Am J Transl Res 2016;8(9):3630-3644
www.ajtr.org /ISSN:1943-8141/AJTR0032291

Original Article

Hepatocyte growth factor promotes proliferation, invasion, and metastasis of myeloid leukemia cells through PI3K-AKT and MAPK/ERK signaling pathway

Jiang-Rui Guo[1*], Wei Li[2*], Yong Wu[1], Lin-Qing Wu[3], Xin Li[1], Ya-Fei Guo[1], Xiao-Hui Zheng[1], Xiao-Lan Lian[1], Hui-Fang Huang[1], Yuan-Zhong Chen[1]

[1]Fujian Institute of Hematology, Fujian Provincial Key Laboratory on Hematology, Fujian Medical University Union Hospital, Fuzhou, China; [2]School of Pharmacy, [3]Department of Immunology, Fujian Medical University, Fuzhou, China. [*]Equal contributors.

Received May 16, 2016; Accepted August 8, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: This study aims to investigate effects of HGF expression on biological behaviors of Kasumi-1 and HL60. Expression of HGF and c-Met gene were detected using qRT-PCR. Short hairpin RNA (shRNA) was used to reduce HGF expression. Silencing effect of shRNA was verified by qRT-PCR and western blot. Cell reproductive capacity, cell clonality and cell cycle (apoptosis) were detected by CCK-8, clone formation, flow cytometry (FCM), respectively. Cell adhesion, cell invasion ability and cell proliferation were also examined. Changes of PI3K-AKT, MAPK/ERK signaling factors were detected by western blot. HGF and c-Met expression in first-vist AML group was significantly higher than in AML-relief and normal control group. HGF shRNA can inhibit cell proliferation, inhibit cloning ability. Compared with control group, apoptosis ratios of Kasumi-1 and HL60 cell in interference groups were significantly higher. After shRNA interference, the number of adherent cells and transmembrane cells were significantly decreased compared with control group. Meanwhile, shRNA also down-regulated Bad, Bcl-XL, Bcl-2, CDK1, Cyclin B, MMP2, MMP9, and up-regulated cleaved caspase9, cleaved caspase3, cleaved PARP, Bax, and P21. Moreover, phosphorylated c-Met, AKT, Erk, and mTOR were also reduced. In conclusion, HGF and c-Met gene highly expressed among first-visit AML patients, but decreased after relief treatment. HGF may promote proliferation, invasion, and metastasis of AML cells through PI3K-AKT and MAPK/ERK signaling pathway. Therefore, proliferation and invasion ability of AML cell can be inhibited by down-regulating HGF gene to retardate cell in G2/M stage.

Keywords: AML, HGF, c-Met, PI3K-AKT, MAPK/Erk

Introduction

Hepatocyte growth factor (HGF) is located in the long arm of No. 7 chromosome (7q2111) and encodes a single-stranded protein in precursor containing 728 amino acids. The specific receptor of HGF is a transmembrane receptor protein with tyrosine kinase activity encoded by a proto-oncogene, c-Met [1]. In physiological status, HGF-c-MET signal path can play a role in the interaction between epithelium and mesenchymal cell, so it has significant importance in aspects of tissue damage repair, anti-inflammation, and immune-regulation [2, 3].

In the studies on solid tumors, it has been found that HGF-c-MET signal pathway get out of control in regulation, which is related to the bad prognosis of distant lymphatic metastasis, tumor recurrence, etc. [4] Therefore, relevant therapies are being positively developed with targets of HGF and c-MET [5].

Studies on acute myeloid leukemia (AML) showed the expression of HGF gene in various AML cell strains, while high level of HGF gene and its protein products can be detected in the plasma and marrow among patients [6]. Kmiecik *et al.* [7] found that HGF can enhance the stimulation of IL-3 and GM-CSF on the proliferation of rat myeloid cell. Kentsis *et al.* [8] found that the proliferation of OCI-AML2 cell is dependent on the abnormal activation of HGF-c-Met pathway. We found that the mRNA expres-

sion level of HGF gene significantly increased with the rise of tumorigenesis rate in nude rat. Therefore, it is speculated that this gene may play a role in AML proliferation and invasion [9]. All above studies showed that HGF-c-MET signal pathway plays an important role in AML, but its specific mechanism requires further illustration. In addition, some key problems, including the clinical significance of abnormal activation in HGF signal path and its relationship with clinical characteristics of patients have never been reported. In this study, the expression levels of HGF gene and c-Met gene in AML patients were detected and its relationship with clinical characteristics of diseases studied. Finally, the role of HGF gene in cell proliferation, invasion, apoptosis, etc. was discussed.

Materials and methods

Sample source

All the samples were collected from patients treated in hematology department of Fujian Medical University Union Hospital from September 2012 to February 2015. There were 132 marrow samples from patients with acute myelogenous leukemia (including 91 patients after the first visit and 41 with relief (with 30 paired samples for the first visit and relief treatment)) and 32 marrow samples from healthy donators for marrow transplantation as normal control. AML patients after the first visit included 54 male patients and 37 female patients in age of 13~72 years old with median age of 43 years old. According to 2008 WHO criteria for the diagnosis and classification of acute myelogenous leukemia, there were 12 patients with t(8;21) genetic abnormality, 9 patients with t(15;17), 3 patients with inv (16), 4 patients with premature AML, 11 patients with AML micronization, 8 patients with mature AML, 2 patients with acute myelomonocytic leukemia, and 42 patients with acute moboblast and monocytic leukemia. Relief and prognosis evaluation criteria followed NCCN guidance. Thirty two patients for normal control included 20 female patients and 12 female patients in age of 38~52 years old with median 41 years old. All of patients signed informed consent.

Cell strain

HL60 (acute myelogenous leukemia cell line), U937 (histiocytic lymphoma cell line), HEL (erythroleukemia cell line), NB4 (acute promyelocytic leukemia cell line), and K562 (chromic granulocytic leukemia cell line) were all strains stored in Fujian Institution of Blood Disease. KG1a cell was purchased from American Type Culture Collection (ATCC). Kasumi-1 (acute myelogenous leukemia cell line) was sent by Professor Lin Donghong from inspection department of medical technician college in Fujian Medical University. All of above cells were cultured in RPMI1640 culture solution (HyClone) containing 10-20% fetal calf serum (Gibco) stored in the incubator (U.S. ThermoCompany) at 37°C and 5% CO_2 saturation humidity. The solution was changed for passage every 2-3 days.

RNA extract and cDNA synthesized by reverse transcription

With bone marrow mononuclear cell and cell line in log production period, total RNA of cells was extracted by TRIzol method (Invitrogen). Then, cDNA was synthesized by steps in specification of reverse transcription kit (Thermo) and stored at -80°C.

Quantitative PCR detection of HGF and c-Met gene expression

As the internal reference, GAPDH gene had following primer sequence: up-stream: 5'-GCACCGTCAAGGCTGAGAAC-3' and down-stream: 5'-TGGTGAAGACGCC AGTGGA-3' with amplified fragment length of 138 bp. The primer sequence of HGFgene was as follows: downstream: 5'-GGGCACTGTCAATACCAT-3' and downstream: 5'-CAGTAGCCAACTCGGATG-3' with amplified fragment length of 189 bp. The primer sequence of c-Metgene was as follows: upstream: a, 5'-GGAGCCAAAGTCCTTTCATCTGTAA-3' and downstream: b, 5'-GCAATGG ATGATCTGGGAAATAAGAAGAAT-3'. By the operation steps in quantitative PCR kit (Roche), the reaction system contains SYBR Green Master (ROX) 10 μl, upstream primer (10 pmol/μl) 0.5 μl, downstream primer (10 pmol/μl) 0.5 μl, cDNA 1 μl, and double distilled water 8 μl. The reaction was conducted in ABI7500 fluorogenic quantitative PCR instrument (Applied Biosystem Company) under conditons: at 50°C for 2 min and 95°C for 10 min, and then at 95°C for 15 sec and 60°C for 1 min as one cycle for 40 times in total. Then, melting curve reaction was conducted under conditions: at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for

15 secas 1 cycle for once. Three ventral orifices were set in every sample. With GAPDH as internal reference, the relative expression quantity of mRNA was represented with RQ value by $2^{-\Delta CT}$ method: RQ = $2^{-\Delta CT}$.

Construction and package of lentiviral vector

According to experimental results in early stage, we planned to synthesize a pair of effective sequences of shRNA targeting forward HGF mRNA according to HGF mRNA (NM_030752.2) sequence (HGF shRNA, forward: 5'-CCGGTGTCTGAAGC ACCCACCAATAC-TCGAGTATTGGTGGGTGCTTC AGACATTTTT-3', reverse: 5'-AATTAAAAATGTCTGAAGCACCCACC-AATAC TCGAGTATTGGTGGGTGCTTCAGACA-3') and a pair of negative shRNA sequences (scrambled control shRNA, forward: 5'-CCG-GTTTCTCCGAACGT GTCACGTTTCAAGAGAACGT-GACACGTTCGGAGAATTTTTG-3', reverse: 5'-AA-TTCAAAAATTCTCCGAACGTGTCACGTTCTCTTG-AAACGTGACACG TTCGGAGAA-3') for control. All the sequences were known without homology with other human genes according to BLAST analysis validation. Two pairs of oligonucleotides, HGF shRNA and scrambled control shRNA were connected with GV248 linearized vectors (GeneChem Co. Ltd.) digested by both AgeI and EcoRI, respectively, to construct lentivirus interference plasmids, LV-HGF-RNAi and LV-scramble-RNAi. After being transformed into Escherichia coli DH5a, positive bacterial colony was selected for sequencing authentication (Shanghai General Biotech Company). Three-plasmid system was used to pack the virus particle, and lentiviral particles (pHelper1.0 and pHelper2.0) were cotransfected into 293T cells with constructed lentiviral interference vectors by Lipofectamine2000. Concentrated virus supernatant was collected 48 hours later to concentrate the virus by super-ventrifugation. Then, virus titer was detected by limiting dilution method.

Kasumi-1 cell and HL60 cell with lentivirus infection

Kasumi-1 and HL-60 in log growth period were collected and inoculated in 96-well plate with 1×10^4 wells. Then, lentivirus with HGF-shRNA or Scrambled-shRNA was added, respectively, for cultivation. After 24 hours, fresh medium with puromycin was replaced, and the expression of green fluorescent protein was observed under florescence microscope to select cell strains with stable transfection. Every cell strain was divided into three groups equally: Control (CON) group (without cell strain with virus transfection), negative control (NC) group (negative control for cell strain with lentivirus Scrambled-shRNA transfection), knockdown (KD) group (transfected cell strain of lentivirus HGF-shRNA).

Detection of cell reproductive capacity

Cells in Kasumi-1-CON group, Kasumi-1-NC group, and Kasumi-1-KD group and cells in HL60-CON group, HL60-NC group, and HL60-KD group were inoculated in 96-well plate in 2×10^3/well and 1×10^3/well, respectively. After 1, 2, 3, 4, and 5 days after inoculation, 10 μl CCK8 (DojindoCompany) was added per well for 3 hours of incubation at 37°C to detect light absorption at 450 nm wavelength. OD value = control group or interference group-blank well, and cell growth curve was drawn with time as horizontal axis and the light absorption value in different groups as vertical axis. Cell proliferation rate was calculated with following formula: cellular proliferation inhibition rate % = (1-average OD value in KD group/average OD value in NC group) ×100%. With 3 ventral orifices in each group, the experiment was repeated for three times.

Clone formation assay

Cells in every group were diluted to 2×10^2/well and inoculated in 24-well plate, and then methylellulose in final concentration of 0.8% (Sigma Company) was added and mixed. After 12 days of incubation in incubator, cell clonal forming amount was observed under inverted microscope. With more than 20 cells as 1 clone, the cloning efficiency = (cloning amount/inoculation quantity) ×100%. With 3 ventral orifices in each group, the experiment was repeated for three times.

Cell cycle detection

Cells in each group were washed with PBS for twice and centrifuged at 2000 rpm for 5 min to regulate the density as 1×10^6/ml. Then, precooling 70% ethyl alcohol was added for fixation overnight at 4°C. On the next day, ethyl alcohol was washed by centrifugation twice at PBS 5000 rpm for 10 min, and 400 μl PI/RNase Staining Buffer (BD Company) was added away from light at room temperature for 15 min. After examination by cytoanalyzer (BD FACS Verse™),

red fluorescence at 488 nm excitation wavelength was recorded for analysis with Flow JO 7.6. The experiment was repeated for three times.

Apoptosis detection

By steps in specification of PE Annexin V Apoptosis Detection Kit I (BD Company), 1-2×10^6 cells were collected from each group, and then 100 μl 1× Binding Buffer suspension cells was added after washing twice with PBS. Then, 5 μl Annexin V-PE and 5 μl 7AAD were added successively and mixed gently away from light for 15 min at room temperature. Finally, 400 ul 1× Binding Buffer was added and mixed gently. Detection by flow cytometry was conducted in 1 hour.

Fibronectin (FN) adhesion experiment

2 μg FN was laid in each well of 96-well plate at dry room ambient. Then, 2% BSA was added to each well for 1 hour of incubator in incubator at 37°C. After being washed with PBS, the fluid was discarded. Cells of each group was suspended in DMEM serum-free culture solution with 0.1% BSA, and 1×10^5 cells were added to each well for 5 hours of incubation in incubator at 37°C. After the medium was discarded, 200 μl PBS was added to each well, and non-adhesive cells were removed by gentle suction and blowing for three times and washing once. After PBS was discarded, CCK8 10 μl was added to each well for 3 hours of incubation in incubator at 37°C. The absorbance at 450 nm wavelength was read in ELIASA. The experiment was repeated for three times.

Transwell chamber invasion assay

By steps in specification, Matrigel (BD Company) was diluted with peridium buffer solution to 300 μg/ml. Then, 100 μl Matrigel was taken to lay in precooling Transwell chamber (Millipore Company) for gel polymerization after 2 hours at 37°C. 2.5×10^5 cells from each group were added to the upper chamber, while 600 μl RPMI 1640 culture solution with 10% fetal calf serum to lower chamber. The chamber was discarded 24 hours after cultivation. The absorbance of living cells in culture solution of lower chamber was detected by above-mentioned CCK8 method to represent the cell number and activity of permeable membrane. The experiment was repeated for three times.

Western blot

Cells from each group were collected, and cell lysis buffer (Roche Company) was added to extract total protein. The protein concentration was detected by BCA method (U.S. Pierce Company). The upper sample of equivalent protein took membrane transfer (Bio-Rad, cat. No. 162-0115) through SDS-PAGE electrophoresis and immunoblotting assay. In the detection, HGF, c-Met, MMP2, MMP7, MMP9, p-mTOR, p-AKT, p-Raf and other anti-actins were purchased from Abcam Company, and p-c-Met (Tyr1349 and Tyr1313), Bcl-xl, bax, Bcl-2, bad, Cleaved caspase-3, Cleaved caspase-9, p-PARP, PI3K-AKTsignal path (p-AKT^{Ser473}, p-mTOR, p-PTEN, p-GSK3β, etc.), MAPK/ERK, cyclins (CDK6, CDK1, CyclinD, etc.) and other anti-actins were purchased from CST Company.

Statistical analysis

GraphPad Prism 5 software package was used in data statistics. The non-normal measurement data were represented by Median. Bilateral Mann-Whitney U test was used in comparison between two groups, Wilcoxon matched pairs test in paring two groups of samples, and Kruskal-wallis test of non-parametric analysis in comparison among three groups. $P<0.05$ means the difference with statistical significance.

Results

Expression of HGF mRNA in AML

The relative transcript level of HGF mRNA in the first-visit AML group (AML-untreated group, 8.096×10^{-3}) was higher significantly compared to that of normal control group (NC group, 2.598×10^{-3}) and relief group (AML-CR group, 1.271×10^{-3}) (**Figure 1A**, P = 0.0149 and 0.0002, respectively). In this study, samples from 30 AML patients were analyzed before and after treatment. According to Wilcoxon matched pairs test, P = 0.0002 (**Figure 1B**). The expression of HGF gene had statistical difference before and after AML treatment. After relief, the expression of HGF mRNA significantly decreases.

The relative transcript level of c-Met mRNA in the AML-untreated group, (3.988×10^{-3}) was higher significantly compared to that of NC group (0.4972×10^{-3}) and AML-CR group

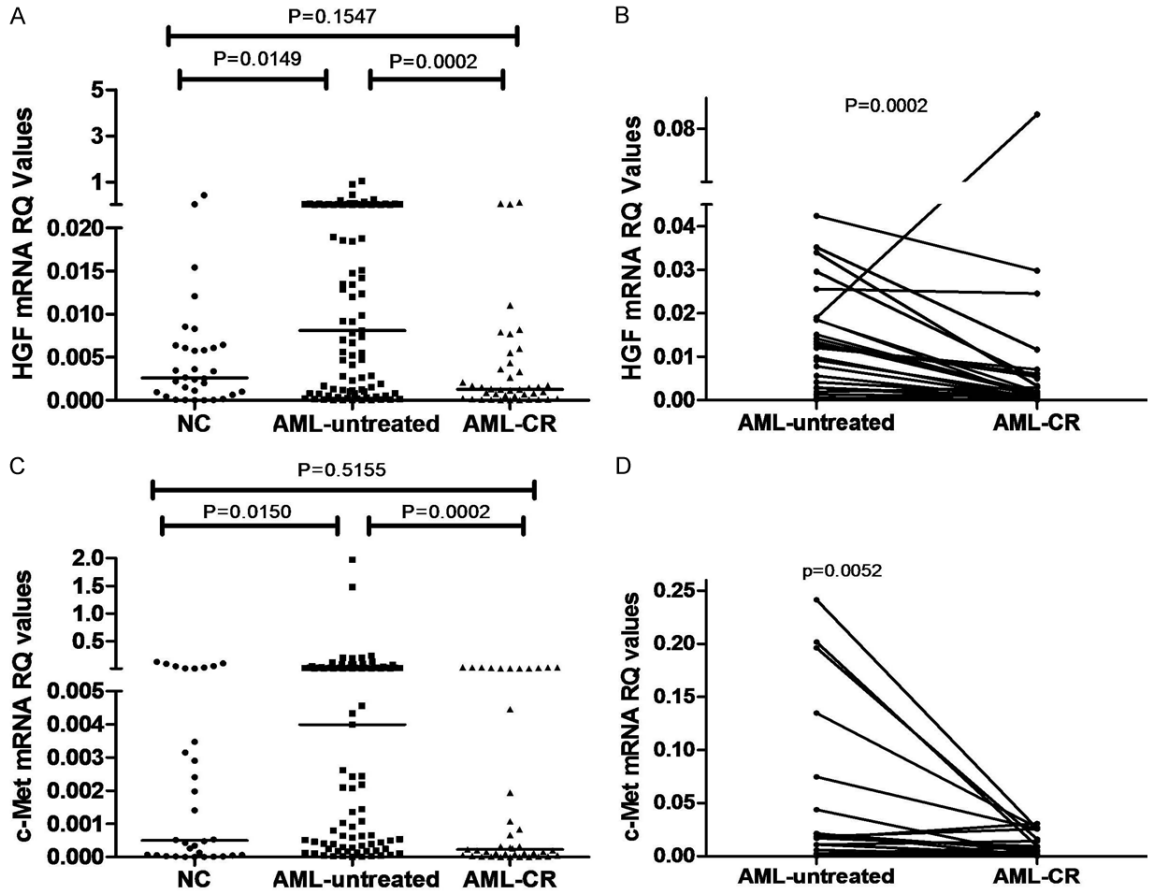


Figure 1. Expression of HGF and c-Met mRNA in AML patients. A. HGF mRNA expression in NC group, AML-untreated group and AML CR group analyzed by Mann-Whitney U test. B. HGF mRNA expression in NC group, AML-untreated group and AML CR group analyzed by Wilcoxon matched pairs test. C. c-Met mRNA expression in NC group, AML-untreated group and AML CR group analyzed by Mann-Whitney U test. D. c-MET mRNA expression in NC group, AML-untreated group and AML CR group analyzed by Wilcoxon matched pairs test. The statistical different *p* values have been added in the images.

(0.2308×10^{-3}) (**Figure 1C**, P = 0.015 and 0.0002, respectively). Meanwhile, according to the Wilcoxon matched pairs test, P = 0.0052 (**Figure 1D**). The expression of c-Met gene had statistical difference before and after AML treatment. After complete relief, the expression level of c-Met mRNA significantly decreased.

Correlation analysis between HGF mRNA expression level and clinical data of AML patients

Partial clinical data of AML patients after the first visit were collected for Mann-Whitney U test. In the marrow of 91 AML patients without treatment, HGF mRNA level showed no correlation to sex, age, hemoglobin, blood platelet, and relief rate in the first course ($P>0.05$). With the median of peripheral blood leucocyte 50×10^9/L as the critical, AML patients were divided into group of high leukocyte and group of low leukocyte. In the first visit, the expression of HGF mRNA in group of high leukocyte was relatively high with difference of statistical significance ($P = 0.0405$). According to NCCN guidance, prognosis layering and Kruskal-Wallis test were conducted for 68 AML patients taking the first visit. The difference among three groups has statistical significance (**Table 1**, $P = 0.009$).

Correlation analysis on expression level of c-Met mRNA and clinical data of AML patients taking the first-visit

Among 91 marrow samples from AML patients without treatment, the level of c-Met mRNA showed no correlation to sex, age, peripheral

Table 1. Correlation between HGF mRNA level and clinical characteristics of AML patients after the first visit

Valuables	Cases	HGF expression Intermediate (min~max)	*p* value
Sex			
Male	54	0.0126 (0.00002~1.050)	0.224
Female	37	0.0056 (0.000008~7.830)	
Age (years)			
<60	80	0.008601 (0.00002~7.830)	0.7378
≥60	11	0.006999 (0.000008~0.899)	
WBC ($\times10^9$/L)			
<50	54	0.006142 (8.940×10^{-6}~0.4399)	0.0405*
≥50	37	0.01987 (4.594×10^{-5}~7.830)	
Hb (g/L)			
<60	19	0.006999 (0.000038~1.050)	0.8337
≥60	72	0.009134 (0.000008~7.830)	
PLT ($\times10^9$/L)			
<50	54	0.005555 (0.0000089~0.4399)	0.1919
≥50	37	0.01418 (0.000020~7.830)	
Remission			
Positive	53	0.007369 (0.000038~1.050)	0.469
Negative	23	0.01201 (0.0000089~7.830)	
Prognosis			
Favorable	11	0.008096 (3.846×10^{-5}~0.2549)	
Intermediate	38	0.004720 (8.940×10^{-6}~7.830)	
Adverse	19	0.01879 (0.001026~1.050)	0.009

*P<0.05 represents the WBC levels in <50 years compared to ≥50 years.

Table 2. Correlation between the expression level of c-Met mRNA and clinical characteristics of AML patients after the first-visit

Valuables	Cases	c-Met expression Intermediate (min~max)	*p* value
Sex			
Male	54	0.003301 (0.000011~1.972)	0.585
Female	37	0.005828 (0.000005~1.476)	
Age (years)			
<60	80	0.002300 (0.0000052~1.972)	0.0617
≥60	11	0.01839 (0.000024~1.476)	
WBC ($\times10^9$/L)			
<50	54	0.004688 (0.0000243~1.972)	0.8116
≥50	37	0.002613 (0.0000052~1.476)	
Hb (g/L)			
<60	19	0.00432 (0.0000052~0.1349)	0.4610
≥60	72	0.00330 (0.00001124~1.972)	
PLT ($\times10^9$/L)			
<50	54	0.002428 (0.0000052~1.972)	0.6023
≥50	37	0.004329 (0.000026~1.476)	
Remission			
Positive	53	0.00218 (0.0000052~0.2415)	0.4975
Negative	23	0.00398 (0.000047~1.476)	
Prognosis			
Favorable	11	0.001080 (5.279×10^{-6}~0.1349)	
Intermediate	38	0.002613 (1.124×10^{-5}~1.972)	
Adverse	19	0.004329 (2.434×10^{-5}~1.476)	0.744

blood leucocyte, hemoglobin and blood platelet level, prognosis layering, and relief rate in the first course (**Table 2**, $P>0.05$).

Construction, package, and transfection into Kasumi-1 and HL-60 cells of lentiviral vector

Lentiviral vector, GV248 connected with HGF shRNA through the linear vector after the digestion of AgeI and EcoRI to obtain recombinant plasmid. After transfection into Escherichia coli DH5a and selection of positive bacterial colony, the results of sequencing authentication showed that the construction of LV-HGF-RNAi lentiviral vector was successful (Supplementary Figure 1A). Co-transfection of 293T cells was conducted through packaging plasmids of lentiviral (pHelper1.0 and pHelper2.0) and constructed lentiviral vector. Virus fluid was collected 48 hours after transfection with virus titer of 5×10^8 TU/mL. According to the results of preliminary experiment for virus transfection, Kasumi-1 and HL60 cells were infected by MOI (multiplicity of infection) = 100 and 150, respectively. After screening with puromycin (Kasumi-1 cell: 1.5 μg/mlpuromycin; HL60 cell: 1.0 μg/mlpuromycin), GFP expression with green fluorescence was seen under fluorescence microscope (Supplementary Figure 1B). According to the analysis on GFP expression of cells in each group by flow cytometry after puromycin screening, the GFP transfection efficiency of cells in each groups with virus transfection was almost 100% (Supplementary Figure 1C). It means that above groups of stable lentiviral transfected strains were constructed successfully.

Expression of HGF mRNA and protein

We detected the expression of HGF mRNA in 6 cell strains of

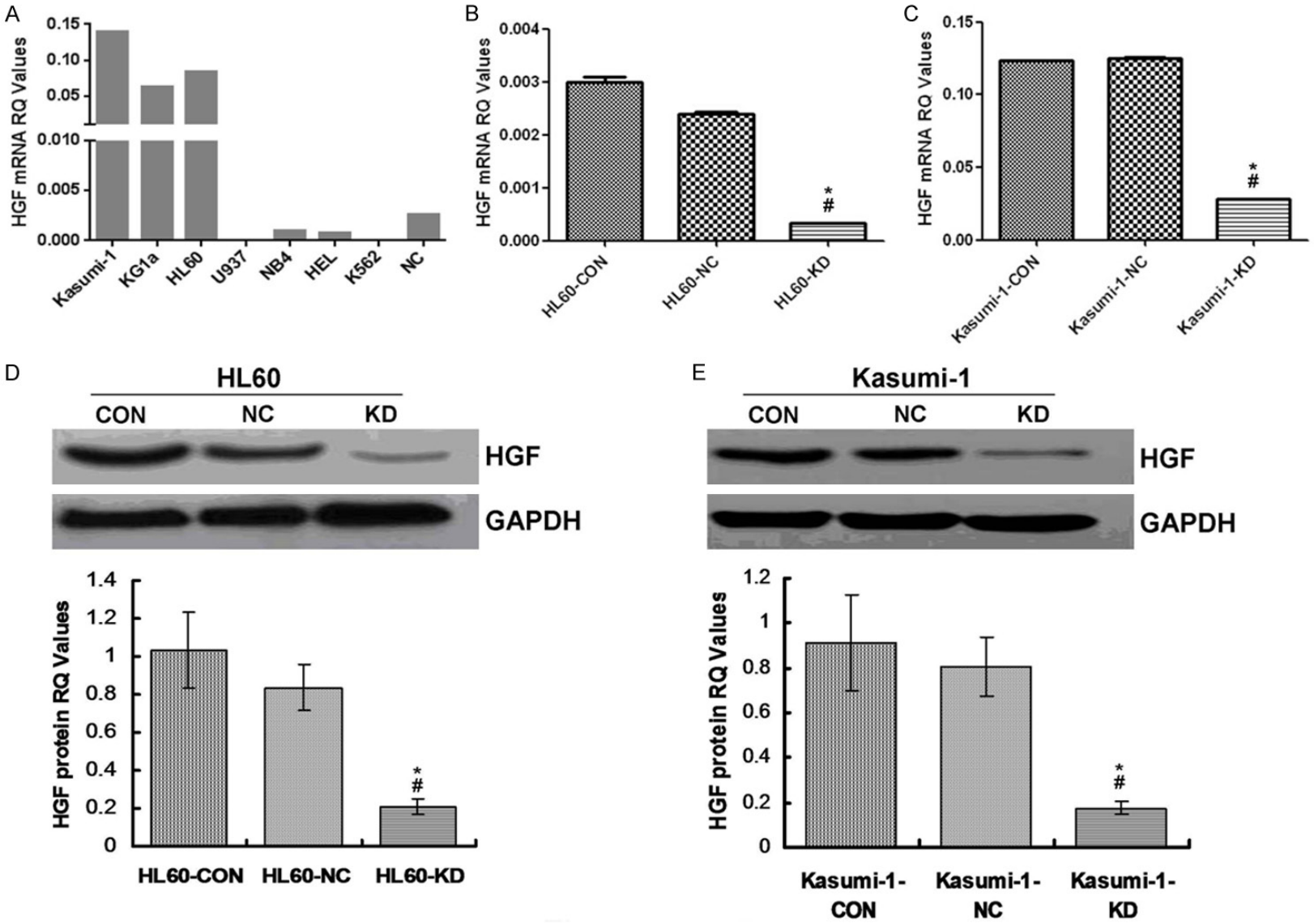


Figure 2. Observation for HGF mRNA expression different cell lines and HGF mRNA (protein) in different groups. A. HGF mRNA expression in 6 cell strains of acute myelogenous leukemia. B. HGF mRNA expression in HL60-KD, HL60-NC, and HL60-CON group. C. HGF mRNA expression in Kasumi-1-KD, Kasumi-1-NC, and Kasumi-1-CON group. D. HGF protein expression detected by western blot and statistical analysis in different groups. E. HGF protein expression detected by western blot and statistical analysis in different groups. *P<0.05 and #P<0.05 represent the mRNA or protein levels in KD group compared to the CON group and NC group, respectively.

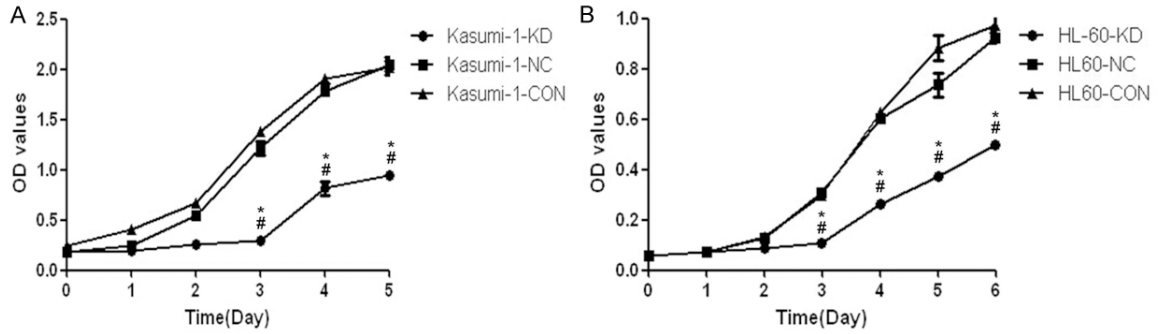


Figure 3. Effect of HGF silencing on proliferation of AML cells. A. Effect of HGF silencing on proliferation of Kasumi-1. B. Effect of HGF silencing on proliferation of HL60. *P<0.05 and #P<0.05 represent the cell proliferation in KD group compared to the CON group and NC group, respectively.

acute myelogenous leukemia, NB4, KG1a, HL60, Kasumi-1, K562, and HEL and the cell strain of histiocytic lymphoma, U937 by qRT-PCR method (**Figure 2A**). With relatively high expression of HGF mRNA, cell strains of myelogenous leukemia, Kasumi-1, HL60, and KG1awere used as target cell strains to study the cell functional changes after HGF silencing (**Figure 2A**).

The mRNA expression of HGF was also examined in the CON (HL60, Kasumi-1), NC (HL60, Kasumi-1) and KD (HL60, Kasumi-1) group. Then, HGF gene silencing level was verified by RT-PCR. The results indicated that the HGF level HL60-KD was lower significantly compared to both HL60-CON and HL60-NC group (**Figure 2B**, P<0.05). Also, the similar result was observed in Kasumi-1 cells (**Figure 3C**, P<0.05).

Meanwhile, the HGF protein gene silencing level was also examined by using the western blot assay. The results showed that HGF protein level in KD group was lower significantly compared to both CON and NC group both in HL60 cells and Kasumi-1 cells (**Figure 2D**, **2E**, P<0.05).

Inhibition effect of silencing HGF on proliferation of Kasumi-1 and HL-60

The change of proliferation capacity of stable cell strains, Kasumi-1 and HL-60 after HGF shRNA lentiviral infection was detected by CCK-8 method. The results showed that the inhibition ratios of cellular proliferation in Kasumi-1-KD group (**Figure 3A**) and HL60 KD group (**Figure 3B**) were 53.6% and 44.4%, respectively, with difference of statistical significance (P<0.05). Above results show that HGF silencing can inhibit the proliferation of Kasumi-1 and HL-60.

Inhibition effect of HGF silencing on cloning formation of Kasumi-1 and HL-60

Compared with Kasumi-1-NC group, the inhibition ratio of cloning formation in Kasumi-1-KD group was 35.95% with difference of statistical significance (**Figure 4A**, P = 0.005). Compared with HL60-NC group, the inhibition ratio of cloning formation in HL60-KD group was 60.12% with difference of statistical significance (**Figure 4B**, P = 0.005).

Effects of HGF silencing on apoptosis and adhesion capacity of Kasumi-1 and HL-60

The cell apoptosis was detected of by using Annexin V-PE/7AAD double-staining method. Compared with Kasumi-1-NC group, the total apoptosis ratio in Kasumi-1-KD group mildly increased (**Figure 5A**, P = 0.001). Compared with HL60-NC group, the total apoptosis ratio in HL60-KD group increased (**Figure 5A**, P = 0.015). However, compared with CON group without transfection, the total apoptosis ratios of two cell strains in NC group showed no difference (**Figure 5A**, P>0.05). It means that the inhibition of HGF expression can induce the apoptosis of Kasumi-1 and HL-60.

Inhibition effects of HGF silencing on adhesion capacity of Kasumi-1 and HL-60

The adhesion capacity in Kasumi-1-KD group was lower significantly compared to Kasumi-1-NC group (**Figure 5B**, P = 0.00). Meanwhile, the

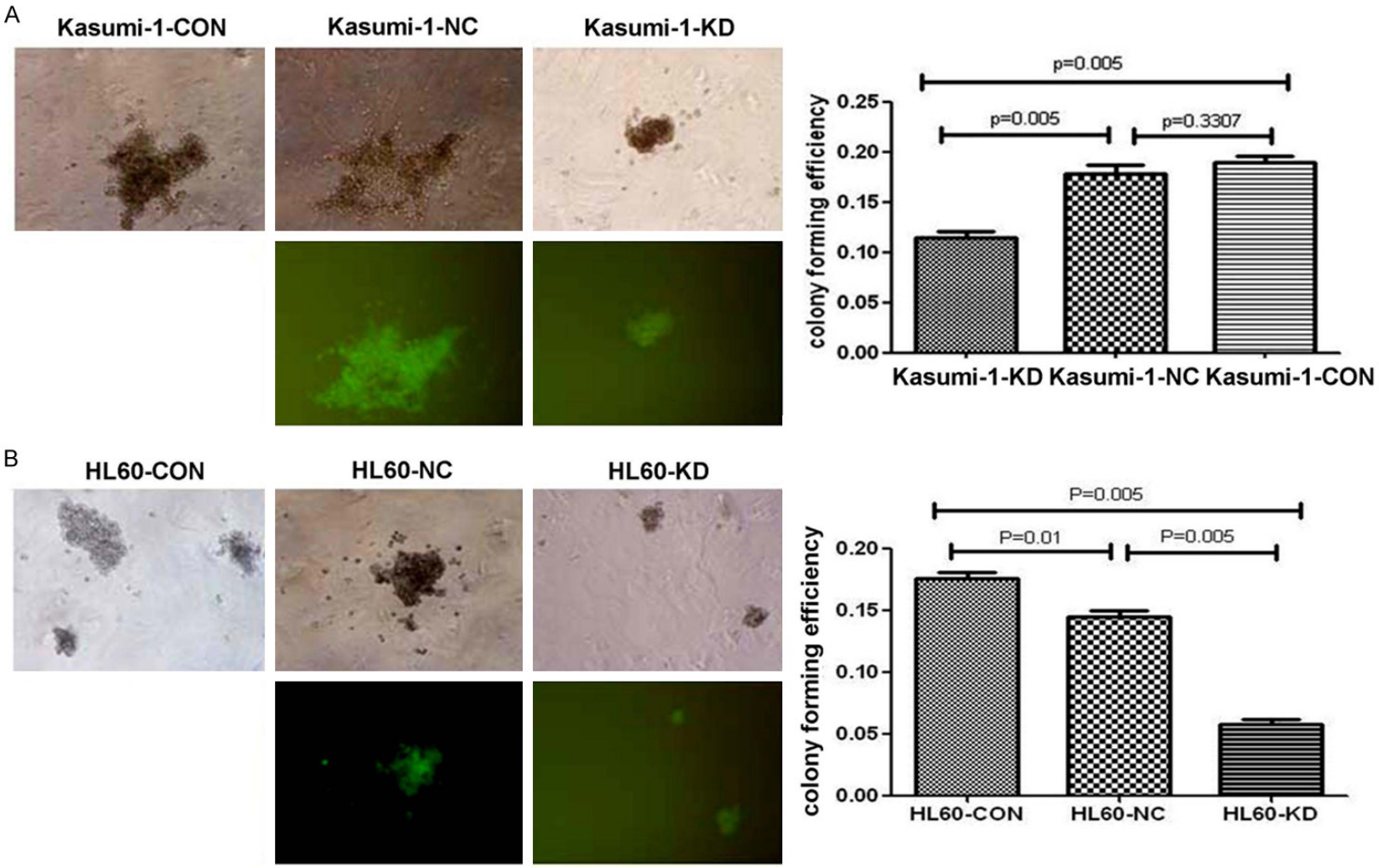


Figure 4. Effects of HGF silencing on cloning formation of Kasumi-1 and HL60 cells. A. Fluorescence microscopy observation for HGF silencing on cloning formation and statistical analysis in Kasumi-1 cells. B. Fluorescence microscopy observation for HGF silencing on cloning formation and statistical analysis in HL60 cells.

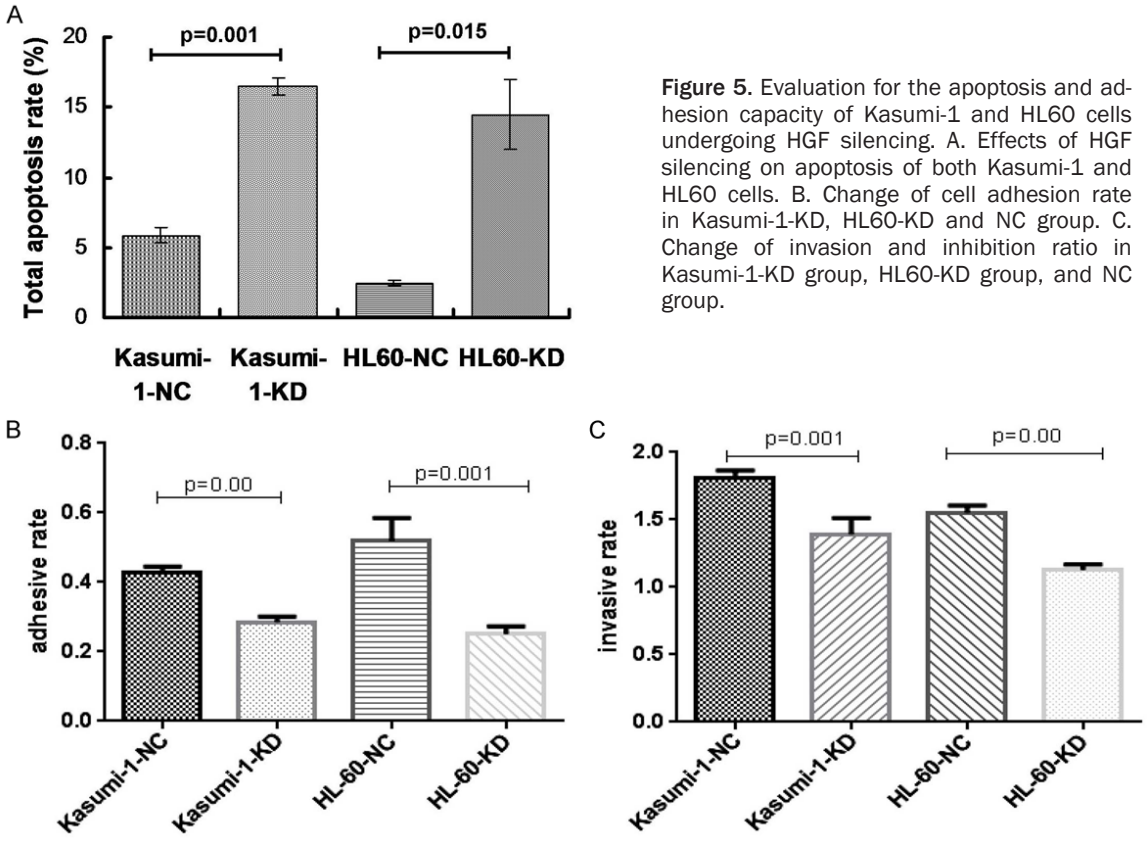


Figure 5. Evaluation for the apoptosis and adhesion capacity of Kasumi-1 and HL60 cells undergoing HGF silencing. A. Effects of HGF silencing on apoptosis of both Kasumi-1 and HL60 cells. B. Change of cell adhesion rate in Kasumi-1-KD, HL60-KD and NC group. C. Change of invasion and inhibition ratio in Kasumi-1-KD group, HL60-KD group, and NC group.

adhesion capacity in HL60-KD group was significantly compared to HL60-NC group (**Figure 5B**, $P = 0.001$). All above values show that the adhesion capacity of Kasumi-1 and HL60 decreased after silencing HGF.

Experiment of Transwell chamber invasion

For the cell invasion, the invasive capacity of the Kasumi-1-KD group lower significantly compared to Kasumi-1-NC group (**Figure 5C**, $P = 0.001$). Meanwhile, the invasive capacity of the HL60-KD group was also lower significantly compared to HL60-NC group (**Figure 5C**, $P = 0.000$). All above values showed that the invasion capacity of Kasumi-1 and HL60 decreased after silencing HGF.

Above results showed that the interference of HGF expression can inhibit the proliferation of Kasumi-1 and HL-60, promote apoptosis. In the following experiments, the change of related signal molecule was detected to discuss the molecular mechanism of effects of HGF silencing on above biological behavior.

Change of Met protein at downstream receptor

As shown in **Figure 6A**, p-Met (Tyr1349 and Tyr1313) in KD group down regulated, and c-Met protein expression had no significant change when comparing Kasumi-1-KD and HL60-KD group with NC group.

Effects of HGF silencing on cyclin

As shown in **Figure 6B**, the expression of cyclin B and Cdk1 in KD group down regulated when comparing Kasumi-1-KD and HL60-KD group with KD group. The expression of P21 upregulated, the phosphorylation level of Chk1 and Cdc25 up-regulated. Meanwhile, the cyclin D and Cdk6 protein changed a little. The down-regulation ratio of cyclin B in Kasumi-1-KD group was significant compared with HL60-KD group (**Figure 6B**).

HGF silencing triggers apoptosis signal pathway

The expression of Bcl-xl and Bax protein in KD group downregulated (**Figure 7**). The expres-

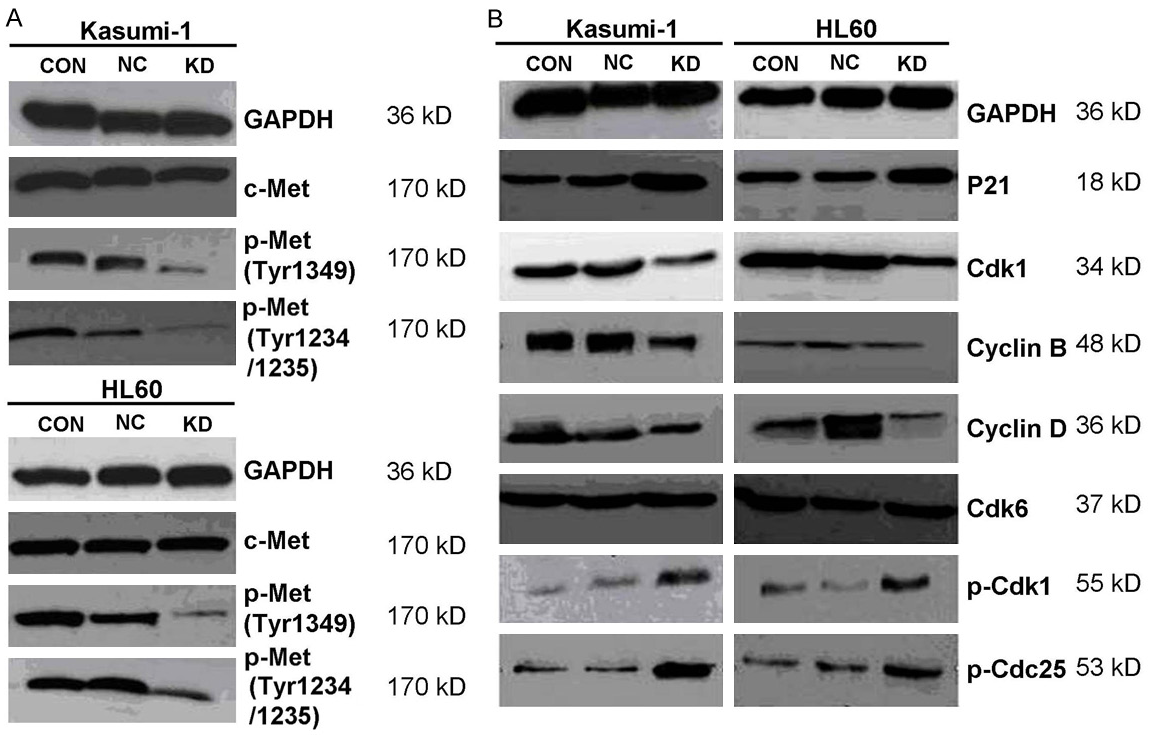


Figure 6. Effect of HGF silencing on Met and cyclin protein expression. A. Western blot analysis for Met expression in Kasumi-1 and HL60 cells. B. Western blot analysis for cyclin expression in Kasumi-1 cells and HL60 cells.

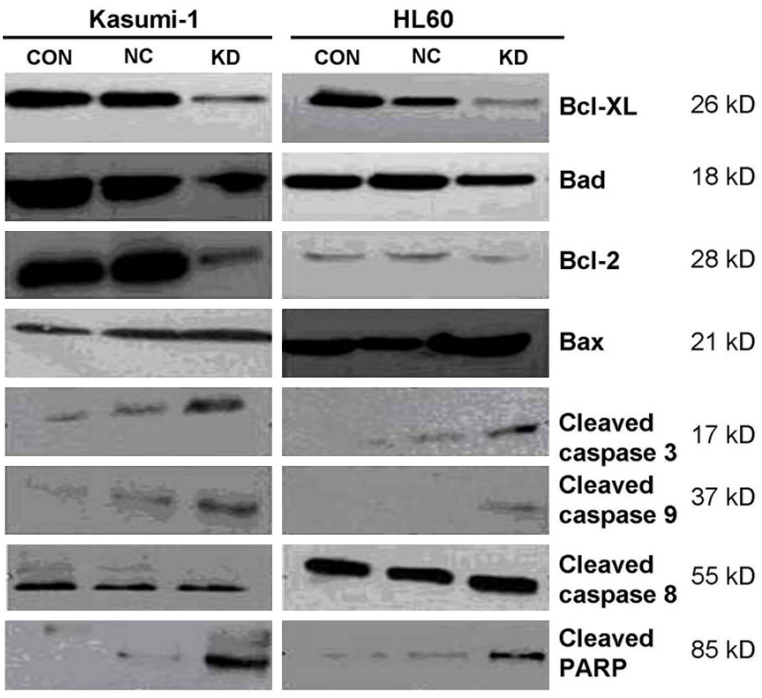


Figure 7. Effect of HGF silencing on the expression of Kasumi-1 and HL60 apoptosis signal protein detecting using western blot.

sion of Bax, Cleaved caspase 9, Cleaved caspase 3, and Cleaved PARP were up-regulated obviously (**Figure 7**). The expression of Bcl-2 in Kasumi-1-KD group was down-regulated obviously, while the change of Bcl-2 expression in HL60-KD was not obviously changed.

Effects of HGF silencing on PI3K-AKT and MAPK/ERK signaling pathway

As shown in **Figure 8A**, the phosphorylation level of AKT (Thr473) and mTOR protein in KD group down regulated. PTEN phosphorylation in Kasumi-1-KD group was up-regulated, while that in HL60-KD group was not obviously up-regulated. GSK3β phosphorylation in HL60-KD group

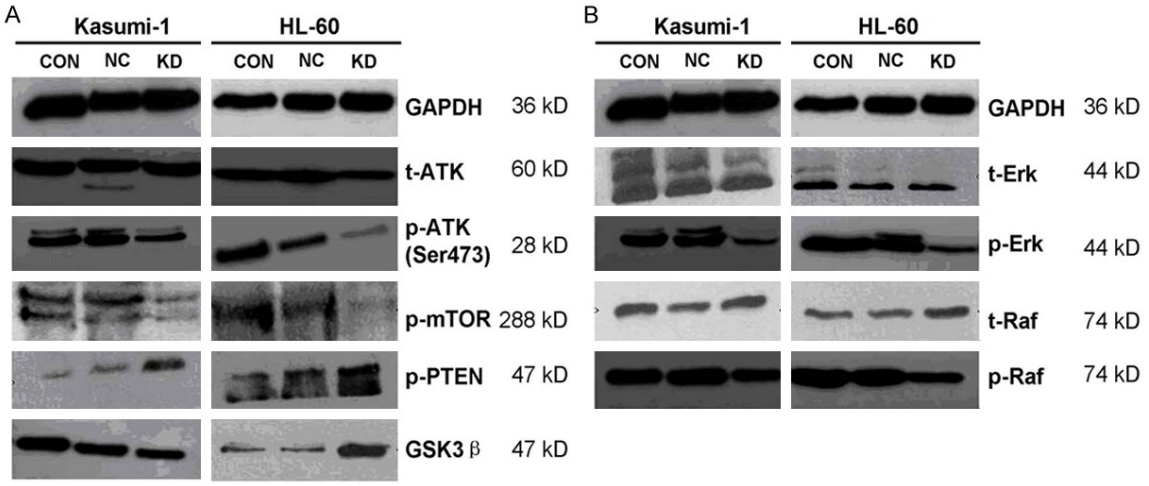


Figure 8. Observation for the HGF silencing on PI3K-AKT and MAPK/ERK signaling pathway. A. Observation for the PI3K-AKT signaling pathway. B. Observation for the MAPK/ERK signaling pathway.

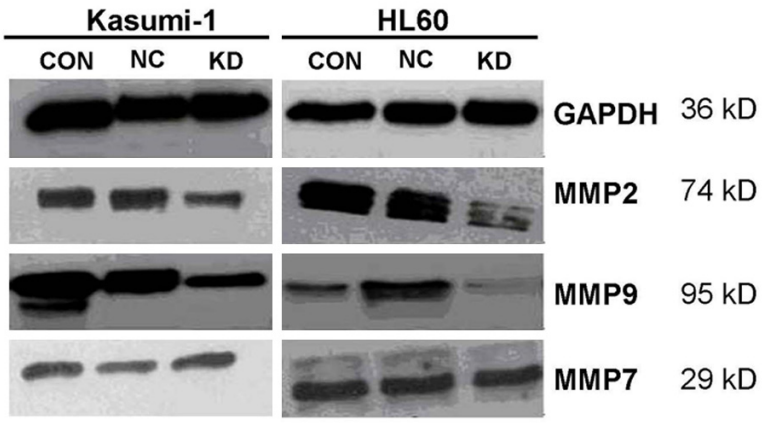


Figure 9. Effects of HGF silencing on protein expression related to invasion of Kasumi-1 and HL60 by suing western blot.

was up-regulated, while that in Kasumi-1-KD group was not changed obviously. Moreover, the t-AKT levels were not changed obviously in both KD groups of cell strains.

As shown in **Figure 8B**, ERK and Raf protein phosphorylation in KD groups was down-regulated significantly when comparing Kasumi-1-KD and HL60-KD groups with NC group. The t-Raf and t-ERK protein had no significant change in KD groups of two cell strains.

Effects of HGF silencing on protein expression related to invasion

The expression of MMP2 and MMP9 protein was down-regulated in KD groups when comparing Kasumi-1-KD and HL60-KD groups with NC group (**Figure 9**). However, MMP7 protein in two cell strains had no significant change in KD groups.

Discussion

Acute myelogenous leukemia is a disease with high heterogeneity. This disease is caused by vicious transformation of normal hematopoietic stem cells in marrow in differentiation stages. During the vicious transformation, the marrow microenvironment of hematopoietic stem cells plays an important role. In the hematopoietic microenvironment, various cell growth factors compose of a complex signal network and precisely regulate the differentiation and growth of hematopoietic stem cells. In the pathological environment, hematopoietic stem cell transforms malignantly because the subtle balance between positive and negative regulatory factors is broken [10].

Hepatocyte growth factor (HGF) is a multi-functional cell growth factor, playing a role of mitogen, motor, morphogenesis promotion, *and etc.* [1] Therefore, it is believed that HGF possibly plays an important role in early hematopoiesis of fetal liver and liver development [11-13]. It is

speculated that HGF only has an indirect effects on normal hematopoiesis. Matsuda-Hashii Y et al. found that the marrow stroma cell can secrete HGF after adding neutral antibody against HGF in marrow stroma cell and $CD34^+$ cell co-culture system. Moreover, $CD34^+$ cloning efficiency can be improved by paracrine [14]. All above research results show that HGF may participate in normal hematopoiesis regulation by autocrine and paracrine.

In early study, we found that the expression level of HGF will up-regulate with the improvement of tumorigenesis rate of HL60 cell [9]. In this study, we extracted bone marrow mononuclear cells from AML patients taking the first visit to quantitatively measure mRNA level and protein level of HGF and made a comparison with the bone marrow mononuclear cells from healthy people. Therefore, we could confirm the HGF secretion of AML cells and its relation with clinical characteristics of AML.

We collected marrow samples from 91 AML patients taking the first visit, and made a comparison with samples of 32 healthy people and 41 AML patients after complete relief. We found that the expression of HGF in first-visit AML group is higher than that in AML relief group and normal control group ($P<0.05$), which is consistent with high HGF expression in plasma and marrow of AML patients reported by Kim J et al. [15]. The study on clinical characteristics of 91 patients showed that the expression level of HGF is relatively high among patients with peripheral blood hyperleukocytois and bad prognosis. In aspect of clinical prognosis, it can be found that the expression level of HGF significantly decreases ($P = 0.0002$) after AML relief in the comparison with 30 samples before and after AML treatment. Therefore, we assumed that if the expression level of HGF can be a reference index in clinical prognosis and relief judgment.

We also studied the quantitative mRNA on the specific receptor c-Met of HGF, which has consistent expression with HGF. The expression of c-Met in first-visit AML group is higher than that in AML relief group and normal control group. Among 30 paired samples before and after AML, the expression of c-Met mRNA also decreases with relief. Matsuda-Hashii Y, et al. found that HGF can promote marrow stroma cell to secrete IL-11, while Goff JP et al. found that IL-11 can promote the expression of c-Met receptor [14-17]. Therefore, we speculated that the high expression of c-Met in AML cell may be related to the increasing HGF content in microenvironment.

In aspect of cell proliferation, CCK-8 result shows that after silencing HGF gene, the proliferation of Kasumi-1 and HL60 is significantly inhibited. The results of FN adhesion experiment and Transwell chamber test also show that after silencing HGF gene, the adhesion and invasion capacity of Kasumi-1 and HL60 cell are also inhibited. According to above results, two cell strains of myeloid leukemia are significantly affected in proliferation, cloning formation, adhesion, invasion and other biological behaviors after silencing HGF.

Previous studies showed that HGF may inhibit apoptosis by regulating endogenous channel [18-20]. This study showed that expression of anti-apoptosis factors Bcl-xl and Bcl-2 decreases, while expression of pro-apoptosis factor Bax mildly increased after silencing HGF in Kasumi-1 cell. The changes of BCL-2 family protein in two cell strains are not consistent, but the ratio of pro-apoptosis/anti-apoptosis members also enlarges. Therefore, two cell strains go apoptosis. In addition, our experiment also shows that the expression of Cleaved Caspase 9, Cleaved Caspase 3, and Cleaved PARP also up-regulates after silencing HGF, further meaning that HGF gene silencing promotes apoptosis by endogenous means.

After combining the specific receptor c-Met of HGF with its ligand HGF, tyrosine residues Tyr1234 and Tyr1235 may have phosphorylation in intracellular domain of β subunit to activate protein kinase domain in cytoplasm. The activated tyrosine kinase can activate tyrosine (Tyr1349 and Tyr1356) at the end of c-Met. Later, the conformation at the end of c-Met changes to recruit different signal trochanter containing SH2 domain for activation of PI3K/AKT, MAPK, and other channels downstream. Therefore, the phosphorylation of c-Met protein which is the specific receptor of HGF in human body, is crucial to the transfer of HGF signal. We also found that the phosphorylated c-Met (Tyr1349 and Tyr1313) shows significantly down-regulation after silencing HGF, while the expression of c-Met protein has no difference between two cell strains. This means that

silencing HGF mainly plays a role in interdicting the phosphorylation of c-Met.

At present, it has been found that HGF can activate PI3K-AKT signal path in various tumor cells. The study by Togo S, et al. found that HGF can activate AKT to reduce the apoptosis of bronchial epithelial cells caused by cigarette. After administering wortmannin or LY294002, PI3K inhibitor, above protection will be converted [21]. We found that after silencing HGF gene, the phosphorylation of AKT (Ser473) of two cell strains will down regulate, while the total protein expression of AKT maintains constant with the phosphorylation of mToR protein in downstream AKT down-regulating. We also found that after silencing HGF gene, the phosphorylation of PTEN protein up regulates, while HL60 cell shows no significant change of PTEN protein phosphorylation. All of these experiments show that HGF gene has multipoint blocking effect on PI3K/AKT, the network of tumor signal path after silencing. Therefore, the blocked PI3K/AKT/mTOR signal path may be one of the reasons to cell growth inhibition after silencing HGF.

MAPK/Erk1/2 signal path is another important channel in downstream of c-Met. Moreover, Ras can activate Erk1/2 through Ras-Raf-Mek1. Siegfried, et al. proved that HGF-c-Met can promote invasion and metastasis of non-small cell lung cancer cells induced by COX2 through signal paths, Erk1/2 and P38MAPK [22]. YH, et al. reported that HGF can reduce pro-apoptosis effect of angiotonin II on vascular endothelial cell through ERk/2 signal path [23]. Our study shows that in two cell strains, kasumi-1 and HL60, the phosphorylation of Raf and Erk protein down-regulates to different extents after silencing HGF gene, so does the expression of MMP2 and MMP9 at downs tream. It may be because the adhesion and invasion capacity of two cell strains decrease after silencing HGF.

In summary, silencing HGF expression can inhibit proliferation, adhesion, and invasion capacity of Kasumi-1 and HL-60 cells in many aspects to induce tumor cell apoptosis. We speculated that HGF can increase the expression of MMP2 and MMP9, promote cell cycle progress, inhibit apoptosis, and promote cell proliferation by activating PI3K-AKT and MAPK/ERK signal pathway. Therefore, it plays a role in the occurrence and development of AML.

Acknowledgements

This study was granted by The National and Fujian Provincial Key Clinical Specialty Discipline Construction Program, P.R.C.; Major project of science and technology of Fujian medical university (Grant No. 09ZD001); Distinguished young university scientists training program of Fujian Province (Grant No. ja13133).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuan-Zhong Chen, Fujian Institute of Hematology, Fujian Provincial Key Laboratory on Hematology, Fujian Medical University Union Hospital, No. 29 Xinquan Road, Fuzhou 350001, Fujian Province, China. E-mail: fuzchenfj@sina.cn

References

[1] Jiang WG, Martin TA, Parr C, Davies G, Matsumoto K, Nakamura T. Hepatocyte growth factor, itsreceptor, and their potential value in cancer therapies. Crit Rev Oncol Hematol 2005; 53: 35-69.

[2] Weidner KM, Di Cesare S, Sachs M, Brinkmann V, Behrens J, Birchmeier W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature 1996; 384: 173-176.

[3] Wang SH, Wu XC, Zhang MD, Weng MZ, Zhou D, Quan ZW. Upregulation of H19 indicates a poor prognosis in gallbladder carcinoma and promotes epithelial-mesenchymal transition. Am J Cancer Res 2015; 6: 15-26.

[4] Graveel CR, Tolbert D, Vande Woude GF. MET: a critical player in tumorigenesis and therapeutic target. Cold Spring Harb Perspect Biol 2013; 5: 1-17.

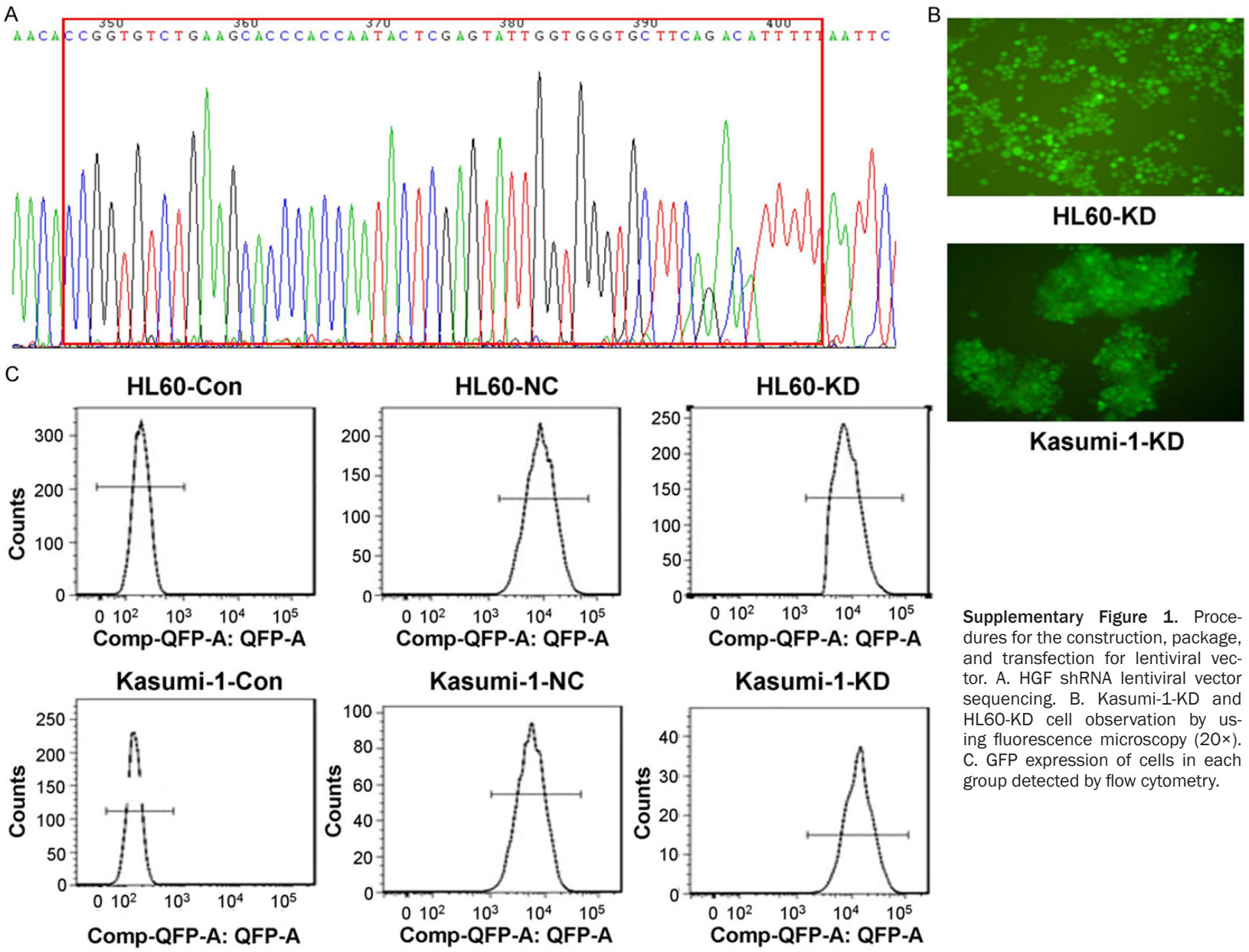
[5] Scagliotti GV, Novello S, von Pawel J. The emerging role of MET/HGF inhibitors in oncology. Cancer Treat Rev 2013; 39: 793-801.

[6] Gohda E, Nakamura S, Yamamoto I, Minowada J. Hepatocyte growth factor-pleiotropic cytokine produced by human leukemia cells. Leuk Lymphoma 1995; 19: 197-205.

[7] Kmiecik TE, Keller JR, Rosen E, Vande Woude GF. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood 1992; 80: 2454-2457.

[8] Kentsis A, Reed C, Rice KL, Sanda T, Rodig SJ, Tholouli E, Christie A, Valk PJ, Delwel R, Ngo V, Kutok JL, Dahlberg SE, Moreau LA, Byers RJ, Christensen JG, Vande Woude G, Licht JD, Kung AL, Staudt LM, Look AT. Autocrine activa-

tion of the MET receptor tyrosine kinase in acute myeloid leukemia. Nat Med 2012; 18: 1118-1122.
[9] Chen JH, Chen YZ, Wu Y. Establishment and characterization of highly tumorigenic leukemia cell line HL-60 transplanted through repeated passages into nude mice. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2010; 18: 350-354.
[10] Kittang AO, Hatfield K, Sand K, Reikvam H, Bruserud Q. The chemokine network in acute myelogenous leukemia: molecular mechanisms involved in leukemogenesis and therapeutic implications. Curr Top Microbiol Immunol 2010; 341: 149-172.
[11] Nishino T, Hisha H, Nishino N, Adachi M, Ikehara S. Hepatocyte growth factor as a hematopoietic regulator. Blood 1995; 85: 3093-3100.
[12] Mizuno K, Higuchi O, Ihle JN, Nakamura T. Hepatocyte growth factor stimulates growth of hematopoietic progenitor cells. Biochem Biophys Res Commun 1993; 194: 178-186.
[13] Takai K, Hara J, Matsumoto K, Hosoi G, Osugi Y, Tawa A, Okada S, Nakamura T. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood 1997; 89: 1560-1565.
[14] Matsuda-Hashii Y, Takai K, Ohta H, Fujisaki H, Tokimasa S, Osugi Y, Ozono K, Matsumoto K, Nakamura T, Hara J. Hepatocyte growth factor plays roles in the induction and autocrine maintenance of bone marrow stromal cell IL-11, SDF-1 alpha, and stem cell factor. Exp Hematol 2004; 32: 955-961.
[15] Kim JG, Sohn SK, Kim DH, Baek JH, Lee NY, Suh JS, Chae SC, Lee KS, Lee KB. Clinical implications of angiogenic factors in patients with acute or chronic leukemia: hepatocyte growth factor levels have prognostic impact, especially in patients with acute myeloid leukemia. Leuk Lymphoma 2005; 46: 885-891.
[16] Sakaguchi H, Seki S, Tsubouchi H, Daikuhara Y, Niitani Y, Kobayashi K. Ultrastructural location of human hepatocyte growth factor in human liver. Hepatology 1994; 19: 1157-1163.
[17] Goff JP, Shields DS, Petersen BE, Zajac VF, Michalopoulos GK, Greenberger JS. Synergistic effects of hepatocyte growth factor on human cord blood CD34+ progenitor cells are the result of c-met receptor expression. Stem Cells 1996; 14: 592-602.
[18] Jia Q, Ha X, Yang Z, Hui L, Yang X. Hepatocyte growth factor protects human mesangial cells against apoptosis induced by lead damage. Biol Trace Elem Res 2014; 162: 80-86.
[19] Skibinski G, Skibinska A, James K. Hepatocyte growth factor (HGF) protects c-met-expressing Burkitt's lymphoma cell lines from apoptotic death induced by DNA damaging agents. Eur J Cancer 2001; 37: 1562-1569.
[20] Date K, Matsumoto K, Shimura H, Tanaka M, Nakamura T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett 1997; 420: 1-6.
[21] Togo S, Sugiura H, Nelson A, Kobayashi T, Wang X, Kamio K, Kawasaki S, Bitterman P, Rennard SI, Liu X. Hepatic growth factor (HGF) inhibits cigarette smoke extract induced apoptosis in human bronchial epithelial cells. Exp Cell Res 2010; 316: 3501-3511.
[22] Siegfried JM, Gubish CT, Rothstein ME, Queiroz de Oliveira PE, Stabile LP. Signaling pathways involved in cyclooxygenase-2 induction by hepatocyte growth factor in non small-cell lung cancer. Mol Pharmacol 2007; 72: 769-779.
[23] Lee YH, Marquez AP, Mungunsukh O, Day RM. Hepatocyte growth factor inhibits apoptosis by the profibrotic factor angiotensin II via extracellular signal-regulated kinase 1/2 in endothelial cells and tissue explants. Mol Biol Cell 2010; 21: 4240-4250.



Supplementary Figure 1. Procedures for the construction, package, and transfection for lentiviral vector. A. HGF shRNA lentiviral vector sequencing. B. Kasumi-1-KD and HL60-KD cell observation by using fluorescence microscopy (20×). C. GFP expression of cells in each group detected by flow cytometry.