RESEARCH ARTICLE

Growth and Differentiation Effects of Homer3 on a Leukemia Cell Line

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Abstract

The Homer protein family, also known as the family of cytoplasmic scaffolding proteins, which include three subtypes (Homer1, Homer2, Homer3). Homer3 can regulate transcription and play a very important role in the differentiation and development for some tissues (e.g. muscle and nervous systems). The current studies showed that Homer3 abnormal expression changes in acute myeloid leukemia (AML). Forced expression of Homer3 in transfected K562 cells inhibited proliferation, influenced the cell cycle profile, affected apoptosis induced by As_2O_3 through inhibition of Bcl2 expression, and also promoted cell differentiation induced by 12-O-tetra decanoylphorbol-acetate (TPA). These results showed that Homer3 is a novel gene which plays a certain role in the occurrence and development of AML.

Keywords: Homer3 - leukemia cell line - cell proliferation - apoptosis - differentiation

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Introduction

The Homer protein family, also known as the family of cytoplasmic scaffolding proteins, which include three subtypes (Homer1, Homer2, Homer3) (Brakeman et al., 1997; Xiao et al., 1998). Homer3 can regulate transcription and play a very important role in the differentiation and development for some tissues (e.g. muscle and nervous systems) (Ishiguro et al., 2004; Shiraishi et al., 2004; Bortoloso et al., 2006). Current studies showed that Homer3 were abnormal expressed in blasts in AML (Stirewalt et al., 2008) and myelodysplastic syndrome (MDS) (data not shown). And decreased expression for Homer3 was associated with a poor outcome in AML (Valk et al., 2004). Our study will try to reveal the function of Homer3 in the leukemia cell line.

Materials and Methods

RNA isolation and RT-qPCR (real time quantitative PCR)

Total mRNAs were extracted from leukemia cell lines (K562, NB4, HL-60, SHI-1) using Trizol (Invitrogen, USA) as described by the manufacturer's instructions. mRNA was quantified by BioPhotometer plus (eppendorf, Germany) at the wavelength of 260 nm. Ensured the concentration of RNA was 0.5 μg/μl, The 2 μg total RNA were reverse-transcribed with an MMLV reverse transcriptase, and the resulted cDNA was subjected for evaluating Homer3 expression, qRT-PCR was performed with an ABI 7500 Real Time PCR System (Applied Biosystems, USA). The PCR reaction conditions were as follows: initially 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. All samples were normalized to the internal control (ABL) and the Homer3 expression level was calculated using the $2^{-\Delta\Delta CT}$ method.

Cell culture and transfection

The K562 leukemia cell line was cultured in IMDM containing 20% FCS. Plasmid of pcDNA-Homer3 was transfected into K562 cells and stable transfectants were selected for their G418 resistance (800 mg/ml). Empty vector was also transfected as a control.

Western blot analysis

Over-expressed Homer3 clones were detected by Western blot analysis. The stable cells were lysed using the IP (immunoprecipitation) lysate in the presence of PMSF proteinase inhibitor (Beyotime, China). After denatured in 5×Laemmli sample buffer at 100°C for 5 min, total proteins were electrophoresed on 15% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk in PBST 2 h before incubation with the primary antibody. The primary antibody used mouse polyclonal to Homer3 (Abcam, USA). GAPDH as a loading control. After incubated with secondary antibody

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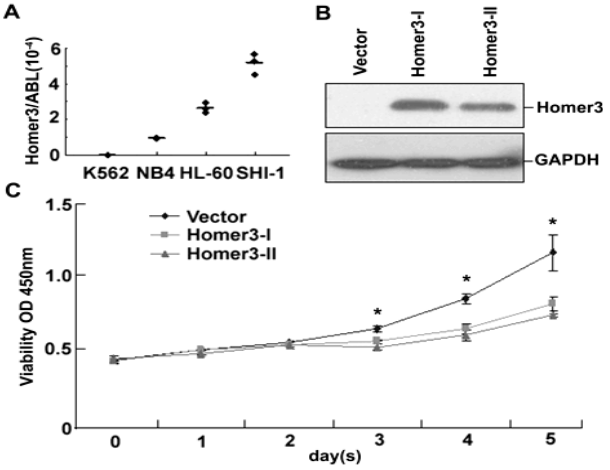

Figure 1. Homer3 Transformed Cells Inhibited Cellular Proliferation. (A). The expression of Homer3 in several leukemia cell lines (K562, NB4, HL-60, SHI-1) detected by RT-qPCR. Data were normalized against ABL expression. (B).Two stable clones expressing Homer3 gene and control clone of K562 cells were detected using Western blot analysis with anti-Homer3 and anti-GAPDH antibodies. (C). Viability of two stable clones expressing Homer3 and control clone of K562 cells were detected by CCK-8. *P<0.05, significant difference compared with Vector

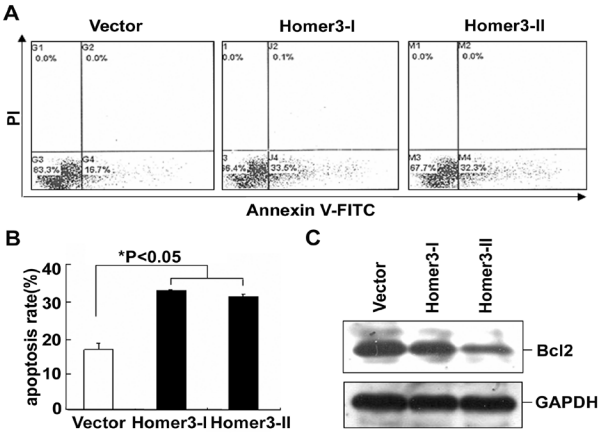

Figure 2. Homer3 Enhanced Apoptosis Induced by As_2O_3. (A). The cells were double stained with FITC-conjugated annexin V and PI, and apoptosis was detected by flow cytometry. Annexin-V binds to cells expressing phosphotidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cell (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin-V) and necrotic cells (stained with both Annexin-V and PI). (B). Results of three experiments showing the rate of apoptosis cells induced by As_2O_3. (C). Western blot showing markedly reduced Bcl2 protein induced by As_2O_3 in two stable K562/Homer3 clones compared to Vector. *P<0.05, significant difference compared with Vector

for 1 h at room temperature, the signals were visualized with enhanced chemiluminescence (ECL) (Beyotime, China).

Cell proliferation in liquid culture assays

The cells were counted using Cell Counting Kit-8 (CCK-8) beginning in 96-well plates at 2×10^3 cells/well; 20 μl CCK-8 solution was added to each well, the final volume of 200 μl and the IMDM containing 20% FCS, after 2 h of incubation with 37°C and 5% CO_2, the optical absorbance at 450 nm was measured. Each experiment was performed three times.

Flow cytometric analysis for cell cycles

After culture without FCS for 3-6 h, 20% FCS were added into the medium followed by continuous culture for 24 h. After harvested and washed three times by cold 1×PBS, the cells were then re-suspended with 20 μg/ml PI, 500 μg/ml RNase and 0.03% NP-40 in wash buffer, and analyzed by flow cytometry (FACScan) (Becton Dickinson, Germany)

Apoptosis assessment by annexin-V staining

Harvest the cells after As_2O_3 induction for 3 h and wash in cold PBS, re-centrifuge the washed cells, discard the supernatant and resuspend the cells in 100 μl 1× annexin binding buffer (~1×10^6 cells/ml) and 5 μl Alexa Fluor 488 annexin V (Invitrogen, USA) and 1 μl 100 μg/ml PI working solution. Following incubating the cells at room temperature for 15 min, cells were analyzed by flow cytometry as soon as possible. Annexin-V binds to cells which express phosphotidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those that have a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin-V) and necrotic cells (stained with both annexin-V and PI).

Flow cytometric analysis for cell differentiation

After culture without FCS for 3-6 h, 20 nmol 12-O-tetra decanoylphorbol-acetate (TPA) (Sigma, USA) and 20% FCS were added into the medium followed by continuous culture for 24 h, then harvest the cells and washed twice with ice-cold PBS before incubation at 4°C 30 min in dark conditions with a monoclonal mouse anti-human CD15-PE antibody, the PE-conjugated anti-mouse-IgG as a control tube.

Statistical analysis

Each experiment was performed three times; data were presented as mean ± standard deviation (SD), where applicable, and differences were evaluated using Student's t-tests. P values < 0.05 were considered statistically significant.

Results

Homer3 can inhibit cellular proliferation of K562 cell

qRT-PCR was used to detect the expression of Homer3 in several cell lines. We found that Homer3 mRNA expression was significantly lower in K562 cell line than in other cell lines (NB4, HL-60, SHI-1) (Figure 1A). For low expressed of Homer3 gene, K562 cell line was selected for our study finally. K562 cell line, also known as acute erythroleukemia (M6) cell line, was isolated from patients with chronic myelocytic leukemia (CML) at blast phase. To examine the effect of Homer3 on cell proliferation, the transfected K562 cell line with Homer3 (K562/Homer3) and control clone (Vector) were selected

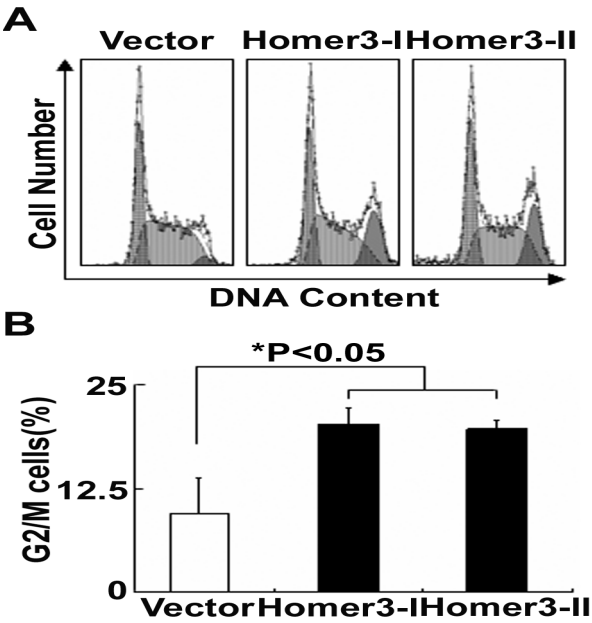

Figure 3. Homer3 Influenced Cell Cycles. (A). Cell cycles were analyzed by flow cytomety. (B). Results of three experiments showing percentage of G2/M cells. *P<0.05, significant difference compared with Vector

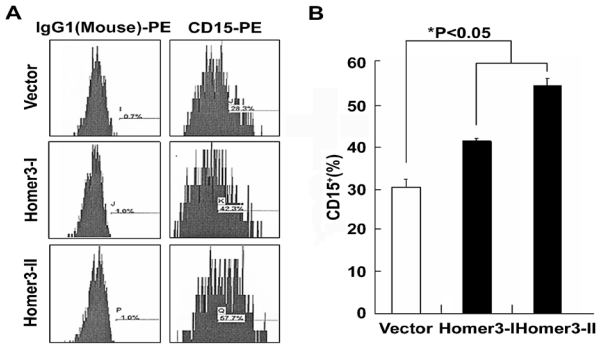

Figure 4. Homer3 Promoted the Differentiation Induced by TPA. (A). CD15-PE and IgG1 (Mouse)-PE were analyzed by flow cytometry. (B). Results of three experiments showing $CD15^+$ cells induced by TPA. *P<0.05, significant difference compared with Vector

by G418. Finally, identification by Western blot, two stable clones which high expressed of Homer3 gene and one control clone were isolated (Figure 1B). The CCK-8 assay clearly showed that Homer3 markedly inhibited the viability of K562 cells (Figure 1C).

Homer3 enhanced apoptosis of K562 cells induced by As_2O_3

In order to define the difference of As_2O_3-induced apoptosis between K562/Homer3 and Vector, the cells were incubated in the presence of As_2O_3 (0.8 μmol) at 37°C for 3 h. The cells were stained by annexin-V and analyzed by flow cytometry. Over thirty-three percent of the cells in the stable transfected K562 clones over-expressed Homer3 were positive for annexin-V staining while very few annexin-V positive stained cells were detected in control clone (Figure 2A).The result is significant (P<0.05) (Figure 2B).

To elucidate the relevance of the Homer3 gene to the apoptosis pathway, we analyzed the protein levels of both Bcl2 and Bax in cells exposed to As_2O_3. In stable K562 clones over-expressed Homer3 the protein Bcl2 was markedly reduced compared to the control clones (P<0.05) (Figure 2C), while no difference was found in the Bax protein level between these two groups (data not shown).

Homer3 influenced cell cycle

The cell cycle distribution was analysised by flow cytometry. The results showed that there were a markedly higher number of the K562/Homer3 clone cells in G2/M phase than Vector (P<0.05) (Figure 3B).

Homer3 promoted cell differentiation distribution upon TPA induction

CD15 is a surface molecule of mature granulocyte and monocyt. The cell differentiation degree analysis by flow cytometry and the cells treated by TPA, results showed a significantly higher proportion of the K562/Homer3 cells than Vector (Figure 4B).

Discussion

Acute myeloid leukemia (AML), a deadly form of hematopoietic malignancies, is a group of heterogeneous diseases with considerable diversity in terms of clinical behavior and prognosis, it is the most common malignant cancer in children and young people (Weltermann et al., 2004; Pasqualucci et al., 2006; Falini et al., 2007; Mills et al., 2009; Burnett et al., 2011). Gene-expression detection technology in the hematopoietic cell of AML patients have been applied in scientific research and clinical testing, in hope of identifying candidate genes which may refer to the development and progression of AML and marker of diagnosis or prognosis (Delaunay et al., 2003; Weltermann et al., 2004; Gonzalez Garcia et al., 2006; Foran, 2010; Flach et al., 2011). For example, in the passed several years, the nucleophosmin (PNM1) mutations with normal cytogenetics has been identified a prognostic factors in AML patients (Schnittger et al., 2005), more and more genes would probably provide biomarkers in the future.

In the present, researching the function of Homer3 mainly in the nervous system and the immune cell. But in some previous data showed that Homer3 were abnormal expression changed in blasts in AML and MDS. Although the significance of Homer3 abnormal expression changes in leukemic blasts is unknown, further analyses found a significant association between Homer3 gene expression levels and prognosis (Stirewalt et al., 2008). The AML patients can be divided into three groups by the abnormal of cytogenetic (i.e. favorable, intermediate, or unfavorable). Homer3 were over expressed in AML samples with normal cytogenetics, while it is decreased expression in AML patients with unfavorable cytogenetic. In addition, EVI1 expression, which has been linked to a poor outcome in AML, was also associated with decreased expression for Homer3 (Jółkowska et al., 2000; Valk et al., 2004). Our study was revealed the function of Homer3 in the leukemia cell line firstly. The CCK-8 assay clearly showed that Homer3 markedly inhibited the proliferation of K562 cells. At same time we demonstrated that the G2/M phase

arrest was the main reason result in slow growth compare with Vector. Also we found without inducing agents, there were no difference of the apoptosis rate between K562/Homer3 and Vector (results not shown). When treated by As_2O_3, more K562/Homer3 cells were apoptosis than Vector cells. Bcl2 was involved in this process (Akao et al., 1998; Qi et al., 2008). The differentiation of K562 cells were detected by flow cytometry nowadays. The CD15 molecule is a very important cell surface marker of mature granulocyte lineage and monocytes lineage. TPA can induce K562 cells differentiation toward monocytes, so we choose CD15 molecule (Sun et al., 2007). We confirmed that the differentiation rate of the K562/Homer3 cells were obviously more than Vector cells induced by TPA. Finally, the results of these experiments showed that Homer3 can promote differentiation in K562 cells.

In our experiments, the forced over-expressed of the Homer3 gene in K562 cells can suppress cellular proliferation, promote cell apoptosis, differentiation and affect cell cycle significantly. So we think that the hematopoietic cell clones in leukemia of favorable groups which over-expressed Homer3 might weaken a growth advantage through these effects. All of these can explain it is over-expressed in MDS and leukemia of the low risk group, while in high-risk group of the patients displayed decreased expression. In conclusion, we found the up-regulation of Homer3 expression might be an important event of pathogenesis and prognosis in AML. We believe that it will become a new ways of gene therapy in AML patients in the future.

Acknowledgements

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