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Induction of Multidrug Resistance of Acute Myeloid Leukemia Cells by Cocultured Stromal Cells via Upregulation of the PI3K/Akt Signaling Pathway

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This study aimed to investigate the role of the PI3K/Akt signaling pathway in multidrug resistance of acute myeloid leukemia (AML) cells induced by cocultured stromal cells. Human AML cell lines HL-60 and U937 were adhesion cocultured with human bone marrow stromal cell line HS-5 cells. Such coculturing induced HL-60 and U937 cells resistant to chemotherapeutic drugs including daunorubicin (DNR), homoharringtonine (HHT), and cytosine arabinoside (Ara-C). The coculturing-induced resistance of AML cells to DNR, HHT, and Ara-C can be partially reversed by inhibition of the PI3K/Akt signaling pathway. Clinically, AML patients with a low level of PTEN and a high level of CCND1 had high relapse rates within 1 year, and newly diagnosed AML patients with extramedullary infiltration had a low level of PTEN. This study confirms the involvement of the PI3K/Akt signaling pathway in multidrug resistance in AML cells induced by stroma and suggests that the expression of PTEN and CCND1 may be a prognostic indicator for AML.

Key words: Acute myeloid leukemia (AML); HS-5 cells; Multidrug resistance; PI3K/Akt signaling pathway

INTRODUCTION

The bone marrow microenvironment can protect acute myeloid leukemia (AML) cells from injury by therapeutic drugs, thus causing AML relapse (1–3). In our previous studies, AML cell lines and primary AML cells were cocultured with the stromal HS-5 cells or mesenchymal stem cells (MSCs) to simulate the growth and maintenance of AML cells in the bone marrow microenvironment. We found that such coculture significantly reduced the sensitivity of AML cells to As_2O_3 and the CD44 monoclonal antibody A3D8 and that the abnormal activation of the PI3K/Akt signaling pathway played an important role in the drug resistance of AML cells (4,5). The PI3K/Akt signaling pathway is an important signal transduction pathway in cells that participate in cell proliferation, differentiation, and apoptosis. The abnormal activation of this pathway can be observed in many hematologic malignant diseases and can reduce the sensitivity of cells to chemotherapeutic drugs (6–8).

Currently, the clinical drugs used for AML treatment include antimetabolism drugs such as cytosine arabinoside (Ara-C), anthracyclines such as daunorubicin (DNR), and alkaloids such as homoharringtonine (HHT). The mechanisms of action of these three groups of drugs are different (9). Ara-C, a cell cycle-specific drug, is phosphorylated after entering the human body and competes with normal nucleotides for DNA polymerases, inhibits DNA synthesis, or intercalates into DNA as a pseudometabolite. Ara-C functions in the G_1 and S phases. DNR, a non-cell cycle-specific drug, can irreversibly bind to DNA to inhibit RNA synthesis and DNA replication through DNA chelation. HHT selectively induces cell apoptosis at the G_1 and G_2 phases; in addition, HHT can block the binding between aminoacyl-tRNA and ribosomes and inhibit peptide chain elongation, thus causing the extensive inhibition of protein synthesis. Until now, studies on drug resistance in leukemia induced by bone marrow stromal cells mainly focused on one single chemotherapeutic drug and its mechanism; however, there are few reports on whether bone marrow cells can induce AML cell drug resistance to many types of existing conventional chemotherapeutic drugs, and on the related mechanisms. We hypothesized that the PI3K/Akt signaling pathway may contribute to the resistance. Therefore, in this study, the AML cell lines HL-60 and U937 cells were cocultured with bone marrow stromal HS-5 cells to compare the changes in drug sensitivity to DNR, HHT, and Ara-C before and after the coculturing. Furthermore, the PI3K/

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Akt signaling pathway-specific inhibitor LY294002 was applied in AML cells to investigate whether the reduction in drug sensitivity after coculturing was associated with the activation of the PI3K/Akt signaling pathway.

Abnormal activation of the PI3K/Akt signaling pathway is one of the indicators of poor prognosis in various tumors, including AML (10–12). However, in the clinical setting, prediction of prognosis through detection of protein expression in primary AML cells is not convenient. The approach to screen gene expression levels of important signaling molecules in the PI3K/Akt signaling pathway for predicting prognosis of AML patients remains to be explored. The PTEN, CCND1, FOXO1, mTOR, and RICTOR genes are important signaling molecule genes in the PI3K/Akt signaling pathway and display abnormal expression levels in acute leukemia patients (13). The present study further investigated changes in the above genes and related proteins in HL-60 and U937 cells after being cocultured with HS-5 cells. The signaling molecules with consistent gene and protein expression were further selected to detect changes of these genes in primary AML cells and to analyze a possible association between clinical presentation and prognosis. It is expected that results obtained from this study will provide experimental evidence for using important signaling molecules in the PI3K/Akt signaling pathway as prognostic indicators of AML patients.

MATERIALS AND METHODS

Cell Culture

U937 and HL-60 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). HS-5 cells that did not express CD45 were purchased from the Biomedicine and Health of the Chinese Academy of Sciences (Guangzhou, Guang Dong, China). The cells were cultured in a humidified incubator at 37°C and 5% CO_2 in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and conventional concentrations of penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

Coculture System and Cell Sorting

HS-5 cells were seeded into 12-well plates and cultured overnight to allow the cells to become adherent. U937 or HL-60 cells were seeded into the plates at a 1:1 ratio and cocultured for 24 h. AML cells were collected by CD45 magnetic beads (Miltenyi Biotec Technology & Trading, Shanghai, China).

Cell Viability Assay (CCK-8 Assay)

HL-60 cells and U937 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated with DNR, HHT, or Ara-C, respectively, for 24 h. Cell proliferation was measured with a CCK-8 Kit (Dojindo, Tabaru, Japan) according to the following protocol: 10% CCK-8 solution was added to each well and incubated at 5% CO_2 and 37°C for 2 h. Absorbance at 450 nm was measured using a microplate reader.

Reagents

DNR (Wanle Pharmaceutical, Shenzhen, China) was diluted with sterile phosphate-buffered saline (PBS) to a concentration of 2 mM and stored at 4°C. HHT (Sanjing Pharmaceutical, Ha Erbing, China) was diluted with PBS to a concentration of 1 μg/ml and stored at −20°C. Ara-C (Hengrui Pharmaceutical, Jiangsu, China) was diluted with sterile PBS to a concentration of 50 mM and stored at −20°C. The PI3K/Akt signaling pathway inhibitor LY294002 was purchased from Cell Signaling Technology (Danvers, MA, USA), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a concentration of 10 mM and stored at −20°C. All drugs were diluted in RPMI-1640 medium (Gibco) to a working concentration immediately prior to use.

Isolation of Primary AML Cells

The 42 cases of primary AML cells were derived from newly diagnosed AML patients [except acute promyelocytic leukemia (APL)] based on WHO classification (2008) in the Department of Hematology in the Union Hospital of Fujian Medical University. The samples were collected with the patients' consent. The normal bone marrow sample was derived from the donor of stem cell transplantation with consent. Ficoll medium was used to separate the mononuclear cells followed by isolation of $CD3^+$ cells with magnetic beads (Miltenyi Biotec Technology & Trading).

Real-Time Polymerase Chain Reaction

Total RNA was extracted with TRIzol reagent (Life Technologies, Waltham, MA, USA) followed by reverse transcription into complementary DNA (cDNA) according to the manufacturer's instruction (Thermo Scientific, Waltham, MA, USA). Real-time quantitative polymerase chain reaction (PCR) was performed with QuantiTect SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA) in ABI PRISM 7500 PCR instrument (Applied Biosystems). The reaction solution (50 μl) contained 25 μl 2× QuantiTect SYBR Green PCR Master Mix, 0.2 μmol/L of each primer, 2.5 μl of cDNA, and RNase-free water. The PCR conditions were as follows: predenaturation at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Each reaction and each experiment were done in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal reference. The primers are presented in Table 1 (13). The relative gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer Sequences for Real-Time PCR

Gene	Primer Sequence	Product Length (bp)
GAPDH	Forward 5′-GAGTCAACGGATTTGGTCGT-3′ Reverse 5′-CATGGGTGGAATCATATTGGA-3′	141
PTEN	Forward 5′-GCTATGGGATTTCCTGCAGAA-3′ Reverse 5′-GGCGGTGTCATAATGTCTTTCA-3′	133
mTOR	Forward 5′-CGCTGTCATCCCTTTATCG-3′ Reverse 5′-ATGCTCAAACACCTCCACC-3′	325
RICTOR	Forward 5′-GGAAGCCTGTTGATGGTGAT-3′ Reverse 5′-GGCAGCCTGTTTTATGGTGT-3′	116
CCND1	Forward 5′-AATGACCCCGCACGATTTC-3′ Reverse 5′-ATGGAGGGCGGATTGGAA-3′	143
FOXO1	Forward5′-GCAGATCTACGAGTGGATGGTC-3′ Reverse 5′-AAACTGTGATCCAGGGCTGTC-3′	192

Western Blot Analysis

Western blot was performed as previously described. The following antibodies were used: anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-Akt, p-Akt^{Ser473}, anti-p-PDK1, anti-PTEN, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology).

Statistical Analysis

SPSS version 11.5 for Windows was used for all analyses. Quantitative data are expressed as the mean ± standard deviation (mean ± SD). Analysis of variance was used to compare group differences. Dunnett's *t*-test was used to compare the means of two specific groups. A value of $p < 0.05$ was considered significant.

RESULTS

Decreased Drug Sensitivity After Coculture

The sensitivity of HL-60 and U937 cells to DNR, HHT, and Ara-C significantly decreased after adhesion cocultured with HS-5 cells, whereas the PI3K/Akt signal pathway inhibitor LY294002 can significantly increase the sensitivity. The growth inhibitory effects of DNR, HHT, and Ara-C on HL-60 and U937 cells were all reduced after the coculture. The inhibition rate of 0.2 μM DNR, 8 ng/ml HHT, and 2.5 μM Ara-C on HL-60 cells was 65.77 ± 2.74%, 59.95 ± 1.54%, and 68.17 ± 3.16%, respectively, at monoculture compared to 40.13 ± 2.19%, 28.13 ± 3.03%, and 48.23 ± 2.82% at coculture. Similar results were obtained with the U937 cells (53.07 ± 3.54%, 59.95 ± 1.54%, and 56.77 ± 4.78% at monoculture compared to 36.43 ± 2.96%, 26.43 ± 3.03%, and 40.37 ± 3.36% at coculture). All the differences of inhibition rate between the monoculture and coculture group were statistically significant ($p < 0.01$). Importantly, the PI3K/Akt signaling pathway inhibitor LY294002 sensitized HL-60 and U937 cells under coculture to DNR, HHT, and Ara-C (Fig. 1). The differences in each group were all statistically significant.

Adhesion Activates PI3K/Akt Pathway

Given that the PI3K/Akt inhibitor could partially reverse coculture-induced resistance, it is reasonable to speculate that coculture may activate the PI3K/Akt signaling pathway. To this end, HL-60 and U937 cells were cocultured with HS-5 cells for 24 h and harvested for Western blot analysis. As shown in Figure 2, both p-Akt^{Ser473} and p-PDK1 were upregulated, but p-PTEN was significantly downregulated, suggesting that the PI3K/Akt pathway was activated. Application of the PI3K/Akt signaling pathway-specific inhibitor LY294002 on HL-60 and U937 cells after adhesion coculture could specifically inhibit the up-regulation of p-Akt^{Ser473} and p-PDK1, suggesting that the activation is specific to the PI3K/Akt signaling pathway.

MDR1 and ABCG2 Displayed no Change After Adhesion Coculture

The transcription levels of the multidrug resistance gene (MDR1) and the drug pump gene adenosine 5′-triphosphate (ATP)-binding cassette transporter G2 (ABCG2) in HL-60 and U937 cells did not display significant changes after adhesion cocultured with HS-5 cells. After adhesion, they were cocultured with HS-5 cells for 24 h; the transcription levels of MDR1 and ABCG2 genes in HL-60 and U937 cells stayed essentially same as the control group (Fig. 3).

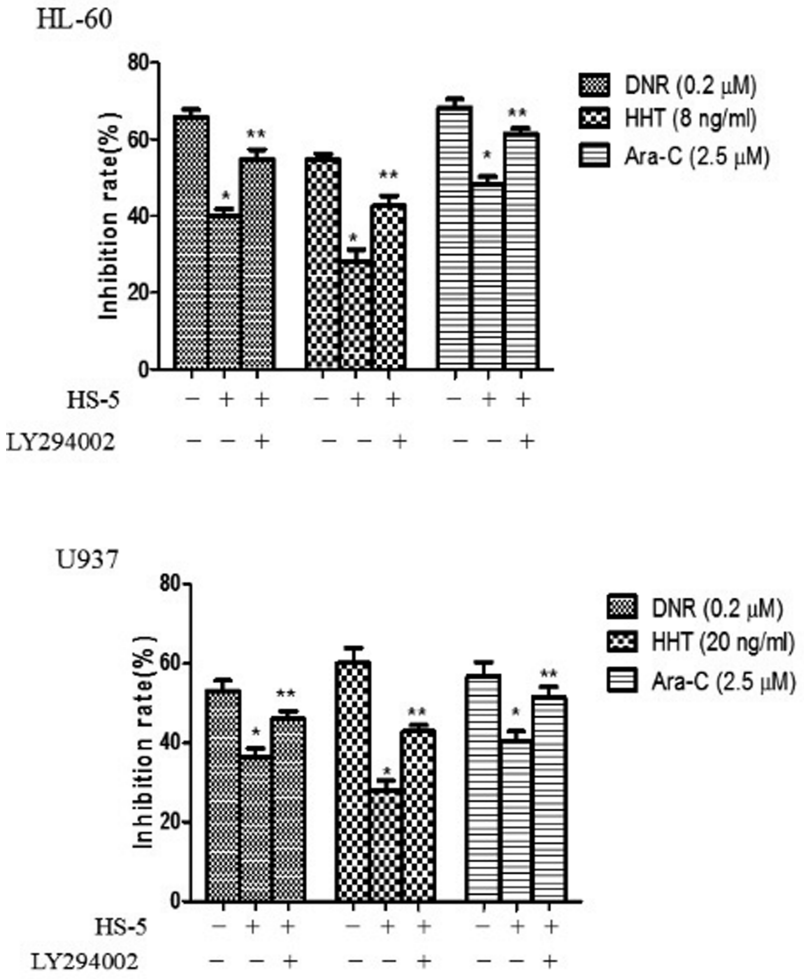

Figure 1. Inhibition rate of DNR, HHT, and Ara-C on AML cells. Adhesion coculture with stromal cells significantly decreased drug sensitivity of HL-60 cells and U937 cells, whereas LY294002 increased the sensitivity. AML cells (1×10^5/ml) cultured in single suspension or adhesion cocultured with HS-5 cells for 24 h were treated with DNR, HHT, and Ara-C, respectively, and the inhibition rate was evaluated by the CCK-8 method. Inhibition rate (%) = (OD value of the single suspension culture group − OD value of the coculture group)/OD value of the single suspension culture group × 100%. The experiments were repeated three times. The results are presented as mean ± SD. *$p < 0.01$, **$p < 0.05$.

Changes of CCND1, FOXO1, mTOR, PTEN, and RICTOR After Adhesion Coculture

CCND1, FOXO1, mTOR, PTEN, and RICTOR are the important molecules in the PI3K/Akt pathway (13). After adhesion, they were cocultured with HS-5 cells for 24 h, and the mRNA expression levels of CCND1 and FOXO1 in HL-60 cells were significantly upregulated to 5.41 ± 0.81 and 5.98 ± 0.95 times that of the control group, respectively. Such coculture also induced 3.69 ± 1.93- and 3.02 ± 0.77-fold higher CCND1 and FOXO1 mRNA expression in U937 cells than in the control group. The mRNA expression of PTEN in HL-60 and U937 cells was downregulated to 0.32 ± 0.10 and 0.26 ± 0.08 times that of the control group, respectively. The expression of mTOR and RICTOR did not change (Fig. 4A). The results suggested that the changes in protein and mRNA expression of CCND1 and PTEN were consistent (Fig. 4B). Therefore, the transcription expression levels of CCND1 and PTEN may be used as prognostic markers for primary AML cells.

Clinical Prognosis of CCND1 and PTEN in AML Patients

Figure 5A shows that among the 42 cases of patients with primary AML, the patients with high level of CCND1 and low level of PTEN had higher relapse rates within 1 year compared to other patients (8/13 vs. 4/29). Newly diagnosed patients with extramedullary infiltration had lower PTEN expression levels than patients without extramedullary infiltration (Fig. 5B).

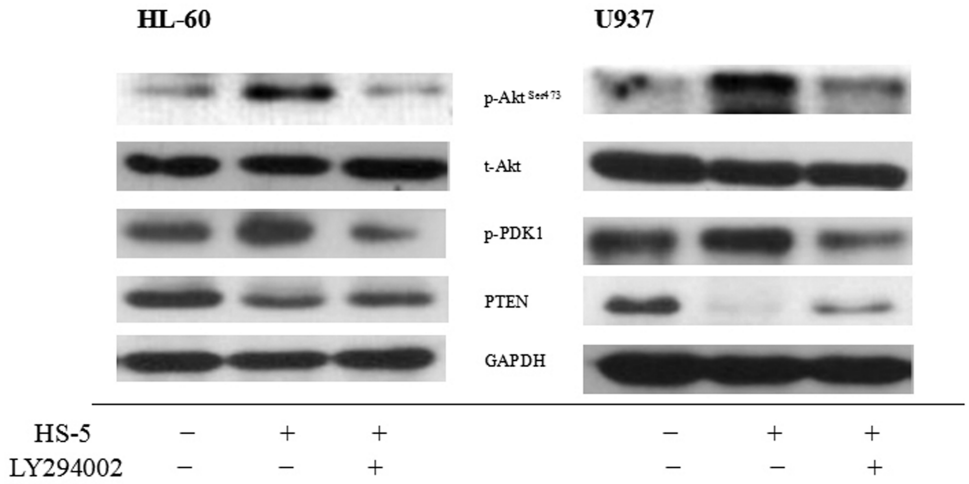

Figure 2. The PI3K/Akt signaling pathway was activated in HL-60 and U937 cells after adhesion cocultured with HS-5 cells, but this activation process could be inhibited by LY294002. Treatment methods for all AML cell groups: single suspension culture, adhesion cocultured with HS-5 cells, and treatment with LY294002 for 12 h after adhesion coculture (HL-60 cells: 10 μM; U937 cells: 20 μM). The amount of protein loaded was 40 μg. The protein levels of all of the important molecules were analyzed by Western blot.

DISCUSSION

Although in recent years, with the application of many new drugs and the development of stem cell transplantation technology, the remission rates and disease-free survival of AML patients have improved, relapse is still a major problem in AML management. Studies have confirmed that the bone marrow microenvironment, which is composed of stromal cells, adhesion molecules, and cytokines, not only provides signals for normal hematopoiesis but also generates a large number of signals to support the growth and maintenance of leukemia cells, particularly leukemia stem cells (LSCs). Therefore, the sensitivity of leukemia cells to conventional chemotherapeutic drugs is compromised, which enables the leukemia cells to escape cytotoxic effects of chemotherapeutic drugs resulting in disease relapse (3,14). Thus, strategies to increase the sensitivity of AML cells in the microenvironment to chemotherapy and to reverse multidrug resistance have become a hot topic in studies of AML treatment.

In our previous studies, primary MSCs or HS-5 stromal cells were cocultured with leukemia cells to simulate the growth and maintenance of leukemia cells in the bone marrow microenvironment. The results demonstrated that the sensitivity to As_2O_3 and the CD44 monoclonal antibody A3D8 was significantly reduced after coculture. Furthermore, this drug resistance was associated with the abnormal activation of the PI3K/Akt signaling pathway (4,5).

The results in the present study showed that adhesion coculture with HS-5 cells could significantly decrease the sensitivity of HL60 and U937 cells to three types of chemotherapeutic drugs: DNR, HHT, and Ara-C. Many transport proteins have been confirmed to be involved in the

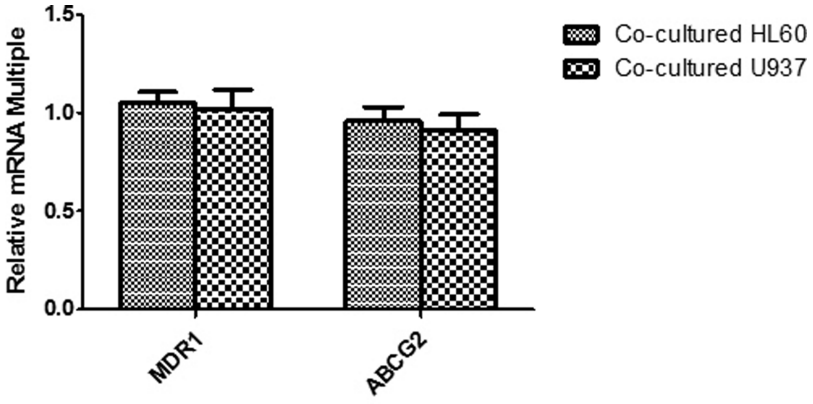

Figure 3. The mRNA expression levels of the MDR1 and ABCG2 genes in HL-60 and U937 cells did not display significant changes after being cocultured with HS-5 cells. Cells (1×10^6) were collected, and the gene expression levels were detected using real-time PCR.

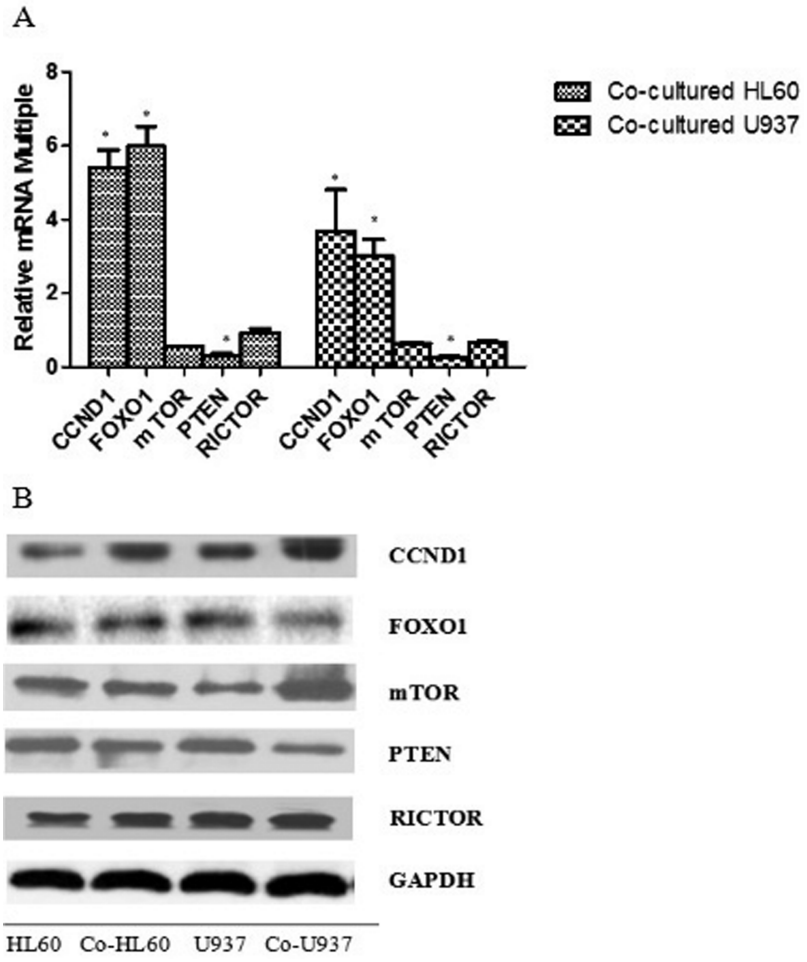

Figure 4. The expression of CCND1, FOXO1, mTOR, PTEN, and RICTOR in HL60 and U937 cells monocultured or adhesion cocultured. (A) mRNA expression detected by real-time PCR. (B) Protein expression detected by Western blot. *$p < 0.01$.

formation of drug resistance in tumor cells, of which the ATP-binding cassette (ABC) superfamily members have been studied more frequently (15–17). Our results showed that the transcription levels of the MDR1 gene (encoding P-glycoprotein, i.e., ABCB1) and the drug pump gene ABCG2 in HL-60 and U937 cells did not undergo significant changes after coculture. These results were similar to those reported by Funayama et al. (18), suggesting that the drug resistance of AML cells induced by stromal cells might not be associated with MDR1 and ABCG2.

The PI3K/Akt signaling pathway is one of the important signal transduction pathways in cells. The abnormal activation of this pathway can be detected in many hematologic malignant diseases, including T-lymphocytic leukemia, mantle cell lymphoma, acute lymphoblastic leukemia, AML, and chronic myeloid leukemia (6–8,10,19). In addition, this pathway participates in the drug resistance of leukemia cells to chemotherapeutic drugs (20,21). Our results showed that pretreatment of the PI3K/Akt signaling pathway-specific inhibitor LY294002 in cocultured HL-60 and U937 cells could partially restore their sensitivity to DNR, HHT, and Ara-C. Western blot results also confirmed that p-Akt^{Ser473} and p-PDK1 were significantly upregulated, and PTEN was significantly downregulated in HL-60 and U937 cells after being cocultured with HS-5 cells, indicating that the PI3K/Akt signaling pathway in these two cells was activated. After LY294002 treatment, p-Akt^{Ser473} and p-PDK1 in HL-60 and U937 cells were significantly downregulated and PTEN was significantly upregulated, indicating that LY294002 could significantly inhibit the activated PI3K/Akt signaling pathway after coculture. These results suggested that PI3K/Akt signaling pathway inhibitors could be combined with clinical chemotherapeutic drugs to enhance the inhibitory function on the proliferation of leukemia cells in the bone marrow microenvironment.

The abnormal activation of the PI3K/Akt signaling pathway is present in many malignant hematologic

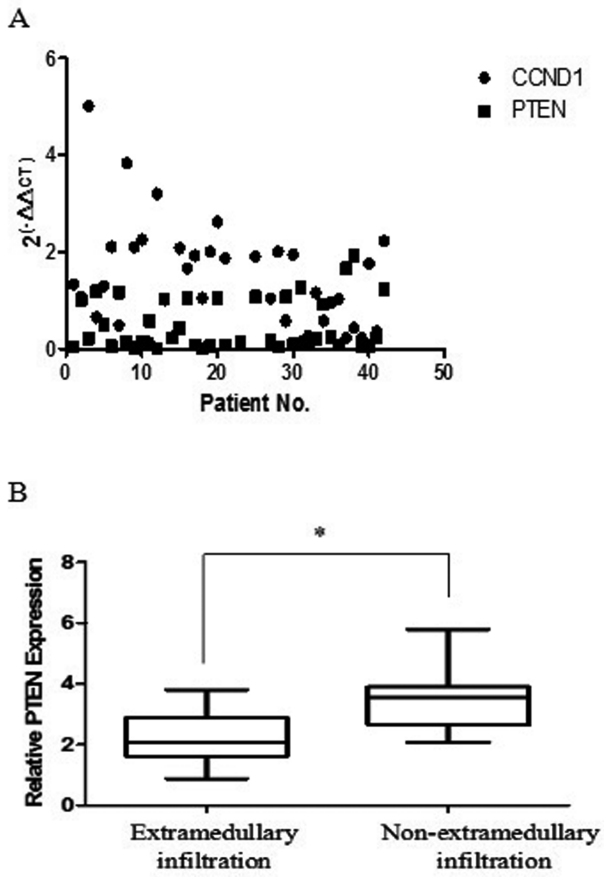

Figure 5. Clinical significance of CCND1 and PTEN in AML patients. (A) CCND1 and PTEN mRNA expression in AML patients. (B) PTEN mRNA expression in newly diagnosed AML patients with or without extramedullary infiltration. AML cells after sorting were collected, and mRNA expression levels were assessed using real-time PCR. Data analysis was performed by the $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta Ct} \geq 3$ was defined as upregulation, and $2^{-\Delta\Delta Ct} \leq 1/3$ was defined as downregulation. $^{*}p < 0.01$.

diseases and is an indicator for poor prognosis. However, a method to rapidly and conveniently detect the activation status of this pathway and apply this finding in clinical works remains unknown. PTEN, CCND1, FOXO1, mTOR, and RICTOR genes are important signaling molecules in the PI3K/Akt signaling pathway and have abnormal expression in acute leukemia patients (13). Coculture altered the expression of these genes at both the mRNA and protein levels. This suggests that a major component of the effect of coculture on these gene expressions is at the transcriptional level. Changes in the mRNA and protein expression levels of CCND1 and PTEN were more consistent. Therefore, the mRNA levels of CCND1 and PTEN in primary AML cells were used to analyze their association with clinical prognosis. The results showed that AML patients with high CCND1 mRNA expression and low PTEN mRNA expression had high relapse rates within 1 year; furthermore, PTEN expression levels were associated with extramedullary infiltration.

The tumor suppressor gene PTEN is located on chromosome 10q23. PTEN dephosphorylates PIP3 to produce PIP2, which antagonizes PI3K activities and participates in the regulatory processes of cell growth and proliferation; therefore, PTEN is an important inhibitory molecule in the PI3K/Akt signaling pathway (22,23). A high frequency of PTEN gene mutations or deletions can be detected in many types of human tumor cells, such as colon cancer, ovarian cancer, and endometrial cancer (24,25). Studies have shown that mice with PTEN mutations or deletions had a presentation similar to myeloproliferative neoplasm (MPN), which subsequently was transformed into acute myeloid or lymphoid leukemia. After the implantation of leukemia cells into severe combined immunodeficient (SCID) mice, the recipient mice developed leukemia with extramedullary infiltration (26–29). Our results also showed that newly diagnosed AML patients with extramedullary infiltration had lower PTEN mRNA levels than patients without extramedullary infiltration. The possible reason for this result is that PTEN inactivation usually results in the activation of the PI3K/Akt pathway, which then catalyzes a series of downstream protein phosphorylation events to promote tumor cell invasion and metastasis.

The CCND1 gene is located at 11q13. Its encoded cyclin D1 is a member of the highly conserved cyclin protein family. As a regulatory subunit of CDK4 or CDK6, cyclin D1 can promote the transition of the cell cycle from the G_1 phase and the S phase (30). The CCND1 mRNA and protein expression levels in HL60 and U937 cells after coculture were both upregulated, suggesting that the activation of the PI3K/Akt signaling pathway after coculture caused the increase of CCND1 to promote cells to enter the cell cycle. However, previous studies by us (4–5) and others (31) have all shown that the G_0/G_1 ratio was upregulated, and the S phase percentage decreased in AML cells after coculture. The presence of this paradox phenomenon requires further experimental studies.

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