Roles of GINS2 in K562 human chronic myelogenous leukemia and NB4 acute promyelocytic leukemia cells

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Abstract. GINS2 has been found to be closely related to cell proliferation, survival and maintenance of genomic integrity in addition to the induction of polyploidy. However, the molecular mechanisms of GINS2-mediated tumor regression have not been fully elucidated. Our study showed that GINS2 interacted with PML-C (a structural domain of PML, which is a cutting product of PML-RARα) as demonstrated by yeast two-hybrid assay and co-immunoprecipitation, and PML (NLS^-) also interacted with GINS2 by co-immunoprecipitation. Following transfection with plasmids expressing GINS2 siRNA, the apoptosis and cell cycle distribution were determined by flow cytometry. The results revealed that K562 chronic myelocytic leukemia cells and NB4 acute promyelocytic leukemia cells were arrested in the G2 phase. Western blot assay indicated that the expression of cyclin A, cyclin D1 and cyclin B1 was decreased, and cyclin B1 was localized in the cytoplasm after GINS2 knockdown as demonstrated by immunofluorescence staining. The expression of the apoptosis-related protein Bcl-2 was decreased but that of Bax was increased in the cells transfected with GINS2 siRNA. The percentage of apoptotic cells was increased as detected by flow cytometry. Our findings demonstrated that GINS2 plays an important role in apoptosis and may function via the p38MAPK signaling pathway.

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Abbreviations: GINS, Japanese Go-Ichi-Ni-San meaning 5-1-2-3, for the four-related subunits of the complex Sld5, Psf1, Psf2 and Psf3; PML, promyelocytic leukemia; RARα, retinoic acid receptor α; APL, acute myelocytic leukemia; HIPK2, homeodomain-interacting protein kinase 2; CK2, casein kinase 2; NE, neutrophil elastase; K562, chronic myelocytic leukemia K562 cells; NB4, acute promyelocytic leukemia NB4 cells; MAPK, mitogen-activated protein kinases; HEK293, human embryonic kidney 293 cells; GFP, green fluorescence protein; FBS, fetal bovine serum; BSA, bovine serum albumin; NC, negative control; shRNA, short haipin RNA

Key words: GINS2, PML (NLS^-), PML-C, K562, NB4, cyclin B1, p38MAPK

Introduction

Acute promyelocytic leukemia (APL) is genetically characterized by the PML/RARα fusion gene that forms as a consequence of the 15;17 translocation (1). PML-RARα expression is required for the development of APL. However, the role of fusion protein PML-RARα as a whole is not unchangeable. For example, some researchers have found that the phosphorylation of promyelocytic leukemia (PML) by mitogen-activated protein kinase plays a key role in PML-dependent apoptosis in response to As_2O_3 exposure (2). PML can activate transcription by protecting homeodomain-interacting protein kinase 2 (HIPK2) and p300 from SCFFbx3-mediated degradation, and casein kinase 2 (CK2) regulates the PML protein levels by promoting its ubiquitin-mediated degradation which depends on direct phosphorylation at Ser517 (3,4). Meanwhile, PML is required for fas and caspase-dependent apoptosis and is involved in non-caspase-dependent apoptosis. PML plays an important role in cell growth by regulating the expression of several cyclin proteins (5). Notably, neutrophil elastase (NE) is important for PML-RARα activity in early myeloid cells, and PML-RARα was found to be rapidly and predictably cleaved by NE in an early myeloid cell line (U937 cells) generating 69- and 53-kDa products (6,7). PML-RARα cleavage products play an important role in the development of APL. Thus, to investigate the interaction among these cleavage products is critical to elucidate the molecular pathogenesis of APL. WT-PML consists of three domains: PML-C (a coiled-coil domain close to carboxyl), PML-B (a B-BOX domain close to amino) and NLS (a nuclear localization signal), which is lost in the pathogenesis of APL instead of binding to RARα, and is named PML (NLS^-).

The yeast two-hybrid assay has demonstrated the interaction between PML-C and GINS2. The plasmids of PML (NLS^-) bait-protein and PML-B exhibit transcription factor activity and toxic effects to yeast cells (8). Thus, the yeast two-hybrid cannot be used to examine whether there is

an interaction between PML (NLS^-) and GINS2. Thus, co-immunoprecipitation was employed to directly identify the interaction between PML (NLS^-) and GINS2, which compensates for the deficient of the yeast two-hybrid assay.

GINS complex subunit 2 (GINS2) is a member of the tetrameric complex termed GINS, composed of GINS1, GINS2, GINS3 and GINS4, which most likely serves as the replicative helicase, unwinding duplex DNA ahead of the moving replication fork (9-11). The GINS complex has been shown to be associated with DNA replication in humans (12-16) and DNA damage (17-19). Several recent reports suggest a role of GINS in cancer cells. For example, GINS has been suggested to be related to cell division, more precisely in chromosome segregation (20,21). However, the manners in which GINS functions in leukemia cells is not yet clear. In the present study, GINS2 expression was knocked down to observe changes in apoptosis and cell division and the signaling pathway mediating these changes.

Materials and methods

Reagents. Lipofectamine™ 2000 (Invitrogen); antibodies against Bcl-2 and Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); antibodies against cyclin B1, cyclin D1 and cyclin A, and anti-p-p38, anti-p-ERK and anti-p-JNK antibodies (Cell Signaling Technology, Inc.); antibodies against cyclin B1 (Epitomics), GINS2 and PML (both from Abcam), and SB203580 (Beyotime) were used in the present study.

Cell culture. Human embryonic kidney (HEK) 293 cells, chronic myelocytic leukemia cells (K562) and acute promyelocytic leukemia cells (NB4) were purchased from ATCC (Rockville, MD, USA). HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (both from HyClone). K562 and NB4 cells were grown in RPMI-1640 (Gibco) containing 10% FBS (HyClone), 10 μg/ml penicillin and streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Cells in passages 3-20 in logarithmic growth phase were collected for the experiments.

Yeast two-hybrid screening. The screening was performed with the interaction mating assay, a variation of the classic yeast TH screening. To obtain the bait plasmid, PML-C was amplified from genomic DNA and cloned into PGBKT7 (our laboratory). The AH109 yeasts with stable expression of BD-PML-C and the Y187 yeasts transformed with human leukemia cell cDNA library (our laboratory) were added to 2X YPDA/Kan liquid medium for hybridization for 20 h. One drop of solution was collected for observation under a phase contrast microscope. Once zygotes were present, hybridization was carried out for another 4 h, and the whole medium was added to SD/-Trp/-Leu/-His/-Ade (QDO) nutrient deficiency medium. Screening by nutrient deficiency and galactosidase was conducted thrice, and the blue colonies were collected as positive colonies which were used for extraction of plasmids of the AD library. The plasmids of the AD library and bait plasmids pGBKT7-PML-C were used to co-transfect AH109 yeasts followed by inverse incubation on a plate pre-coated with SD/-Trp/-Leu/-His/-Ade/X-α-gal at 30˚C for 7-10 days. The bacterial growth and presence of blueness were observed. The positive colonies were collected for sequencing and analysis of biological information.

Plasmids and constructs. Fusion protein eukaryotic expression vectors pCMV-HA-PML-C/pCMV-HA-PML and pCMV-Myc-GINS2 were constructed and then co-transfected into HEK293 cells. The plasmids of 5 groups of GINS2 short hairpin RNA (shRNA) were constructed and contained a reporter gene such as green fluorescence protein (GFP) and the neomycin-resistant gene, respectively. These vectors were designed for the cloning and expression of shRNA in mammalian cells under the control of the human U6 promoter.

Reverse transcription-polymerase chain reaction and western blot assay. For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), the total cellular RNA was isolated using TRIzol reagent (Invitrogen). Total RNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio, Inc.). The cDNA was diluted 1:10, and SYBR Premix Ex Taq™ analysis was performed with a Bio-Rad CFX Manager, using primers for GINS2 (Invitrogen): forward, 5'-AATGCCCAGC CCTTACTA-3' and reverse, 5'-GGATTTCGTCTGCCTTCG-3'. Following qRT-PCR, the shRNA possessing the highest interfering efficacy was used for further experiments. The relative expression of messenger RNA (mRNA) was determined using β-actin as an endogenous control. Primers for β-actin (Invitrogen) were; forward, 5'-ACGAGACCACCTTCAACTCC ATC-3' and reverse, 5'-TAGAAGCATTTGCGGTGGACGA-3'. Each experiment was performed twice with triplicate samples, and data were averaged for further analysis.

For the western blot assay, aliquots of total cell lysates were separated on sodium dodecyl sulfate polyacrylamide gels and were then transferred onto PVDF membranes (Beyotime). The membranes were blocked in 5% skim milk and then probed with the corresponding antibody overnight at 4˚C (GINS2, 1:1,000; Abcam) and the antibody against β-actin (1:4,000; Cell Signaling Technology, Inc.). The secondary antibody was goat-anti-mouse IgG (1:3,000; Santa Cruz Biotechnology, Inc.). Visualization was carried out with the ECL kit.

Immunofluorescence staining. Immunofluorescence staining for cyclin B1 was performed using standard procedures. Cells on coverslips were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min, and the background was excluded by blocking with 5% bovine serum albumin (BSA) in PBS for 1 h before incubation with the primary and secondary antibodies. Primary antibody rabbit against cyclin B1 (1:50; rabbit anti-cyclin B1; Epitomics) was diluted in 0.5% Triton X-100, and incubation with the primary antibody was performed for 90 min at 37˚C. The secondary antibody was TRITC-conjugated goat anti-rabbit IgG (1:100; Santa Cruz Biotechnology, Inc.) and diluted in 0.5% Triton X-100. Incubation with the secondary antibody was performed for 60 min at room temperature. Cells were then stained with DAPI for 3 min. Rinsing with PBS was carried out at each step.

For immunofluorescence staining of HA-PML (NLS^-) and Myc-GINS2, HEK293 cells were cultured on coverslip

in a 24-well plate and stained using the same procedures as described above. Primary antibodies against HA-PML-C (1:100, rabbit anti-HA polyclone antibody) and Myc-GINS2 (1:100, mouse anti-Myc monoclonal antibody) (both from Clontech) were diluted in 0.5% Triton X-100, and incubation with the primary antibody was performed for 90 min at 37˚C. The secondary antibody was TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (1:100; Santa Cruz Biotechnology, Inc.). Cells were then stained with DAPI for 3 min. Rinsing was carried out with PBS at each step.

Protein extraction. Cells were lysed in RIPA containing 1% PMSF on ice for 30 min and shaking was conducted once every 5 min. After centrifugation at 12,000 x g for 30 min at 4˚C, the supernatant was collected.

Transfection, immunoprecipitation (IP) and western blot assay. To evaluate the interaction between PML-C and GINS2 by co-immunoprecipitation, pCMV-HA-PML-C and pCMV-Myc-GINS2 were co-transfected with Lipofectamine™ 2000 into HEK 293 cells. pCMV-HA-PML and pCMV-Myc-GINS2 were independently transfected into HEK 293 cells as the control groups. Protein extracts, aliquots (500 μl) of the platelet suspension (10^9 cells/ml) were immunoprecipitated by incubation with 2 μg/ml rabbit anti-HA antibody (Clontech) overnight at 4˚C and 40 μl of protein A-agarose. Immunoprecipitates were resolved in 12% SDS-PAGE. Separated proteins were electrophoretically transferred onto PVDF membranes for subsequent incubation with mouse anti-myc antibody (1:1,000) overnight at 4˚C. The primary antibody was detected by incubation with horseradish peroxide-conjugated goat anti-mouse IgG antibody (1:3,000) for 1 h and exposed to ECL solution. Consistently, the interaction between PML (NLS^-) and GINS2 was also detected by co-immunoprecipitation using the same protocol.

Four groups of plasmids of GINS2 shRNA were respectively named 1, 2, 3, 4 and then transfected independently into K562 and NB4 cells. siRNA sequences are as follows: siGINS2: 1, forward, 5'-CACCGCTGGCGATTAACCTGAA ACATTCAAGAGATGTTTCAGGTTAATCGCCAGCTTT TTT-3' and reverse, 5'-GATCCAAAAAAGCTGGCGATTAA CCTGAAACATCTCTTGAATGTTTCAGGTTAATCGCC AGC-3'; 2, forward, 5'-CACCGGATCATGAACGAAAGGA AGATTCAAGAGATCTTCCTTTCGTTCATGATCCTTT TTT-3' and reverse, 5'-GATCCAAAAAAGGATCATGAAC GAAAGGAAGATCTCTTGAATCTTCCTTTCGTTCATG ATCC-3'; 3, forward, 5'-CACCGCCCTTACTACATGGAAC TTATTCAAGAGATAAGTTCCATGTAGTAAGGGCTTTT TTG-3' and reverse, 5'-GATCCAAAAAAGCCCTTACTAC ATGGAACTTATCTCTTGAATAAGTTCCATGTAGTAA GGGC-3'; 4, forward, 5'-CACCGAGCGCTCAACCACTAG TACAATTCAAGAGATTGTACATGTGGTTGAGCG CTTTTTTTG-3' and reverse, 5'-GATCCAAAAAAAGCGC TCAACCACATGTACAATCTCTTGAATTGTACATGTGG TTGAGCGCTC-3'; negative control, forward, 5'-CACCGTT CTCCGAACGTGTCACGTCAAGAGATTACGTGACACG TTCGGAGAATTTTTTG-3' and reverse, 5'-GATCCAAAA AATTCTCCGAACGTGTCACGTAATCTCTTGACGTGA CACGTTCGGAGAA-3'.

Cells with low transfection efficiency were treated with G418 until most of the non-transfected cells were removed to prepare a population of cells stably expressing the siRNA. The GINS2 siRNA is essential for survival, and thus the cells transfected with the plasmid target which effectively reduced the expression of the target gene may die. Thus, a less stringent antibiotic selection was performed for 21 days. The surviving cells were maintained and assessed for target gene expression. The interfering efficiency was detected by immunoblot assay and qRT-PCR.

Western blot assay was employed to measure the protein expression of GINS2, cyclin B1, cyclin D1, cyclin A, p-p38, t-p38, p-ERK, t-ERK, p-JNK and β-actin according to the above mentioned procedures.

Cell cycle distribution by flow cytometry. To investigate the effect of GINS2 siRNA on cell cycle distribution, PI staining assay was performed. K562 cells ($8x10^5$ cells/ml) were seeded in 6-well plates and transfected with the plasmid. After screening, K562 cells were collected by centrifugation, followed by washing, fixation and PI staining. The cell cycle distribution was examined by flow cytometry (Becton-Dickinson). Data were analyzed using the Modfit program (Becton-Dickinson).

Detection of apoptosis by Annexin V/PI staining. K562 cells were transfected with GINS2 siRNA. Cells were harvested, washed and resuspended in PBS. Apoptotic cells were identified by staining with Annexin V and PI, using the Annexin V/PI Apoptosis Detection kit (KeyGene) according to the manufacturer's instructions. Flow cytometry was performed immediately after supravital staining. The percentages of early apoptotic and late apoptotic/necrotic cells were determined, respectively, as the percentage of Annexin V^+/PI^- or Annexin V^+/PI^+ cells. Data acquisition and analysis were performed using a Becton-Dickinson flow cytometer with CellQuest software.

Statistical analysis. Data are presented as the means ± SD, and Student's t-test was used for comparisons among the different groups. A value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Yeast two-hybrid assay and co-immunoprecipitation for evaluating the interaction between PML-C and GINS2. Our group had applied yeast two-hybrid assay to primarily indicate the interaction between PML-C and GINS2. To further confirm this interaction, the plasmid of the bait protein PML-C and GINS2 protein were co-transformed into AH109 yeasts which were inversely grown in SD/-Trp/-Leu/-His/-Ade (QDO) at 30˚C for 7-10 days. The blue bacteria were continuously observed. Sequencing showed that transformation was successful. These positive plasmids and blank pGBKT7 were then used to co-transform AH109 yeasts as a control, which were also inversely grown in SD/-Trp/-Leu/-His/-Ade (QDO) at 30˚C for 7-10 days; while the blue bacteria were absent (Fig. 1A). Whether PML-C interacts with GINS2 in mammalian cells is still unclear.

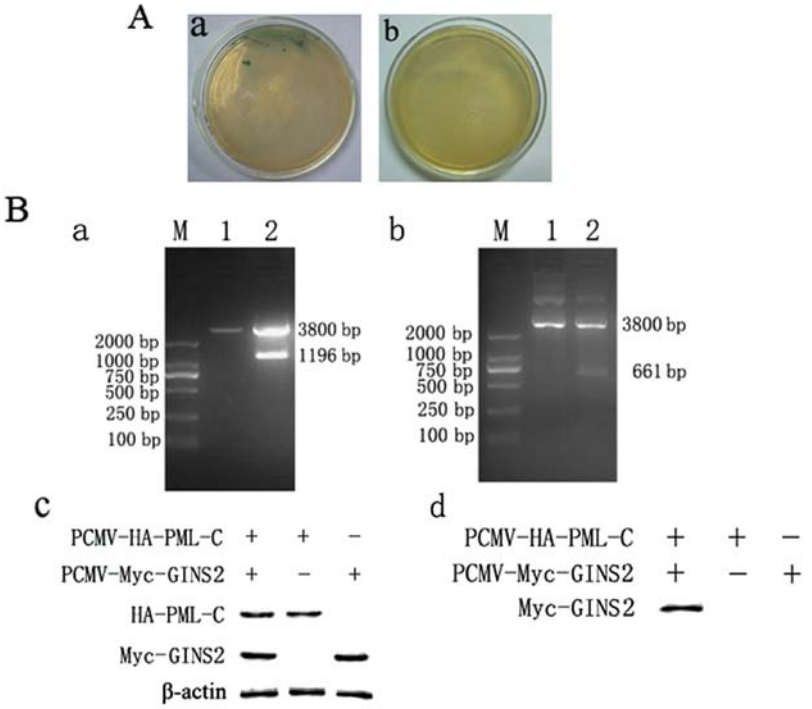

Figure 1. Interaction between PML-C and GINS2 as determined by the yeast two-hybrid assay and co-immunoprecipitation. (A) (a) Positive colonies in the QDO plate. (b) Control group. (B) (a) Restriction enzyme analysis of pCMV-HA-PML-C with *Sal*I and *Eco*RI. (b) Restriction enzyme analysis of pCMV-Myc-GINS2. (c) Expression of PML-C and/or GINS2 via western blot assay in the cell lysate. (d) Interaction between PML-C and GINS2 via co-immunoprecipitation.

We then constructed eukaryotic expression vectors, pCMV-HA-PML-C and pCMV-Myc-GINS2, both of which were transfected into HEK293 cells. The HEK293 cells which were transfected with one of the vectors served as a negative control. Protein bands were present in cells undergoing co-transfection but absent in cells transfected with one of the vectors. The expression of the target protein was also found in the cell lysate. These findings together with those of the co-immunoprecipitation and western blot assay demonstrated the interaction between PML-C and GINS2 (Fig. 1B).

Immunofluorescence and coimmunoprecipitation evaluating the interaction between PML (NLS$^-$) and GINS2. The plasmids of PML (NLS$^-$) and PML-B were toxic to AH109 yeasts and underwent self-activation, and thus these plasmids were not used in the yeast two-hybrid screening. In the present study, we aimed to directly investigate the interaction between PML (NLS$^-$) and GINS2 by immunofluorescence staining and co-immunoprecipitation. The HEK293 cells undergoing co-transfection were then subjected to immunofluorescence staining with the rabbit anti-HA polyclonal and mouse anti-myc monoclonal antibodies. Results showed that HA-PML (NLS$^-$) and Myc-GINS2 were expressed in both the nucleus and cytoplasm (Fig. 2A). The vectors pCMV-HA-PML (NLS$^-$) and pCMV-Myc-GINS2 were used to co-transfect HEK293 cells, and cells transfected with one of the vectors served as a negative control. Results from the co-immunoprecipitation indicated an interaction between PML (NLS$^-$) and GINS2 (Fig. 2B), which provides evidence for the interaction between PML (NLS$^-$) and GINS2, which may serve as a supplement to the results from the yeast two-hybrid screening. This method also breaks through the limitations of yeast two-hybrid screening and provides a basis for the investigation of the role of GINS2 in leukemia cells. In order to validate the endogenous interaction between PML (NLS$^-$) and GINS2 in leukemia cells, the PML (NLS$^-$) protein was immunoprecipitated by the anti-PML polyclonal antibody, and GINS2 protein was measured by western blotting with the anti-GINS2 polyclonal antibody from the immunoprecipitared complex. Results from co-immunoprecipitation indicated an interaction between PML and GINS2 (Fig. 2B).

Validation of GINS2 interference effect in K562 and NB4 cells. K562 and NB4 cells were independently transfected with different GINS2 plasmids. Forty-eight hours after transfection, a few green fluorescent proteins were found under inverted fluorescence microscope. G418 was then used to select positive clones. Three weeks after G418 screening, polyclonal cell lines were collected and the expression level of green fluorescent GINS2 was ~70% in cells which underwent transfection and 90% in the NC group (only one interference group and NC were presented herein) (Fig. 3A). Four weeks after screening, the expression level of fluorescence protein was markedly reduced, but the number of apoptotic cells increased. These findings were consistent with the finding that GINS2 silencing may increase the number of dead polyploid cells and inhibit cell growth and activity. However, the cell growth remained unchanged in the NC group. Thus, the polyclonal cell lines at 3 weeks were collected for further study. In addition, fluorescent quantitative PCR was employed to detect the GINS2 expression, and the results demonstrated the reduction of interference efficacy. The interference efficacy in K562

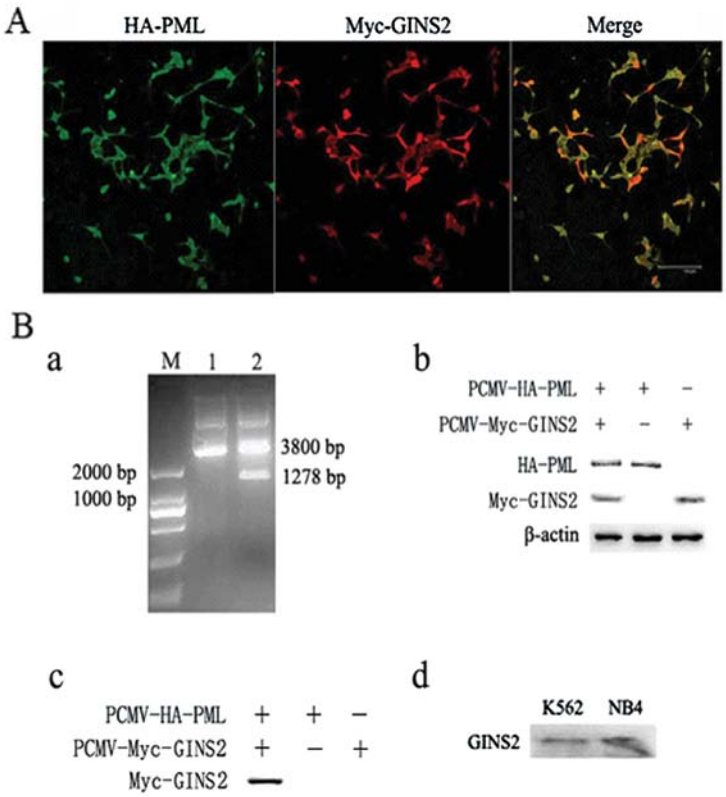

Figure 2. Interaction between PML (NLS$^-$) and GINS2 by co-immunoprecipitation. (A) HA-PML (NLS$^-$), analysis of localization of HA (NLS$^-$)-PML with anti-HA and anti-rabbit IgG antibodies (green). Myc-GINS2, analysis of the localization of Myc-GINS2 with anti-Myc and anti-mouse IgG antibodies (red). (B) Interaction between PML (NLS$^-$) and GINS2 via co-immunoprecipitation. (a) Restriction enzyme analysis of pCMV-HA-PML (NLS$^-$) with *Sal*I and *Eco*RI. (b) Expression of PML (NLS$^-$) and/or GINS2 via western blot assay in the cell lysate. (c) Interaction between PML (NLS$^-$) and GINS2 via co-immunoprecipitation. (d) Interaction between endogenous PML and endogenous GINS2 in K562 and NB4 cells via co-immunoprecipitation.

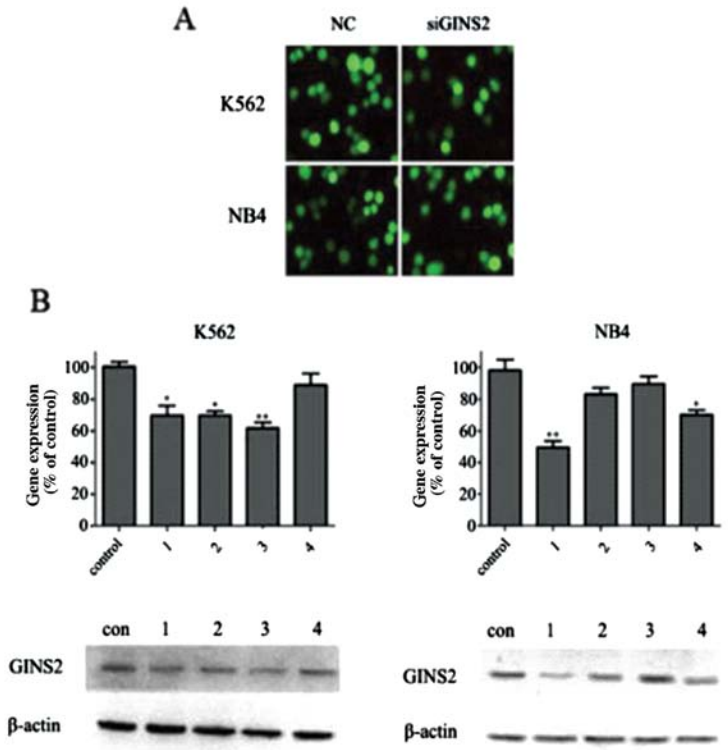

Figure 3. Effect of GINS2 gene silencing on K562 and NB4 cells. (A) Screening of clones stably expressing siGINS2 by using G418. (B) In cells with the highest silencing efficiency, GINS2 mRNA expression was reduced by 38 and 50% in the K562 and NB4 cells, respectively. Western blot assay indicated that the GINS2 protein expression was decreased significantly in groups 3 and 1 in the K562 and NB4 cells, respectively. All data are expressed as means ± SEM from three independent experiments. *P<0.05 and **P<0.01 vs. control.

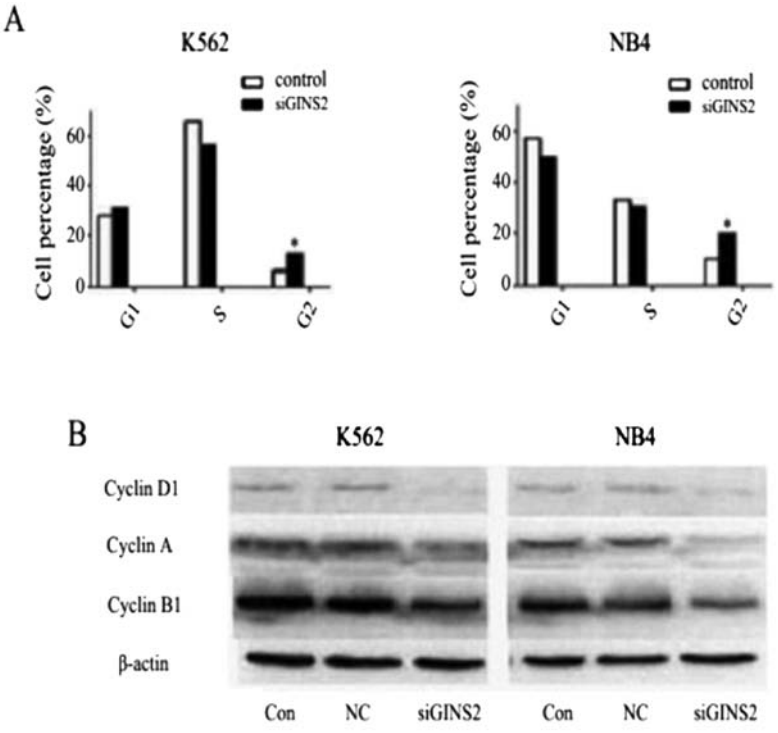

Figure 4. Changes in the cell cycle distribution and cell cycle-related proteins in K562 and NB4 cells following GINS2 knockdown. (A) The cell cycle was arrested in the G2 phase in K562 and NB4 cells as demonstrated by flow cytometry. (B) Expression levels of cylin D1, cyclin A and cyclin B1 were decreased when compared with levels in the control and NC group as demonstrated by western blot assay.

cells was 29, 30, 38 and 5%, and that in NB4 cells was 50, 17, 9 and 28% (Fig. 3B). Western blot assay was used to measure the GINS2 protein expression and results were consistent with those of PCR (Fig. 3B). The K562 and NB4 cells with the best interference efficacy were employed for subsequent experiments.

Changes in cell cycle distribution and expression of cell cycle-related proteins in K562 and NB4 cells after GINS2 knockdown. To confirm the role of GINS2 in leukemia, K562 and NB4 cells were employed, and changes in both types of cells were observed after GINS2 gene silencing. Flow cytometry was carried out to detect cell cycle distribution. Results showed that the proportion of both types of cells in G2 phase was markedly increased ($P<0.05$). The proportion of K562 cells in G1 phase was increased while that of NB4 cells in G1 phase was reduced but a significant difference was absent (Fig. 4A). To further explore the effect of GINS2 silencing on the cell cycle, western blot assay was employed to detect the protein expressions of cyclin B1, cyclin D1 and cyclin A. Results showed that the protein expression of the three proteins was markedly reduced when compared with the control and the NC group (Fig. 4B). Of note, cyclin B1 aggregates in the cytoplasm and is a regulator of G2 phase. Thus, immunofluorescence staining was performed to locate cyclin B1 in the K562 and NB4 cells using rabbit anti-cyclin B1 monoclonal antibody. Results showed that cyclin B1 exhibited high expression in the cytoplasm and nuclei in the control group but high expression was only found in the cytoplasm following GINS2 silencing (Fig. 5). These findings were consistent with arrest in the G2 phase in both types of cells. These results demonstrated that GINS2 gene silencing inhibits the transport of cyclin B1 from the cytoplasm into the nucleus resulting in aggregation of cyclin B1 in the cytoplasm. This may be the cause of cell cycle arrest in the G2 phase.

Detection of apoptotic cells and proteins. The dysregulation of the cell cycle has been reported to correlate with apoptosis induction. Based on the G2 peak in the cell cycle, apoptosis was analyzed by Annexin V/PI staining. We further characterized the GINS2 siRNA-induced apoptosis by Annexin V/PI staining. The proportions of apoptotic cells in the GINS2 siRNA groups were 26.99 and 32.54% in the K562 and NB4 cells, respectively, and the proportions in the NC group were 7.85 and 9.36% in K562 and NB4 cells, respectively; that in the control group was 6.53 and 7.07% in K562 and NB4 cells, respectively. A statistically significant difference was noted between the GINS2 siRNA groups and the control group ($P<0.001$) but no marked difference was noted between the NC group and the control group (Fig. 6A). In attempt to further clarify the mechanisms underlying the inhibitory effect of GINS2 siRNA on leukemia cells, the levels of Bcl-2 family members were detected by western blot assay. A significant decrease in Bcl-2 was noted while an increase in Bax was noted in the K562 cells. Similar findings were observed in NB4 cells (Fig. 6B). These results suggest that GINS2 siRNA induces apoptosis of K562 and NB4 cells by upregulating Bax expression and downregulating Bcl-2 expression.

p38MAPK signaling transduction pathway. Some reports suggest that the MAPK signaling pathway mediates cell

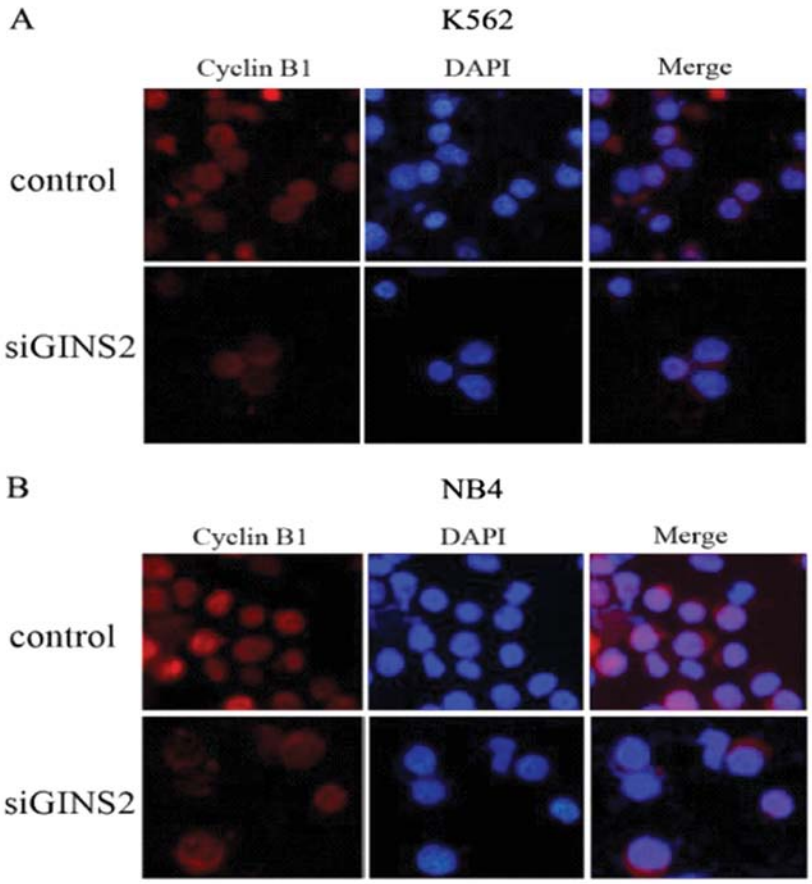

Figure 5. Localization of cyclin B1 in K562 and NB4 cells. Cyclin B1 was expressed in the nucleus and cytoplasm of the control cells, whereas cyclin B1 was expressed weakly in the nucleus but strongly in the cytoplasm after GINS2 knockdown (Red, cyclin B1; blue, nucleus).

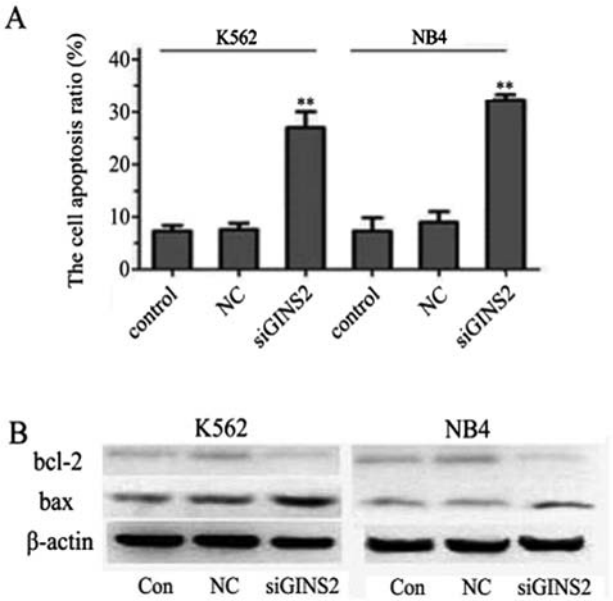

Figure 6. (A) The percentage of apoptotic cells increased as demonstrated by flow cytometry in the K562 and NB4 cells. (B) Western blot assay was employed to detect the expression of Bcl-2 and Bax in K562 and NB4 cells. A significant decrease in Bcl-2 expression was observed while an increase in Bax expression was noted when compared with expression levels in the NC and control groups. All data are expressed as means ± SEM from three independent experiments. $^{**}P<0.01$ vs. control.

cycle arrest. To study the relationship between the MAPK signaling pathway and cell cycle arrest, the activation of MAP kinases was detected by western blot assay using a specific anti-phospho-antibody. The K562 cells expressing GINS2

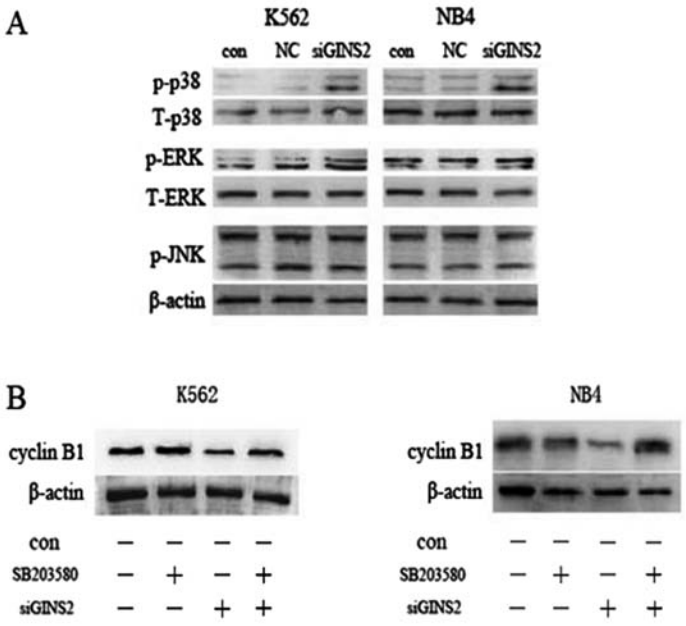

Figure 7. p38MAPK signaling pathway mediates cell cycle arrest. (A) Western blot assay was employed to detect the expression of p-p38, T-p38, p-ERK, T-ERK, p-JNK and β-actin in K562 and NB4 cells. The expression of p-p38 was increased in both K562 and NB4 cells, while that of phosphorylated JNK and ERK expression remained unchanged in the K562 and NB4 cells. (B) Western blot assay was conducted to detect the expression of cyclin B1 and β-actin in the K562 and NB4 cells. Western blot assay indicated that SB203580 attenuated the decrease in cyclin B1 expression in the GINS2 siRNA groups, but had no significant effect on cyclin B1 expression in the control group.

siRNA had increased expression of phosphorylated p38, and a slight increase in phosphorylated ERKl/2 expression, while phosphorylated JNK expression was not affected. Similarly, the NB4 cells expressing GINS2 siRNA had increased expression of phosphorylated p38, while the expression of phosphorylated ERKl/2 and phosphorylated JNK remained unchanged (Fig. 7A).

To further investigate whether p38MAP kinase activation is associated with GINS2 siRNA-induced G2 phase arrest, specific p38MAPK inhibitor, SB203580 (10 nM, 1 h), was used to inhibit p38. Western blot assay indicated that SB203580 attenuated the decrease in cyclin B1 expression in the GINS2 siRNA groups, but had no significant effect on cyclin B1 expression in the control group (Fig. 7B). Moreover, SB203580 had no evident influence on cell morphology and growth. These results suggest that GINS2 knockdown influences the cell cycle through activation of p38MAPK.

Discussion

Extensive research has been conducted aiming to discover novel and effective strategies for the treatment of leukemia. At present, the therapeutic strategies for leukemia mainly include traditional chemotherapy and bone marrow transplantation. However, chemotherapy has poor specificity and its side effects often compromise the therapeutic efficacy of chemotherapy. In addition, donors are insufficient, which significantly limits the wide application of bone marrow transplantation. Thus, increasing attention has been given to find new chemopreventive and key role targets and develop novel methods for molecular-targeted therapy. In the present study, our results demonstrated an interaction between PML-C and GINS2 by the yeast two-hybrid assay and co-immunoprecipitation, and co-immunoprecipitation was employed to identify the interaction between PML (NLS^-) and GINS2. It is well known that the yeast two-hybrid assay requires bait-protein without transcription factor activity and toxic effects to yeast cells, yet the plasmid of PML (NLS^-) bait protein itself has transcription factor activity and toxic effects to yeast cells (8). Consequently, through the interaction between PML-C and GINS2 by the yeast two-hybrid, we further investigated the interaction between PML (NLS^-) and GINS2 by co-immunoprecipitation. This suggests that GINS2 may be a therapeutic target for leukemia treatment.

Several previous reports have confirmed the roles of GINS. For example, GINS components were found to be over-expressed in aggressive melanoma (22) and upregulation of GINS1 promoted the growth of breast cancer cells (23). It has been reported that DNA replication-associated proteins have diverse functions in different cells; however, the role of its components in mammalian cells is not yet clear. In the present study, GINS2 knockdown was found to result in growth inhibition and induction of apoptosis in NB4 and K562 cells by suppressing G2 phase progression, indicating that GINS2 may affect, in addition to DNA replication initiation essential for S-phase progression (in the GINS complex), cell division and probably chromosome segregation in human leukemic cells. Consistent with other studies, our results demonstrated that GINS2 exerts obvious effects on cell survival. Cell cycle analysis revealed an increased proportion of cells in the G2 phase following GINS2 silencing. To further clarify the effect of GINS2 knockdown on the cell cycle, the expression of cyclin A, cyclin D1 and cyclin B1 was measured. The results showed that the expression of these proteins was significantly decreased. Moreover, it is well acknowledged that the complex of cdc2 and cyclin B1 [M-phase-promoting

factor (MPF)] is a key regulator of the G2/M cell cycle transition (24,25). Since cyclin Bl is known to localize in the cytoplasm in G2 phase and to be transported into the nucleus during the M phase (26), we examined the subcellular localization of cyclin B1 after GINS2 knockdown. In control cells, cyclin Bl was expressed in both the cytoplasm and nucleus. However, cyclin B1 accumulated in the cytoplasm after GINS2 knockdown. These findings suggest that the nuclear transport of cyclin B1 is inhibited by GINS2 knockdown, which further leads to cell cycle arrest in the G2 phase.

Some studies have suggested that the MAPK signaling pathway mediates cell cycle arrest. For example, ERK kinase is required for G2/M phase arrest induced by DNA damage, and p38 is associated with the G2/M checkpoint (27-30). Therefore, the activation of MAP kinase was detected by western blot assay. The expression of phosphorylated p38 was increased after GINS2 knockdown in both K562 and NB4 cells, while that of phosphorylated ERK and phosphorylated JNK remained unchanged in the K562 and NB4 cells.

Taken together, our results demonstrated an interaction between PML and GINS2 by co-immunoprecipitation which is a breakthrough and widens the scope of application of the yeast two-hybrid assay. In addition, GINS2 siRNA inhibits the cell survival and apoptosis by activating p38MAP kinase and altering the expression of Bax/Bcl-2 in K562 and NB4 cells. Our findings suggest that GINS2 siRNA can exert potent inhibition on leukemia cell growth by inducing apoptosis. Thus, GINS2 siRNA may be developed as a promising chemotherapeutic method for the treatment of leukemia.

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