

Cell adhesion downregulates the expression of Homer1b/c and contributes to drug resistance in multiple myeloma cells

JIE TANG¹, HONGXUAN ZHOU¹, CHUN WANG¹, XIAODONG FEI¹, LIQUN ZHU¹,
YUEJIAO HUANG³, YUNHUA HE², JING LIU², XIAOBING MIAO^{2,3}, YAXUN WU^{2,3} and YUCHAN WANG²

¹Liyang People's Hospital, Liyang, Jiangsu 213300; ²Jiangsu Province Key Laboratory for Inflammation and Molecular Drug Target, Medical College of Nantong University, Nantong, Jiangsu 226001;

³Nantong University Cancer Hospital, Nantong, Jiangsu 226001, P.R. China

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Abstract. Previous studies have demonstrated that Homer1b/c plays an important pro-apoptotic role through classical mitochondrial apoptotic pathway. The present study was undertaken to determine the expression and functional significance of Homer1b/c in multiple myeloma (MM). We found that Homer1b/c was lowly expressed in MM cell apoptotic model induced by doxorubicin. The positive role of Homer1b/c in cell apoptosis was further confirmed by knocking down Homer1b/c. Further study confirmed that Homer1b/c was able to affect the CAM-DR via pro-apoptotic activity regulating the ability of cell adhesion. Collectively, these data indicate that Homer1b/c may represent a good candidate for pursuing clinical trial in MM.

Introduction

Multiple myeloma (MM) is a clonal disease of plasma cells that remains, for the most part, incurable despite recent advances in treatment strategies and new molecular-targeted compounds (1-3). Most patients with MM eventually become resistant to the chemotherapeutic agents and die of disease progression within 10 years (4). To improve the prognosis of patients of MM, it is important to overcome drug resistance (DR). It has been demonstrated that the marrow microenvironment plays a critical role in myeloma DR (5-9). The marrow microenvironment consists of hematopoietic cells, stromal cells and extracellular matrix (ECM). Interactions between the MM cells and stromal cells/ECM could alter drug response by blocking apoptosis. This phenomenon was termed 'cell adhesion-mediated drug resistance' (CAM-DR), which is believed to play a crucial role in MM cells escape the cytotoxic effects of chemotherapeutic agents (10-13).

However, the underlying molecular mechanisms involved are unclear to date.

The Homer proteins, which belong to post-synaptic density (PSD) families, have been shown to be the products of alternative splicing of an immediately early gene (14,15). This family of proteins is composed of two major groups: the short-form proteins (Homer1a and Ania3) and the long-form proteins (Homer1b/c, Homer2 and Homer3) (16,17). With a conserved N-terminus of an Enabled/vasodilator-stimulated phospho-protein (Ena/VASP) homology 1 (EVH-1) domain, they are capable of binding to a proline-rich consensus sequence (PPXXFR) in various other scaffolding and signal transduction molecules, including group 1 metabotropic glutamate receptor (mGluRs) and 1,4,5-trisphosphate receptors (IP3Rs) (18-20). Additionally, with a coiled-coil (CC) structure in the carboxyl-terminal regions, Homer1b/c self-multimerize and in turn couple group 1 mGluRs and IP3Rs to form multi-protein complexes and facilitate signal transduction to down-stream pathways (21). The knock-down of Homer1b/c using specific siRNA protects cortical neurons, as shown against glutamate-mediated excitotoxicity via anti-apoptotic activity (14). Treatment of cortical neurons with glutamate resulted in an increase in Bax with a decrease in Bcl-2, followed by activation of caspase-9, representing the classical mitochondrial pathways. Based on the above research, we speculated that Homer1b/c might be closely related to apoptosis, however, no report exists on the relationship between Homer1b/c and cell apoptosis in hematological malignancies.

In the present study, we first investigated the expression patterns of Homer1b/c at the protein level in the doxo-mediated MM cell apoptosis, and then we detected the changing of Homer1b/c protein expression in cell adhesion model. This study was conducted to gain greater insight into the pro-apoptotic effects of Homer1b/c and its functions in CAM-DR of myeloma cells, possibly providing potential therapies for clinical trials.

Materials and methods

Cell cultures and stimulation. The human MM cell lines RPMI-8226, U266 and bone marrow stromal cell line HS-5

Correspondence to: Dr Yuchan Wang, Jiangsu Province Key Laboratory for Inflammation and Molecular Drug Target, Medical College of Nantong University, Nantong, Jiangsu 226001, P.R. China
E-mail: ntwangyc@126.com

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were obtained from Cell Library, China Academy of Science. The cell lines RPMI-8226, U266 were cultured in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) and the HS-5 cultured in F12 (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin-streptomycin mixture (Gibco-BRL) at 37°C and 5% CO₂. To study apoptosis, cells were seeded onto a 60-mm dish and incubated in a low concentration of serum (1% FBS) for 24 h prior to treatment with doxorubicin for different concentrations.

Western blot analysis. Western blot experiments were used to measure certain proteins. Briefly, the cells were lysed in lysis buffer [120 mM Tris (pH 7.4), 135 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS, 1 mM Na₃VO₄, 1 mM aprotinin and 1 mM PMSF]. An equivalent amount of protein from each sample was electrophoresed by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane. After blocking with phosphate-buffered saline (PBS) containing 5% non-fat milk and 0.1% Tween-20 overnight, the membrane was incubated with primary antibody at 4°C overnight. After washing with PBS containing 0.1% Tween-20 three times, each for 5 min, the membrane was incubated with HRP-labeled secondary antibody for another 2 h at room temperature. The membrane was then developed using the ECL detection systems. The antibodies used in the present study included: anti-Homer1b/c (anti-rabbit, 1:500; Santa Cruz Biotechnology), cleaved caspase-3 (anti-rabbit, 1:500; Santa Cruz Biotechnology), anti-Bax (anti-rabbit, 1:1,000; Cell Signaling Technology), anti-Bcl-2 (anti-mouse, 1:500; Santa Cruz Biotechnology) and anti-GAPDH (anti-rabbit, 1:1,000; Sigma).

Preparation of siRNA and transient transfection. Double-stranded small interfering RNA (siRNA) transfection was used to knock down Homer1b/c expression. The siRNA was commercially synthesized (Shanghai GenePharma Co., Ltd., Shanghai, China). For controls, scrambled RNA oligonucleotides were used. For each well, 33.3 nM of each of the three oligos was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 6 h, the medium was replaced with RPMI-1640 containing 10% FBS. Transfected cells were used for the subsequent experiments 48 h after transfection.

Cell viability assay. To evaluate the effect of transfection of Homer1b/c siRNA, cells were seeded on a 96-well cell culture cluster (Corning Inc., Corning, NY, USA) at a concentration of 5x10⁴/well in a volume of 100 µl and grown overnight. Cell Counting kit-8 (CCK-8) reagents (Dojindo Laboratories, Kumamoto, Japan) were added to the different subset wells, including control, control siRNA-transfected and Homer1b/c siRNA-transfected cells and then incubated at 37°C and 5% CO₂. The absorbance was quantified using an automated plate reader at a test wavelength of 450 nm at different times.

Adhesion assays and detection of adhesion rate. Adhesion of MM cell lines to FN was done as previously described. In co-culture experiments, HS-5 stromal cells were seeded first and incubated overnight at 37°C and 5% CO₂. The next

morning, stromal cells were washed once with serum-free medium, and MM cell lines were allowed to adhere for 2 h in serum-free RPMI-1640. Adhered MM cells were incubated overnight at 37°C and 5% CO₂, non-adhered cells were removed and RPMI-1640 supplemented with 10% FBS was added for an additional 24 h. MM cells were incubated with 5 µM of Calcein-AM (Santa Cruz Biotechnology) for 30 min, washed and incubated for 45 min to allow unbound dye to diffuse out of the cells. Labeled cells were allowed to adhere for 2 h and non-adherent cells were removed with three washes in PBS. The absorbance was quantified using an automated plate reader at a test wavelength of 490 nm.

Drug cytotoxicity assay. For drug cytotoxicity assays, MM cells were washed once after transfected with siRNA and adhered to FN or stromal cells as previously described. After 24 h, drugs or vehicle control was added to each well and incubated for 48 h, then medium containing drugs was removed, suspended and the attached MM cells were collected. The survival cells were assessed by CCK-8 assay.

Flow cytometry-based Annexin V/PI staining. The flow cytometry assay was performed to measure the degree of apoptosis and necrosis using an ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. Briefly, RPMI-8226 and U266 cells were digested by 0.1% trypsin and resuspended in cold binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, 0.1% BSA) at concentrations between 1x10⁶ and 1x10⁷ cells/ml. Labeled Annexin V (10 µl) was added to 100 µl of the cell suspension. After 15-min incubation on ice, 380 µl binding buffer and 10 µl propidium iodide (PI) solution were added to the cell suspension. Subsequently, the number of stained cells was assessed by a flow cytometer (BD FACSAriaII).

Statistical analysis. All experiments were repeated at least three times per condition. The data are described as mean ± SEM. Data were analyzed using the two-tailed t-test for statistical analysis. P-value <0.05 was considered to indicate a statistically significant result.

Results

Increased Homer1b/c expression in doxo-mediated MM cell apoptosis. The knockdown of Homer1b/c using specific siRNA has been reported to protect cortical neurons against glutamate-induced apoptosis via classical mitochondrial apoptotic pathway (14). First, we examined the cell viability after incubation for 48 h with doxorubicin for different concentrations. As shown in Fig. 1A and B, both RPMI-8226 and U266 cell viability decreased along with the increased concentration of doxorubicin. Herein, we examined whether Homer1b/c also participated in the regulation of doxorubicin-induced MM cells apoptosis. Exposing RPMI-8226 and U266 cells to gradually increasing concentration of doxorubicin for 48 h induced an apparent increase in the protein level of cleaved caspase-3, one of the key executioners of apoptotic cell death, measured by western blot analysis. Concomitantly with an increase in cleaved caspase-3 expression, doxorubicin also resulted in an increase in Homer1b/c expression (Fig. 1C-F).

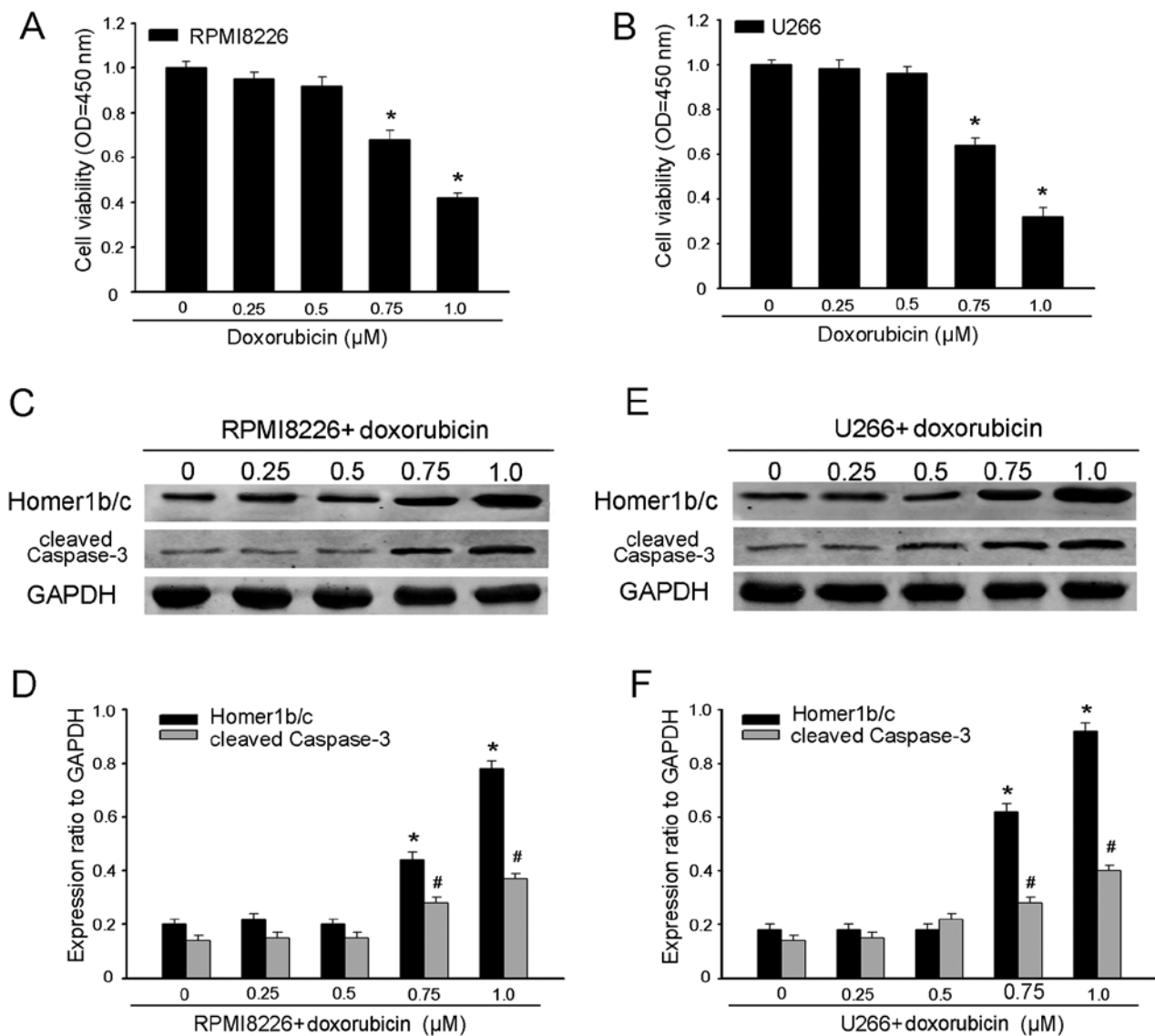


Figure 1. The expression of Homer1b/c and cleaved caspase-3 in doxo-mediated MM cell apoptosis. Cell viability of (A) RPMI-8226 and (B) U266 after incubated for 48 h with doxorubicin (* $P < 0.05$ compared with the group of 0 μM doxorubicin). Western blot analysis of expression of Homer1b/c and cleaved caspase-3 in (C) doxo-mediated RPMI-8226 and (E) U266 cells apoptosis. (D and F) The bar chart demonstrates the ratio of Homer1b/c and cleaved caspase-3 to GAPDH for the above by densitometry. The data are mean \pm SD of three independent experiments (* $P < 0.05$ compared with the group of 0 μM doxorubicin).

To further confirm the involvement of the mitochondria pathway, we investigated the expression levels of Bcl-2 and Bax, and the caspase-9 activity. As expected, treatment of MM cells with doxorubicin resulted in an increase in Bax with a decrease in Bcl-2 and activation of caspase-9 (Fig. 2). These results suggested that Homer1b/c participated in the process of the doxorubicin-mediated MM cell apoptosis. Possibly it promotes doxorubicin-induced apoptosis via classical mitochondrial apoptotic pathway, which should be further proved.

Homer1b/c acts as pro-apoptotic factor in MM cells. To further determine the function of Homer1b/c in MM cell apoptosis, Homer1b/c siRNA was transfected into RPMI-8226 cells, which significantly downregulated the expression of Homer1b/c in cells, confirmed by western blot analysis (Fig. 3A and B). We subsequently evaluated the expression of cleaved caspase-3 by western blot analysis, and found that the expression of cleaved caspase-3 was downregulated in the cells at the

protein level (Fig. 3C and D). To investigate whether the pro-apoptotic functions of Homer1b/c is mediated by mitochondrial pathways, we detected the expression levels of Bcl-2 and Bax and the caspase-9 activity after RPMI-8226 cell transfection with Homer1b/c siRNA by western blot analysis and found that transfection with Homer1b/c siRNA resulted in an increase in Bcl-2 with a decrease in Bax and cleaved caspase-9 (Fig. 3C and D). These results confirmed the pro-apoptotic effects of Homer1b/c. In order to further detect whether Homer1b/c is the direct cause of cell apoptosis, Homer1b/c siRNA and control siRNA cells were incubated for 48 h with 1 μM doxorubicin. In the presence of the chemotherapy drugs, the expression of cleaved caspase-3, Bax and cleaved caspase-9 decreased more significantly in the Homer-1b/c siRNA cells than in those from the control group. At the same time, the Bcl-2 increased more in Homer1b/c siRNA cells (Fig. 3C and D). Cell viability assay also suggested that the cell activity was significantly increased in Homer1b/c siRNA cells and knocking Homer1b/c down

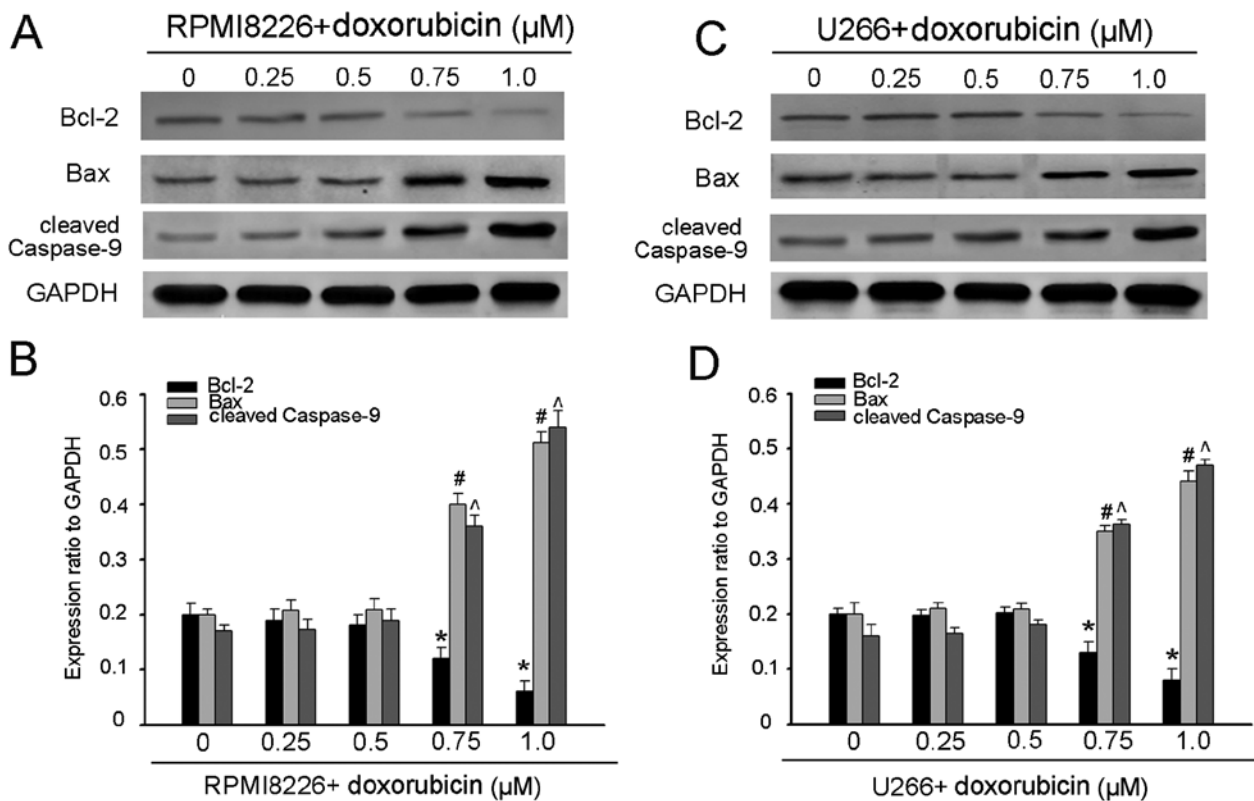


Figure 2. Doxo-mediated MM cell apoptosis involves the mitochondria pathway. Western blot analysis of expression of Bcl-2, Bax and cleaved caspase-9 in (A) doxo-mediated RPMI 8226 and (C) U266 cells apoptosis. (B and D) The bar chart demonstrates the ratio of Bcl-2, Bax and cleaved caspase-9 to GAPDH for the above by densitometry. The data are mean \pm SD of three independent experiments (* $^{\#}$ P<0.05 compared with the group of 0 μ M doxorubicin).

protected MM cells against drug-induced apoptosis (Fig. 3E). These results supported previous research that promotion of MM cell apoptosis by Homer1b/c was through the mitochondrial pathways (14), but whether there are other possible mechanisms included still needs further research.

The expression of Homer1b/c associated with cell adhesion in MM cells. Cell adhesion-mediated drug resistance (CAM-DR) is thought to be a major obstacle in the treatment of myeloma (13,22-25). To investigate the role of Homer1b/c in CAM-DR, we built the MM cell adhesion model, in which RPMI-8226 and U266 cells were adherent to the FN and HS-5 cells (3). After 48-h incubation, Homer1b/c expression was detected by western blot analysis. As shown in Fig. 4A and B, the quantity of Homer1b/c expression was decreased in adherent cells, as compared to that in suspended cells, which suggested that Homer1b/c might participate in the process of cell adhesion. The expression of cleaved caspase-3, Bax, Bcl-2 and cleaved caspase-9 was detected by western blot analysis and we found that the protein level of Bax, cleaved caspase-9 and cleaved caspase-3 increased while Bcl-2 decreased (Fig. 4C and D). All these results indicated that Homer1b/c might play a part in the process of CAM-DR. To further verify the role of Homer1b/c in CAM-DR, cell adhesion assay was performed following Homer1b/c siRNA transfection. RPMI-8226 and U266 cells were transiently transfected with Homer1b/c-siRNA or control siRNA for 48 h and stained with calcein for 30 min. Then they were cultured in suspension, or plated on FN coated surface or pre-established stromal cells and cultured for another 2 h.

Cell adhesion assay revealed cell adhesion rate was significantly increased after knockdown of Homer1b/c (Fig. 4E and F). Taken together these data suggested that Homer1b/c expression might negatively correlate with cell adhesion. The relationship between the pro-apoptotic role of Homer1b/c and the MM CAM-DR has not been fully shown, and more studies should be carried out.

The role of Homer1b/c in MM CAM-DR. In the process of CAM-DR, cell adhesion ability is directly relevant to cell tolerance effects of chemotherapy agents. To further confirm the role of Homer1b/c in CAM-DR, we tested the sensitivity of MM cells to chemotherapeutic agents. Consistent with the existing studies, the sensitivity of adherent RPMI-8226 and U266 cells to doxorubicin and mitoxantrone is lower than that of cells in suspension (Fig. 5A and B) (2,12,26). The drug resistance was further increased in both RPMI-8226 and U266 cells after interference with Homer1b/c siRNA (Fig. 5C and D). We also used a flow cytometry assay to detect the apoptotic cells adherent to stromal cells by assessing the levels of Annexin V-positive cells. We found that meddling with Homer1b/c promoted the chemotherapy drug-mediated cell apoptosis (Fig. 5E). In conclusion, Homer1b/c was a pro-apoptotic factor in MM.

Discussion

The present study analyzed the role of Homer1b/c in MM and described that the expression of Homer1b/c was associ-

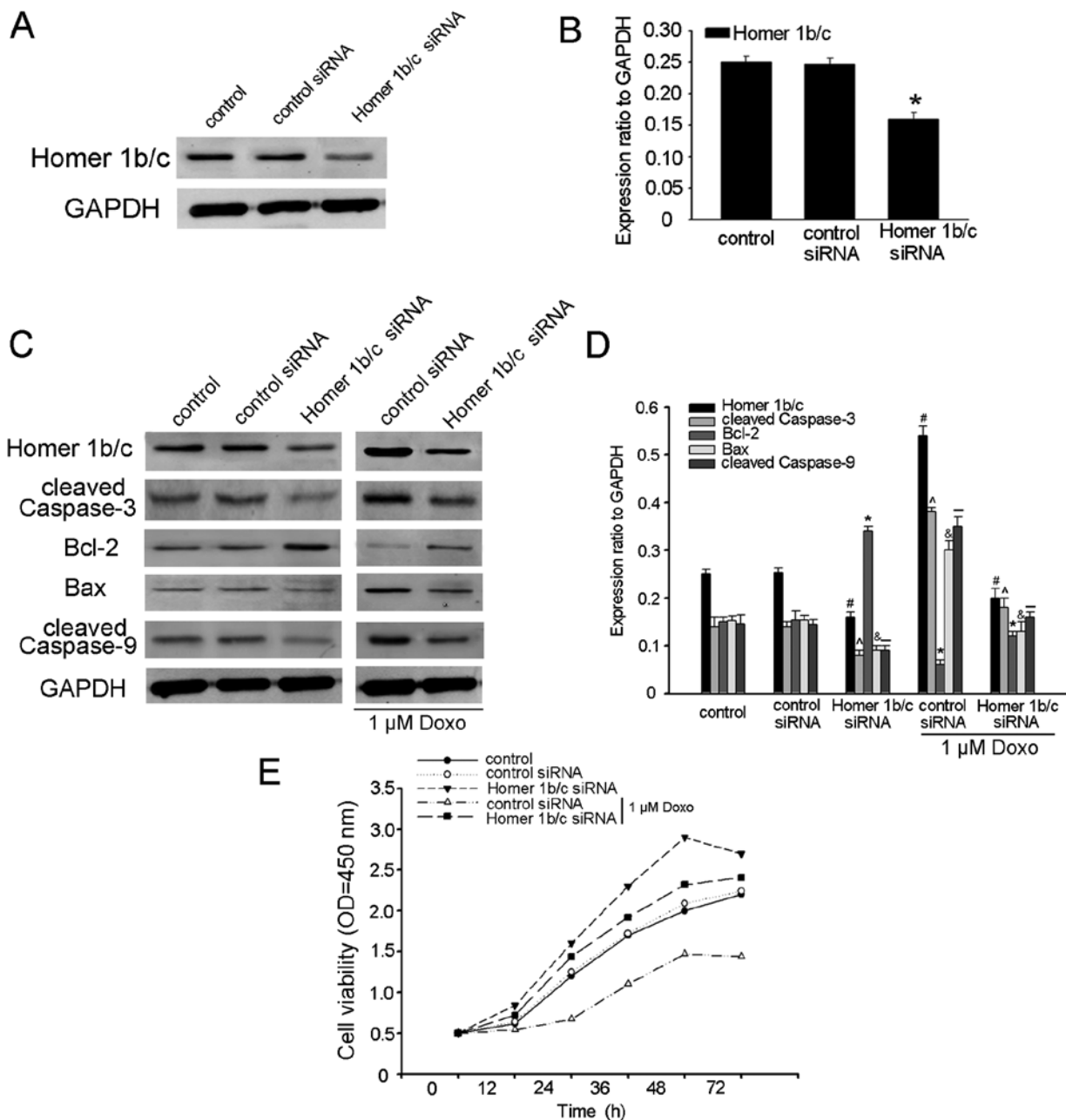


Figure 3. Homer1b/c acts as a pro-apoptotic factor in MM cells. (A) RPMI-8226 cells were transiently transfected with siRNA targeting either Homer1b/c or a scrambled sequence (control siRNA) as described above for 48 h and immunoblot analysis of Homer1b/c and GAPDH was performed. (B) The bar chart demonstrated the ratio of Homer1b/c protein to GAPDH by densitometry after control-siRNA or Homer1b/c-siRNA determined by western blot analysis. (C) After incubated with or without 1 μ M doxorubicin, normal cells and cells transfected with Homer1b/c siRNA or control siRNA were lysed, then analyzed by western blot analysis using antibodies against cleaved-caspase-3, Bcl-2, Bax, cleaved caspase-9 and GAPDH. (D) The bar chart demonstrates the ratio of cleaved-caspase-3, Bcl-2, Bax, cleaved caspase-9 to GAPDH for the above by densitometry. The data are mean \pm SD of three independent experiments (#, ^, *, & - P<0.05 compared with the control group). (E) *In vitro* cell viability was examined by CCK-8 assay at the indicated time. Data show mean \pm SD of triplicates from one experiment representative of three experiments performed (*P<0.05 compared with control or cells transfected with control siRNA).

ated with the CAM-DR. Based on existing research, the cell apoptosis model was established to study the potential functions of Homer1b/c at cellular and molecular levels. Western blot analysis documented that the expression of Homer1b/c in doxorubicin-medicated MM cells increased apoptosis. We also evaluated the activity of caspase-3, an apoptosis marker, using western blot analysis (Fig. 1A and B). The results suggested that Homer1b/c was involved in cell apoptosis after being incubated with drugs, but the exact roles of Homer1b/c remained unclear. We also documented the expression of

Bcl-2 and Bax, and the caspase-9 activity with the increased concentration of doxorubicin. Treatment of MM cells with doxorubicin resulted in an increase in Bax with a decrease in Bcl-2 and activation of caspase-9 (Fig. 2). All these changes following doxorubicin treatment were attenuated by knocking down Homer1b/c (Fig. 3C and D), indicating that the protective effects of Homer1b/c knockdown might be mediated by the mitochondrial pathways (27-30). We hypothesized, based on the present study, that Homer1b/c might play a negative role in MM CAM-DR. Next, MM cell adhesion

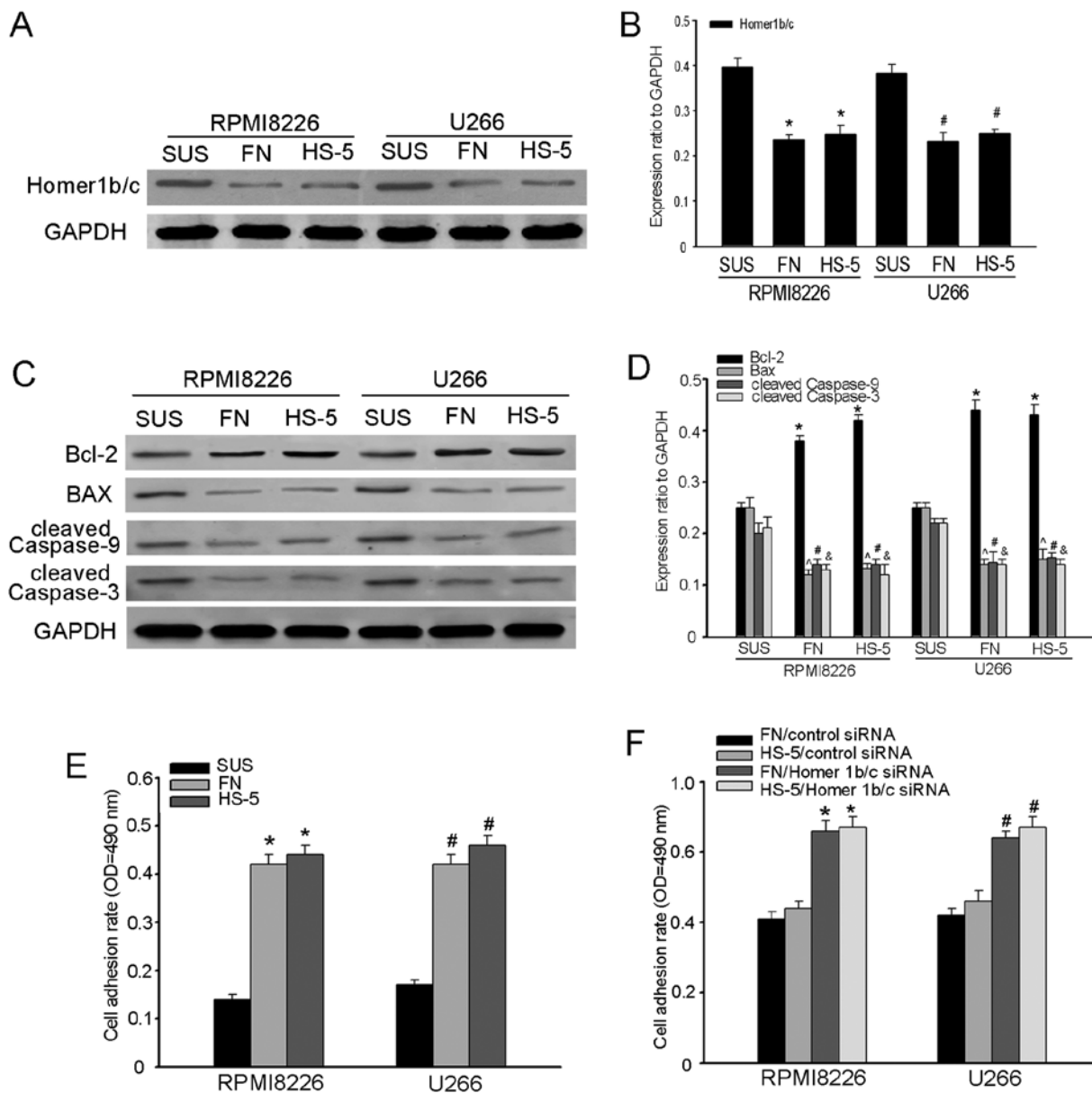


Figure 4. The expression of Homer1b/c and cell adhesion in MM cells. (A and C) Western blot analysis of expression of Homer1b/c, Bcl-2, Bax, cleaved caspase-9 and cleaved caspase-3 in a cell adhesion model of RPMI-8226 and U266 cells adhered to FN or HS-5 cells. Cells in suspension were used as negative control. (B and D) The bar chart demonstrates the ratio of Homer1b/c, Bcl-2, Bax, cleaved caspase-9 and cleaved caspase-3 to GAPDH for the above by densitometry. (E) RPMI-8226 and U266 cells pre-incubated with 5 μ M of Calcein-AM were adhered to FN or HS-5 cells for 2 h. After 2 h, non-adherent cells were removed and the cell adhesion rate was measured on an automated plate reader. (F) RPMI-8226 and U266 cells transfected with siRNA targeting either Homer1b/c or a scrambled sequence (control siRNA) as described above for 48 h and the cell adhesion rate was measured as described above. In (A-F), A representative of three independent experiments is shown (* P <0.05 compared with the control group). FN, fibronectin; SUS, suspension.

model was constructed. As expected, the protein expression level of Homer1b/c decreased after MM cells had adhered to FN or HS-5 cells. Also, the expression of Bcl-2 increased and Bax, cleaved caspase-9, cleaved caspase-3 decreased in the cell adhesion model. Cell adhesion assay revealed that cell adhesion rate was significantly increased after knockdown of Homer1b/c. In cell adhesion model, we confirmed that the MM cell adhesion-mediated drug resistance did exist and found that CAM-DR could be increased after knocking Homer1b/c down. All these results suggested that Homer1b/c might play an essential pro-apoptotic role and be at least partially relevant to CAM-DR through mitochondrial pathways.

Multiple myeloma is a clonal B-cell malignancy characterized by the infiltration and growth of malignant plasma cells in the bone marrow microenvironment (4,31). The extracellular matrix and stromal cells in bone marrow microenvironment protecting MM cells from drug-induced apoptosis are one of the most important reasons for drug resistance and relapse, which is the so-called CAM-DR. Interactions of MM cells with BM microenvironment play an important role in the pathogenesis of myeloma and in the development of drug resistance. In the myeloma cells after chemotherapy, the expression of both anti-apoptosis and pro-apoptosis proteins was induced. Bcl-2, a key apoptosis-suppressor protein, has gained more attention (28,32). On the contrary, Bax as a pro-apoptotic

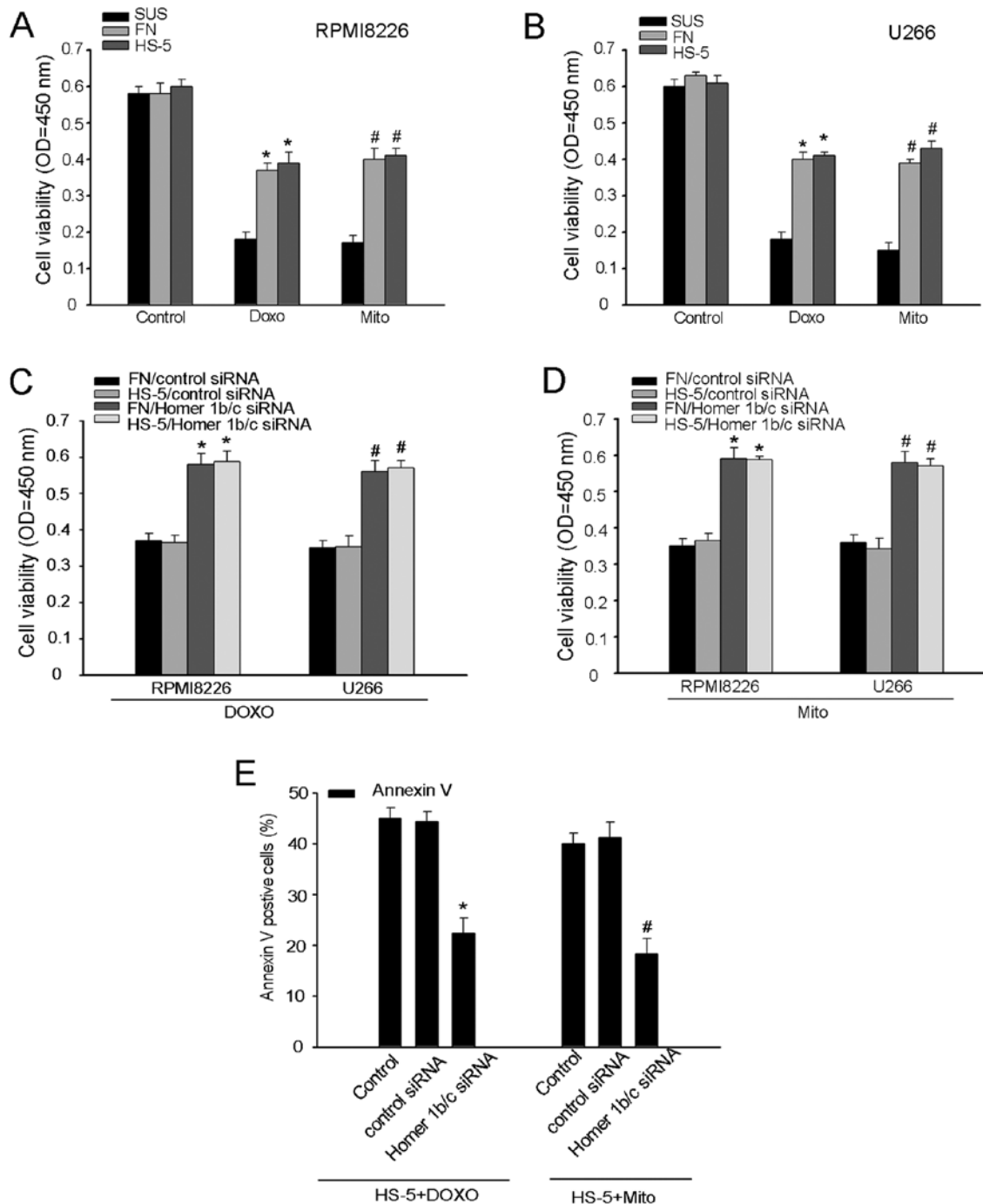


Figure 5. The role of Homer1b/c in MM CAM-DR. (A and B) RPMI-8226 and U266 cells were adhered to FN or HS-5 for 24 h. After 48 h, samples were treated with 1 μ M doxorubicin (doxo), 2 μ M mitoxantrone (mito) for 48 h. Then, CCK-8 reagents were added to the different subset wells and incubated at 37°C and 5% CO₂. Cell viability assay was analyzed using an automated plate reader. Data were collected from three independent experiments (*[#]P<0.05 compared with the cells in suspension). (C and D) RPMI-8226 and U266 cells transfected with siRNA targeting either Homer1b/c or a scrambled sequence (control siRNA) were adhered to FN or HS-5 for 24 h. Cell viability was analyzed after treated with 1 μ M doxo, and 2 μ M mito for 48 h. (E) Quantification of the results from the flow cytometry based study showed that Homer1b/c siRNA induced significant decreases in the number of Annexin V-positive cells after drug stimulation, whereas neither control nor non-specific siRNA had a notable impact. (*[#]P<0.05 compared with the control group). All these data are representative of at least 3 independent experiments.

member of Bcl-2 family can induce destructive changes in mitochondria (33,34). It has been confirmed that mitochondria could mediate intrinsic apoptotic pathways through release of cytochrome *c*, regulation of Bcl-2 family proteins, and cleavage of caspase-9 and -3. So the Bcl-2/Bax ratio is important to MM cell fate after chemotherapy. However, it has been reported that downregulation of Homer1b/c using specific siRNA protects

neurons against neurotoxicity-induced apoptosis through an increase in Bcl-2 with a decrease in Bax (14,35,36). Thus, we suspected that, after chemotherapy, increased expression of Homer1b/c might promote cell apoptosis through the classical mitochondrial apoptotic pathway. Our follow-up research confirmed our suspicions. Doxorubicin is a broad-spectrum chemotherapy drug. It plays a role in embedding the DNA and

inhibiting the synthesis of nucleic acid. Clinically, it is used for the treatment of various tumors, such as MM, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), lung cancer, ovarian cancer, and breast cancer (37-40). So in the model of the present study, we used doxorubicin to induced cell apoptosis. We found that the alteration of Homer1b/c was related to Bcl-2, Bax, cleavage of caspase-9 and -3, which was consistent with our hypothesis that Homer1b/c might play an essential pro-apoptotic role.

Some studies have shown that Homer1b/c could regulate diverse cell functions being able to assemble signaling complexes (41). However, the role of Homer1b/c in development of MM has remained unclear. In the present study, we have demonstrated that the expression of Homer1b/c was significantly decreased in MM cells adhered to FN or HS-5 cells, which suggested that Homer1b/c might be involved in the processes of CAM-DR. All the data were compatible with the hypothesis that decreased levels of Homer1b/c in the adhered cells might have an impact on cell survival through the mitochondrial pathway.

In conclusion, in the present study, we delineated the role of Homer1b/c in multiple myeloma (MM). We found that Homer1b/c plays an important pro-apoptotic role through classical mitochondrial apoptotic pathway. Since cell adhesion-mediated drug resistance remains a major obstacle for treatment of MM, we built a cell adhesion model in MM and detected the change of Homer1b/c protein expression. Homer1b/c siRNA reversed the high rate of MM cell adhesion to either FN or HS-5 cells. Consistent with the decreased adhesion rate, the cell also exhibited decreased drug resistance. Further study should be performed to determine whether there might be other signaling pathways for Homer1b/c during MM cell apoptosis and CAM-DR. Aside from the pro-apoptosis effect, it still calls for further study to confirm the clinical relevance of Homer1b/c in the disease progression.

Acknowledgements

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