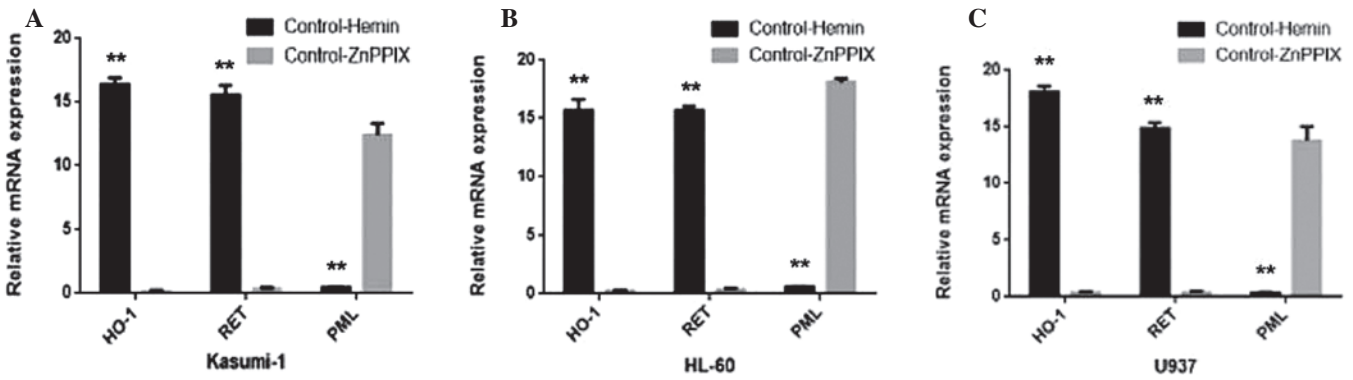

Figure 1. Correlation between mRNA expression of RET and PML with HO-1 in (A) Kasumi-1; (B) HL-60; and (C) U937 cells. HO-1 and RET exhibited higher expression following treatment with control-Hemin compared with control-ZnppIX, however, PML expression exhibited the opposite expression pattern in all 3 cell lines. Thus, RET was positively associated with HO-1 and PML was negatively associated with HO-1. The data is represented as the mean ± standard deviation from 3 independent experiments. **P<0.01 vs. control-ZnPPIX. HO-1, heme oxygenase-1; Hemin, the specific inductor of HO-1; ZnPPIX, the specific inhibitors of HO-1; Control, without any intervention.

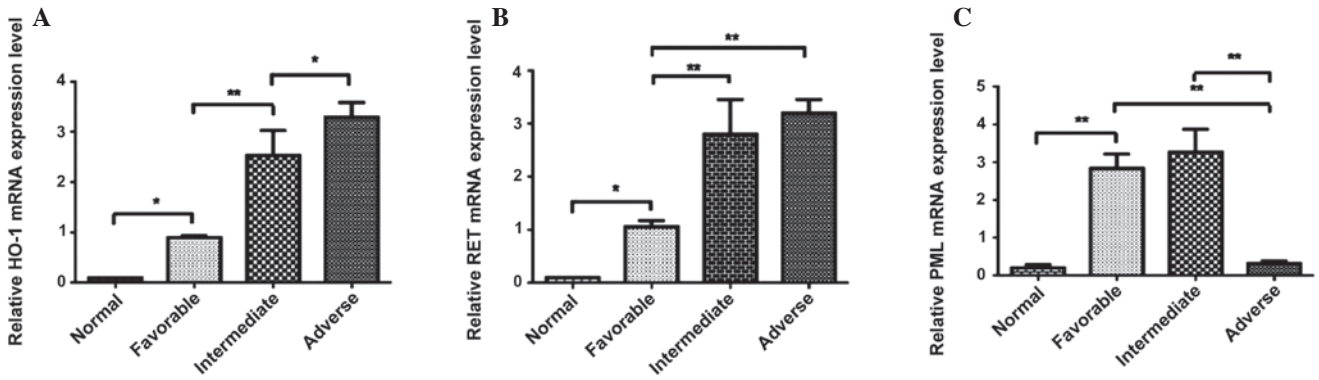

Figure 2. (A) The expression of HO-1 mRNA in normal, favorable, intermediate and adverse risk patient groups. (B) The expression of RET mRNA in normal, favorable, intermediate and adverse risk patient groups. (C) The expression of PML mRNA in normal, favorable, intermediate and adverse risk patient groups. Statistical significance was determined by the one-way ANOVA analysis and *q*-text. The data is represented as the mean ± standard deviation from 3 independent experiments. HO-1, Heme oxygenase-1. RET, a tyrosine kinase receptor. PML, Promyelocytic leukemia gene. *P<0.05; **P<0.01.

(0.0825, P<0.0001; Fig. 2B). No significant difference in the mRNA expression levels of RET were observed between the adverse and intermediate groups (Fig. 2B). By contrast, the PML mRNA expression level in the adverse patient group (0.2056±0.0122) was lower compared with those in the intermediate group (3.2017±0.9004, P<0.0001) and favorable group (2.8002±0.5201, P<0.0001). The PML mRNA expression level of the favorable group was higher compared with the normal group (0.7807±0.004, P<0.0001). No significant differences in the mRNA expression of PML were observed between the favorable and intermediate groups (Fig. 2C).

Protein expression levels of HO-1, RET and PML in favorable, intermediate and adverse AML patient groups. The protein level of HO-1 in the adverse group was notably higher compared with the intermediate group (P<0.01); the protein level of HO-1 in the intermediate group was notably higher compared with the favorable group (P<0.01); the protein level of HO-1 in the favorable group was notably higher compared with the normal group (P<0.01). No significant difference in the protein expression levels of PML were observed between the adverse group and intermediate group (P>0.05; Fig. 3A). Significantly higher Ret protein levels were expressed in intermediate patients compared with those in the favorable group (P<0.01) and significantly increased levels of Ret protein were expressed in favorable patients compared with the normal group (P<0.01), No significant differences in RET protein expression levels were observed between the intermediate and adverse groups (P>0.05) (Fig. 3B). The PML protein expression level of adverse patients was reduced compared with the intermediate group (P<0.01). The PML protein expression level was increased in the favorable group compared with the normal group (P<0.01). No significant differences in the mRNA expression of PML were observed between the favorable and intermediate groups (Fig. 3C).

Survival analysis. Survival curves were obtained using Kaplan-Meier analysis and the log-rank test was used to compare differences in survival among the 3 groups. According to the survival analysis, it was determined that the 3-year overall survival (OS) rate of the group with low levels of HO-1 protein expression was increased in comparison with the group with high levels of HO-1 protein expression (log-rank=9.517; P=0.0086; Fig. 4Aa), and the difference between each group was significant (P<0.05 for medium level vs. high level; P<0.05 for medium levels vs. low level).

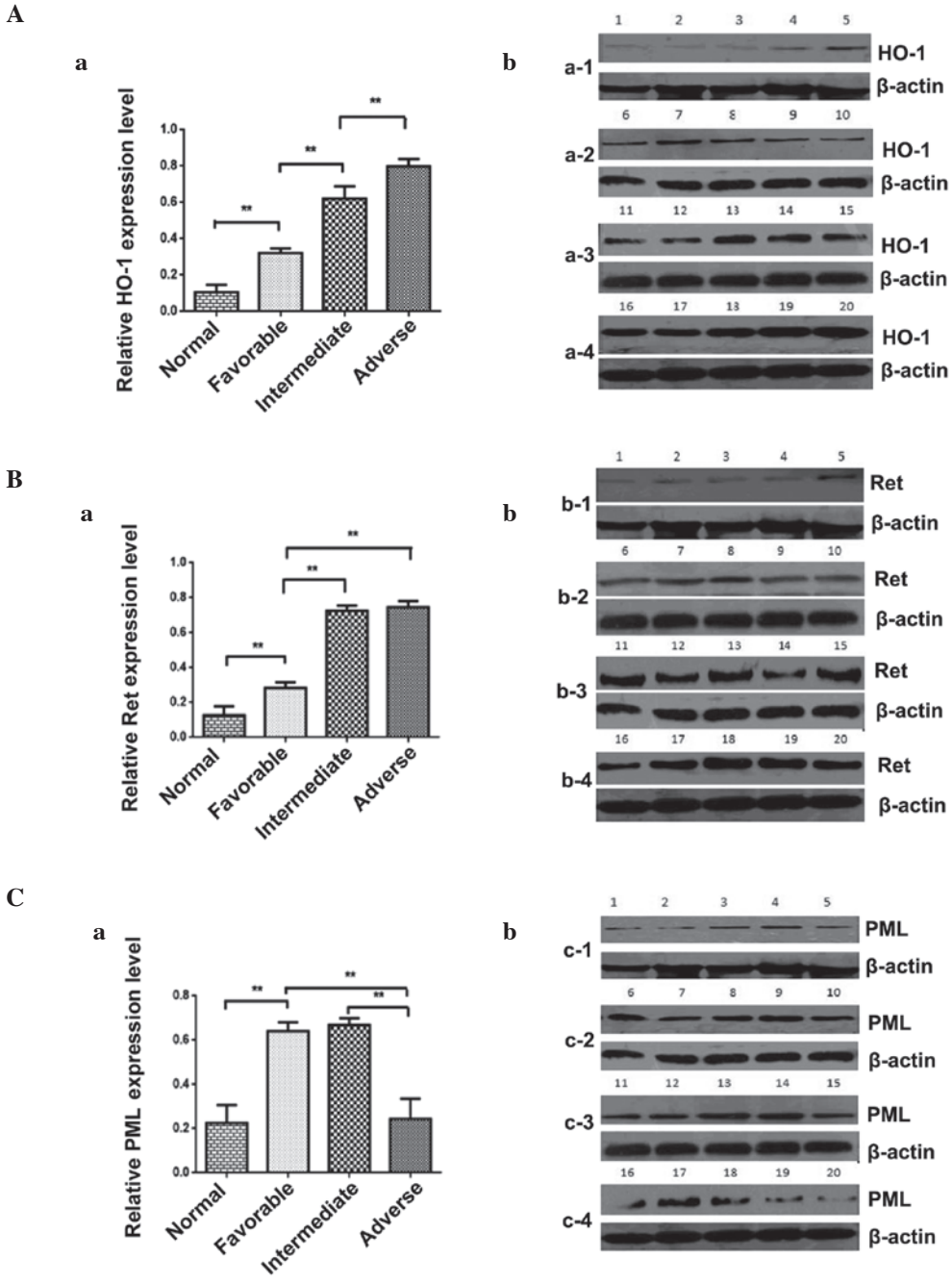

Figure 3. (a) Quantification of western blot analysis and (b) original blots of (A) HO-1; (B) Ret; (C) PML protein expression in normal, favorable, intermediate and adverse risk patients. Data is represented as the mean ± SD from 3 independent experiments. **P<0.01. HO-1, Heme oxygenase-1. RET, a tyrosine kinase receptor. PML, Promyelocytic leukemia gene.

The 3-year relapse-free survival (RFS) rate of the group with low levels of HO-1 protein expression was higher compared with the group with low levels of HO-1 protein expression (log-rank=8.861; P=0.0119; Fig. 4Ab); the RFS rate of the group with medium levels of HO-1 protein expression was higher compared with the group with high levels of HO-1 protein expression (P<0.05; Fig. 4Ab); and the RFS rate of the group with low levels of HO-1 protein expression was higher compared with the group with medium levels of HO-1 protein expression (P<0.05; Fig. 4Ab). However, OS and RFS were not influenced by high vs. low Ret and PML expression levels in AML [Ret, OS: hazard ratio (HR) 1.833; 95% confidence interval (CI), 0.7227-4.839; P=0.2109 (Fig. 4Ba); RFS: HR, 1.200; 95% CI, 0.5296-2.818; P=0.6546 (Fig. 4Bb). PML, OS: HR, 1.714; 95% CI, 0.7112-4.297; P=0.2374 (Fig. 4Ca); RFS: HR, 1.111; 95% CI, 0.4564-2.757;P=0.8106) (Fig. 4Cb)].

Discussion

Conducting accurate risk stratification for AML patients upon first visit may markedly prolong survival and improve the quality of life for the patient. Despite successful risk stratification and prognostic evaluation by examining gene mutation (21), cell genetic characteristics and age (22), AML remains difficult to treat successfully. In the present study, the association between expression levels of HO-1, RET and PML in AML cell lines and

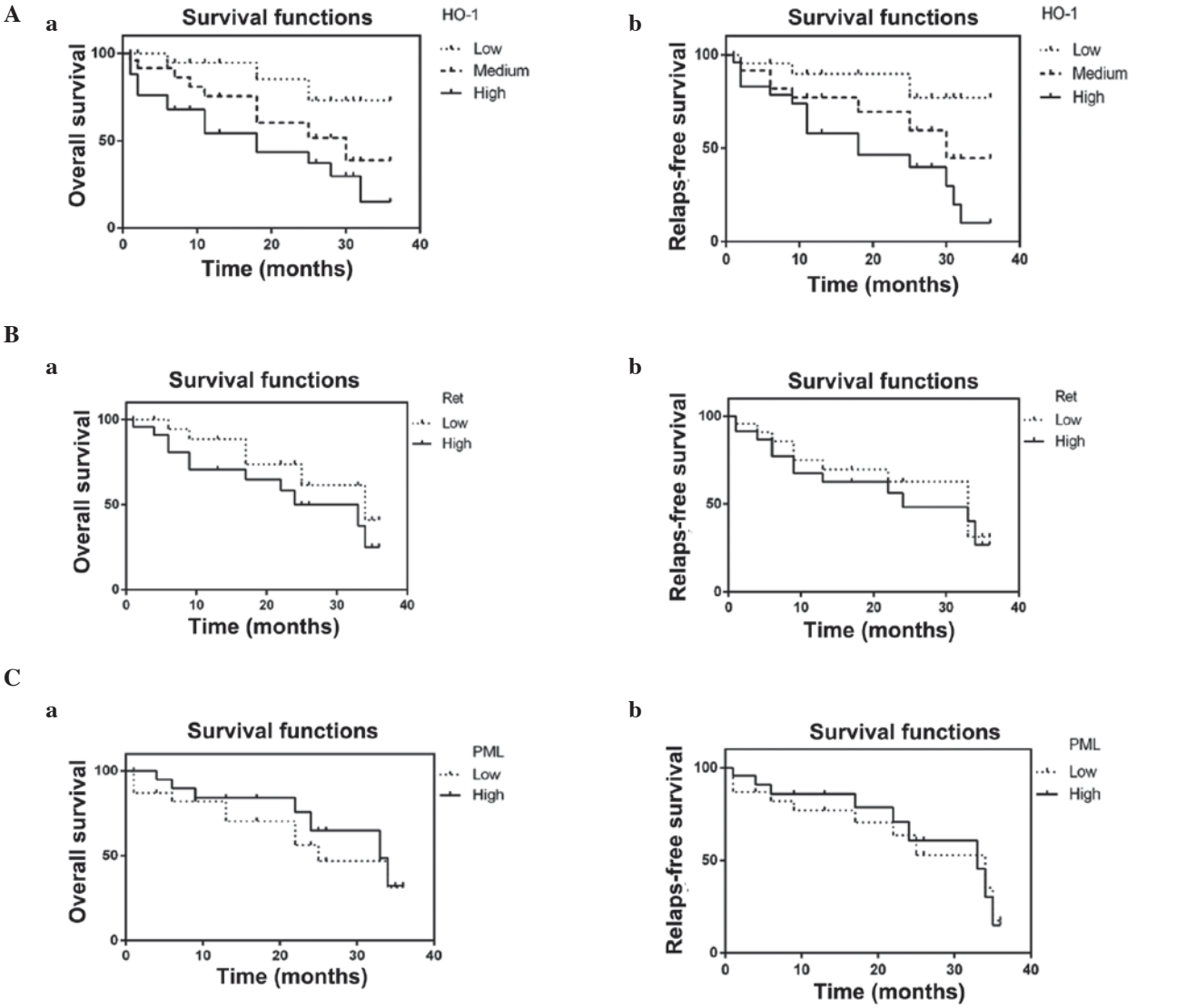

Figure 4. Correlation between low, medium and high (A) HO-1; (B) Ret; and (C) PML expression in AML patients and (a) 3-year overall survival and (b) relapse-free survival rates. HO-1, heme oxygenase-1; RET, a tyrosine kinase receptor. PML, Promyelocytic leukemia gene.

AML patients with different risks were studied. The HO-1 gene is located on human chromosome 22q12 and comprises 5 exons spanning 14 kb, and the molecular weight of HO-1 protein is 32.8 kDa. In general, HO-1 is expressed at higher levels in tumor cells, including cells from solid tumors, compared with normal tissue cells (23-25). When HO-1 is expressed at high levels, the expression of the tumor suppressor gene p53 is inhibited, and the proliferation capacity of tumor cells is notably enhanced; when HO-1 expression is inhibited, the expression of P53 is enhanced, and tumors cells proliferate at a reduced rate (9). In addition, HO-1 expression in CLL cells exceeds that in normal lymphocytes. In response to disease or stress, HO-1 expression results in resistance to cell apoptosis and promotes cell proliferation (26). Stuart *et al* (27) demonstrated that HO-1 was involved in the regulation of leukemia cell apoptosis, proliferation and differentiation through NF-κB, ROS and Nrf2. Mayerhofer *et al* (28) reported that inducing high HO-1 expression levels resulted in a reduced number of cells undergoing apoptosis and increased resistance to imatinib. However, there remains a lack of available studies regarding the expression status of HO-1 in the risk stratification and prognosis of AML. In the present study, RT-qPCR and western blot analysis were used to demonstrate that AML patients exhibited increased HO-1 expression levels compared with normal subjects. HO-1 expression levels increased with increasing AML risk. Moreover, high HO-1 expression levels were associated with a shorter OS and RFS rate in AML patients. This was consistent with the regulatory effects of HO-1 on the apoptosis, proliferation, differentiation and drug resistance of leukemia cells. The RET proto-oncogene is localized to chromosome 10 (10q11.2) and contains 21 exons spanning about 60 kb. The RET gene encodes Ret protein. Gattei *et al* (12) observed that RET expression in human AML was maturation-associated, which may indicate its involvement in developmental regulation during the differentiation of normal hematopoietic cells. In the present study, RT-qPCR demonstrated that RET expression may be correlated with HO-1, and the RT-qPCR and western blot analysis results indicated that the expression levels of RET gene increased with increasing risk amongst the AML patients, although no significant difference in the mRNA and protein expression levels of RET were observed

between the adverse and intermediate groups. OS and RFS were not influenced by the level of Ret expression in AML patients. The PML gene is ~35 kB, is localized to chromosome 15 and contains 9 exons (29). The PML protein has been verified by *in vivo* and *in vitro* studies as a tumor growth inhibitory factor. Rego *et al* (30) demonstrated that deactivating the PML gene apparently triggered the onset of leukemia. Szendefi *et al* (31) were the first to report that PML expression was closely associated with increasing degrees of severity in cervical cancer. Subsequently, Gurrieri *et al* (15) reported that PML expression, which was not expressed in a number of types of tumor, including lung and breast cancer tumor tissues, was associated with tumor grade and stage. However, PML has not previously been associated with AML prognosis or risk stratification. In accordance with these previous findings, the present study demonstrated no association between PML expression and OS or RFS in AML patients. The PML protein expression level of adverse patients was reduced compared with those within the favorable group, whereas the PML expression levels of the intermediate-risk group were not lower than that of the low-risk group (32,33). The findings of the present study do not mimic the close association between PML expression and the degree of severity in cervical cancer (15,31). However, further studies based on larger sample sizes are required to investigate further.

In conclusion, the present study identified that there was a significant difference in the mRNA and protein expression levels of HO-1, RET and PML between low-risk and high-risk patients. In addition, high HO-1 expression was associated with shorter OS and RFS in AML patients. These results may be associated with AML risk stratification and prognosis, which may allow them to be used as suitable markers for evaluation in the future. However, in order to fully elucidate the exact association between HO-1, RET and PML with risk-stratification and prognosis, more samples need to be analyzed and further, larger studies are required.

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