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SIAH1 induced apoptosis by activation of the JNK pathway and inhibited invasion by inactivation of the ERK pathway in breast cancer cells

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Seven in absentia homolog 1 (SIAH1), a homologue of *Drosophila* seven in absentia (Sina), has emerged as a tumor suppressor and plays an important role in regulating cell apoptosis. To investigate the role and possible mechanism of SIAH1 in breast cancer cells, we up-regulated the expression of SIAH1 using pcDNA3-myc-SIAH1 and knocked down SIAH1 using SIAH1 siRNA. We found that the overexpression of SIAH1 induced cell apoptosis by up-regulating the level of Bim through the activation of the JNK signaling pathway, and the suppression of SIAH1 expression increased cell invasion via the activation of the ERK signaling pathway in breast cancer cells. All these results indicate that the JNK and ERK signaling pathways may play an important role in the SIAH1-dependent biological behavior of breast cancer, and it may be a good molecular therapeutic target to increase the expression level of SIAH1 through promoting cell apoptosis and inhibiting cell invasion in human breast cancer. (*Cancer Sci* 2010; 101: 73–79)

Breast cancer is one of the most common malignancies worldwide. Although several novel therapeutic methods have been utilized, curative effects in advanced breast cancer remains poor. Thus, the acquisition of new target molecules that play important roles in breast carcinogenesis will be essential for improving therapeutic intervention and prognosis of breast cancers.

SIAH (seven in absentia homolog) proteins are homologues of *Drosophila* seven in absentia (Sina) protein.[1] Sina has two human homologues, SIAH1 and SIAH2, which have significant amino acid sequence homology, and differ significantly at their N termini.[2,3] It has been suggested that the SIAH1 protein plays a key role in biological processes such as the cell cycle, programmed cell death, and oncogenesis.[4] Some reports showed that SIAH1 might induce cell apoptosis and suppress tumor growth through ubiquitination and proteasomal degradation of some target proteins.[4–11] Recently, it is reported that SIAH1 triggers cell apoptosis through activation of the c-jun-NH2-kinase (JNK) signaling pathway.[12] Our previous studies showed that SIAH1 could induce apoptosis of breast cancer cells (Wen, Yang, Song *et al*, unpublished data), but what may be responsible for this course is still not clear.

The BH3-only protein Bim (Bcl-2-interacting mediator of cell death) is a member of the Bcl-2 family, which has at least three isoforms including BimEL, BimL, and BimS. It is a critical mediator of cell apoptosis in various cell types.[13–17] Many studies have demonstrated that JNK signaling pathways participate in Bim-dependent apoptosis.[18–23]

MAPK (mitogen-activated protein kinase) plays an important role in the proliferation, differentiation, and apoptosis of various cells. There are three members of MAPKs: extracellular signal-regulated kinases (ERKs), JNKs, and P38. It has been well established that JNK and P38 are involved in the apoptotic response of cells exposed to UV irradiation, heat shock, etc., whereas ERK is primarily associated with proliferative responses.[24–27] Our present study showed that SIAH1 increased the expression of P-JNK and decreased the expression of P-ERK, but had no influence on the expression of P-P38. These results suggest that both the JNK and ERK signal pathways may play important roles in the malignant phenotype of human breast cancer.

Materials and Methods

Cell culture. Human breast cancer cell lines MCF-7 and MDA-MB-435S were maintained in Dulbcco's Modifed Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL streptomycin, and 100 units/mL penicillin in a humidified atmosphere with 5% CO_2. Cells were cultured to subconfluence until protein and RNA extraction.

Plasmid, small RNA interference, and transfection. The *siah1* gene expression vector pcDNA3-myc-SIAH1 and the empty vector pcDNA3-myc were kindly provided by Dr Matsuzawa Shu-ichi (Burnham Institute, La Jolla, CA, USA). Three small interfering RNA (siRNA) duplexes were synthesized and purified by Qiagen (Cambridge, MA, USA). The SIAH1 target sequence was as follows: 1, 5′-AACTCCTGCCTCCTTATGTATTT-3′; 2, 5′-GAUAGGAACACGCAAGCAA-3′; 3, 5′-GUUGCAUGUAGUAACACUA-3′. The nonsilencing siRNA (control siRNA) sequence was 5′-AAGAGCCGTCAGACTGCTACA-3′. Considering relative effectiveness and stability, SIAH1 siRNA 1 was selected by comparing our pilot experiments.

For transient transfections, cells were transfected with pcDNA3-myc-SIAH1, pcDNA3-myc, SIAH1 siRNA, or control siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). For incubation with an inhibitor, cells were transfected with pcDNA3-myc-SIAH1/SIAH1 siRNA for 24 h, then cultured for 24 h in the presence or absence of the specific-JNK inhibitor SP600125 (Sigma, St Louis, MO, USA)/specific-ERK inhibitor PD98059 (Sigma) at 5 μmol/L,10 μmol/L, and 20 μmol/L; then cells were collected for studying.

Western blotting. The protein was extracted with lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/mL aprotinin, 1 mM PMSF) for 1 h at 4°C. The supernatants were centrifuged at 10 000*g* for 30 min at 4°C. The supernatants containing total protein were harvested. Aliquots containing 60 μg of proteins were separated on a 12% SDS–polyacrylamide gel and transferred to polyvinylidene fluoride membranes. After blocking, blots were respectively incubated with primary antibody

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directed against SIAH1 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bim (1:1000; Cell Signaling Technology, Danvers, MA; USA), P-JNK (1:1000; Cell Signaling Technology), JNK (1:1000; Cell Signaling Technology), P-ERK (1:400; Santa Cruz), ERK (1:400; Santa Cruz), P-P38 (1:400; Santa Cruz), P38 (1:400; Santa Cruz), or β-actin (1:1000; Zhongshan Golden Bridge Biotechnology, Beijing, China) overnight at 4°C and followed by each corresponding second antibody at room temperature for 1 h at 37°C. Then the results developed by ECL (Pierce Biotechnology, Rockford, IL, USA). The protein bands were then analyzed using the BioImaging System (UVP, Upland, CA, USA). The grayscale values of the SIAH1 and Bim bands were normalized to the values of the corresponding β-actin band to determine the expression level of the protein. The experiments were repeated at least three times independently.

RNA isolation and reverse transcriptase–PCR. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. SIAH1 and Bim RT-PCRs were performed using a Takara RNA PCR Kit (AMV) version 3.0 (Takara, Shiga, Japan) according to the manufacturer's protocol. β-Actin served as an internal control. The primers used in this study were: SIAH1: forward, 5′ -TCCAACAATGACTTGGC-GAGT-3′, and reverse, 5′-CTTT TTCTGTGTGTGGCAGAG-3′; Bim: forward, 5′-CTGCAGATA TGCGCCCAGAGAT-3′, and reverse, 5′-CACCAGGCGGACAATGTAACG-3′; and β-actin: forward, 5′-AAATCGTGCGTGACATTAA-3′, and reverse, 5′- CTCGTC ATACTCCTGCTTG -3′. The PCR product sizes were 253 bp (SIAH1), 167 bp (Bim), and 513 bp (β-actin). After electrophoresis, the PCR products were stained with ethidium bromide and analyzed using the BioImaging system. Relative band intensities were determined using NIH image software. Each experiment was done at least three times independently.

Flow cytometry. A total of 1×10^5 cells/mL were collected by trypsinization after transfection at 48 h and fixed in 75% cold ethanol. Cells treated with 10 μg/mL RNase (Sigma) and 10 μg/mL propidium iodide (Sigma) in PBS were detected by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) and analyzed with Modifit LT version 3.0 software (Verity Software House, Topsham, ME, USA). Nontransfected cells served as the control. Accumulation of sub-G1 cells, a known indicator of DNA fragmentation and apoptosis, was used to quantify apoptosis. All experiments were repeated at least three times independently.

Matrigel invasive assays. Cell invasive ability was examined using a 24-well Transwell with 8-μm pore polycarbonate membrance inserts (Corning, Corning, NY, USA) according to the manufacturer's protocol. The Matrigel (100 μg/mL) was applied to the upper surface of the membranes. After transfection for 24 h, cells were seeded on the upper chamber (5×10^4 cells/well) and incubated for 24 h. Nontransfected cells served as the control. Cells that had invaded the surface of the membrane were fixed with methanol and stained with hematoxylin. Five random high-magnification microscope fields per filter were counted. Each experiment was done at least three times independently.

MTT assay. The transfected cells were seeded in 96-well plates (1×10^4 cells/well). Cell proliferation was evaluated each day for 4 days after transfection using MTT method. The absorbance, which was directly proportional to the number of living cells in culture, was measured at 570 nm using a

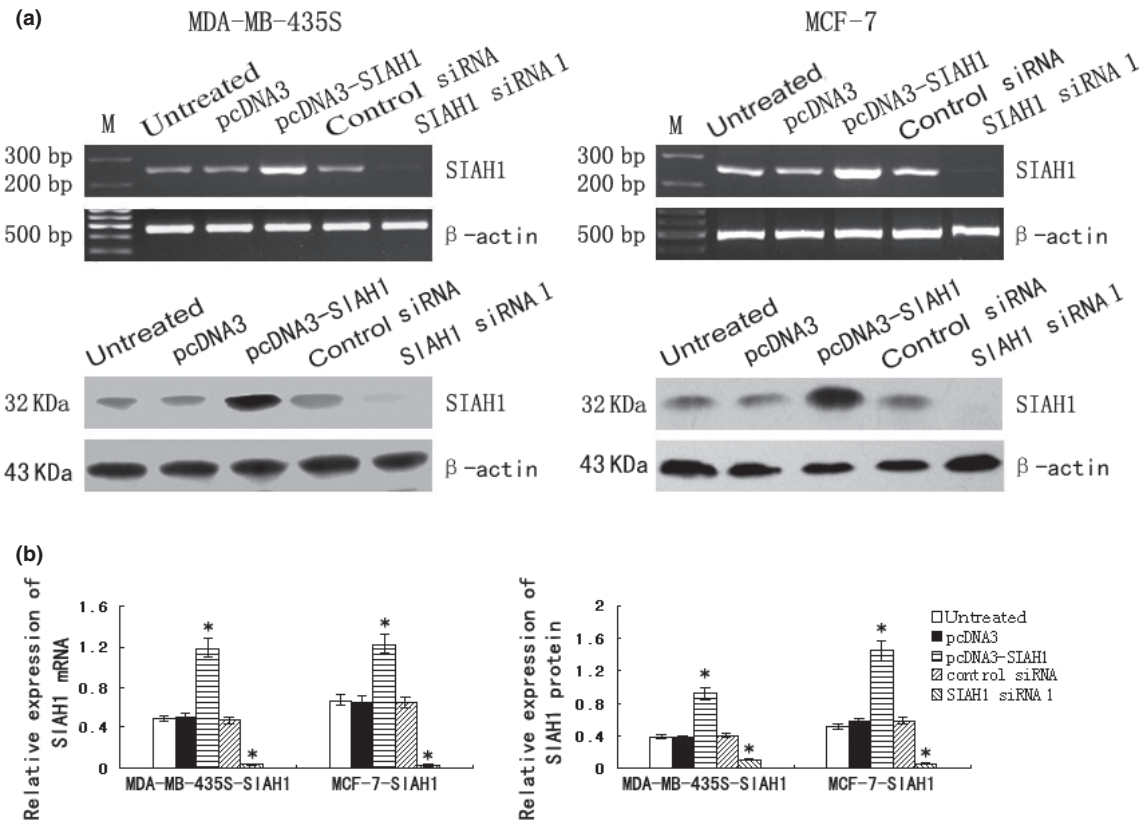

Fig. 1. The expression of seven in absentia homolog 1 (SIAH1), in MDA-MB-435S and MCF-7 cell lines with introduced- or knocked-down SIAH1. (a) RT-PCR and Western blotting showed that the level of SIAH1 was increased in cells transfected with pcDNA3-myc-SIAH1, and decreased in cells transfected with SIAH1 siRNA 1. (b) The expression of SIAH1 was statically analyzed. Data represent the mean ± SD of three independent experiments. Columns, mean (n = 3); bar, SD; *P < 0.05.

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microplate reader (Model 550; Bio-Rad, Hercules, CA, USA). A blank with dimethyl sulfoxide alone was measured and subtracted from all values. All the assays were done at least three times independently.

Statistical analysis. The Mann–Whitney *U*-test and the Kruskal–Wallis test were used to analyze the results of RT-PCR, Western blot, flow cytometry, MTT, and the Matrigel invasive assay. All statistical analyses were performed using SPSS 13.0 for Windows (SPSS, Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Overexpression of SIAH1 after transfection with pcDNA3-myc-SIAH1 and reduction of SIAH1 expression after transfection with SIAH1 siRNA. RT-PCR and Western blot results showed that the expression of SIAH1 was increased after the cells were transfected with pcDNA3-myc-SIAH1, while it was decreased after cells were transfected with SIAH1 siRNA 1. Empty vector pcDNA3-myc and control siRNA were introduced into the cancer cells as controls (Fig. 1).

SIAH1 regulated the expression of MAPKs in MDA-MB-435S and MCF-7 cell lines. Western blot results showed that overexpression of SIAH1 up-regulated the expression of P-JNK but down-regulated the expression of P-ERK. Meanwhile, SIAH1 siRNAs down-regulated the expression of P-JNK but up-regulated the expression of P-ERK, and SIAH1 siRNA 1 was the most effective in the three SIAH1 siRNAs. The expression of P-P38 had no change after the cells were transfected with pcDNA3-myc-SIAH1 or SIAH1 siRNAs (Fig. 2).

Overexpression of SIAH1 induced apoptosis of MDA-MB-435S and MCF-7 cells by the JNK/Bim pathway. RT-PCR and Western blot results showed that overexpression of SIAH1 significantly up-regulated the expression of Bim and P-JNK, but SP600125 at 10 and 20 μmol/L significantly inhibited overexpression of SIAH1-regulated expression of Bim and P-JNK (Fig. 3a,b). The flow cytometry results showed that overexpression of SIAH1 significantly increased apoptosis in MDA-MB-435S and MCF-7 cell lines (17.96% and 19.31%, respectively, $n = 3$), but SP600125 at 10 μmol/L significantly inhibited overexpression of SIAH1-induced apoptosis (0.05% and 0.02%, respectively, $n = 3$) (Fig. 3c,d).

SIAH1 inhibited cell invasion through the ERK pathway. We found that the expression of P-ERK was down-regulated by pcDNA3-myc-SIAH1 and up-regulated by SIAH1 siRNA 1 (Fig. 2). Here Western blot results further confirmed that the expression of P-ERK up-regulated by SIAH1 siRNA 1 was suppressed by the ERK inhibitor PD98059 at 10 and 20 μmol/L (Fig. 4a,b). And invasion assays showed that both MDA-MB-435S and MCF-7 cells transfected with SIAH1 siRNA 1 (47.78 ± 6.47 and 34.11 ± 5.64, respectively, $P < 0.05$, $n = 3$) had greater numbers of cells which invaded onto the lower

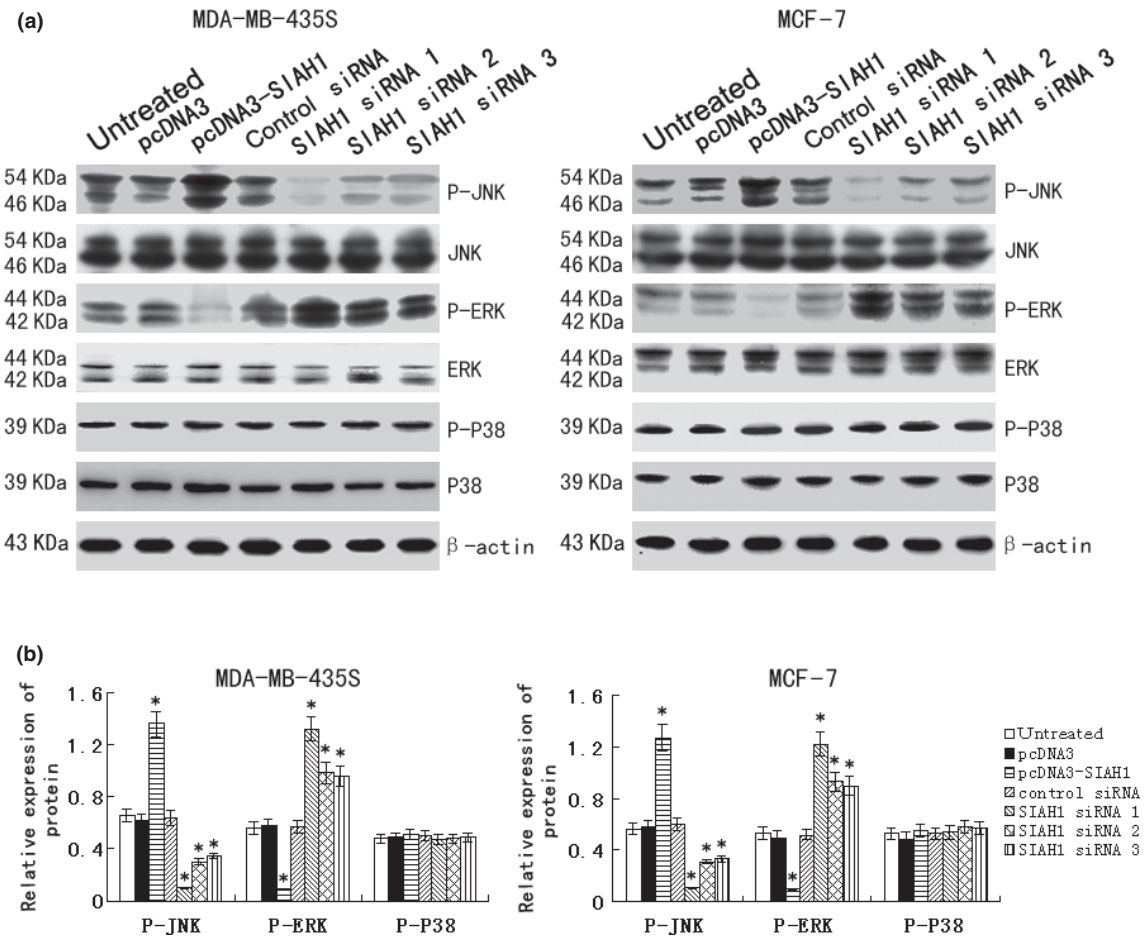
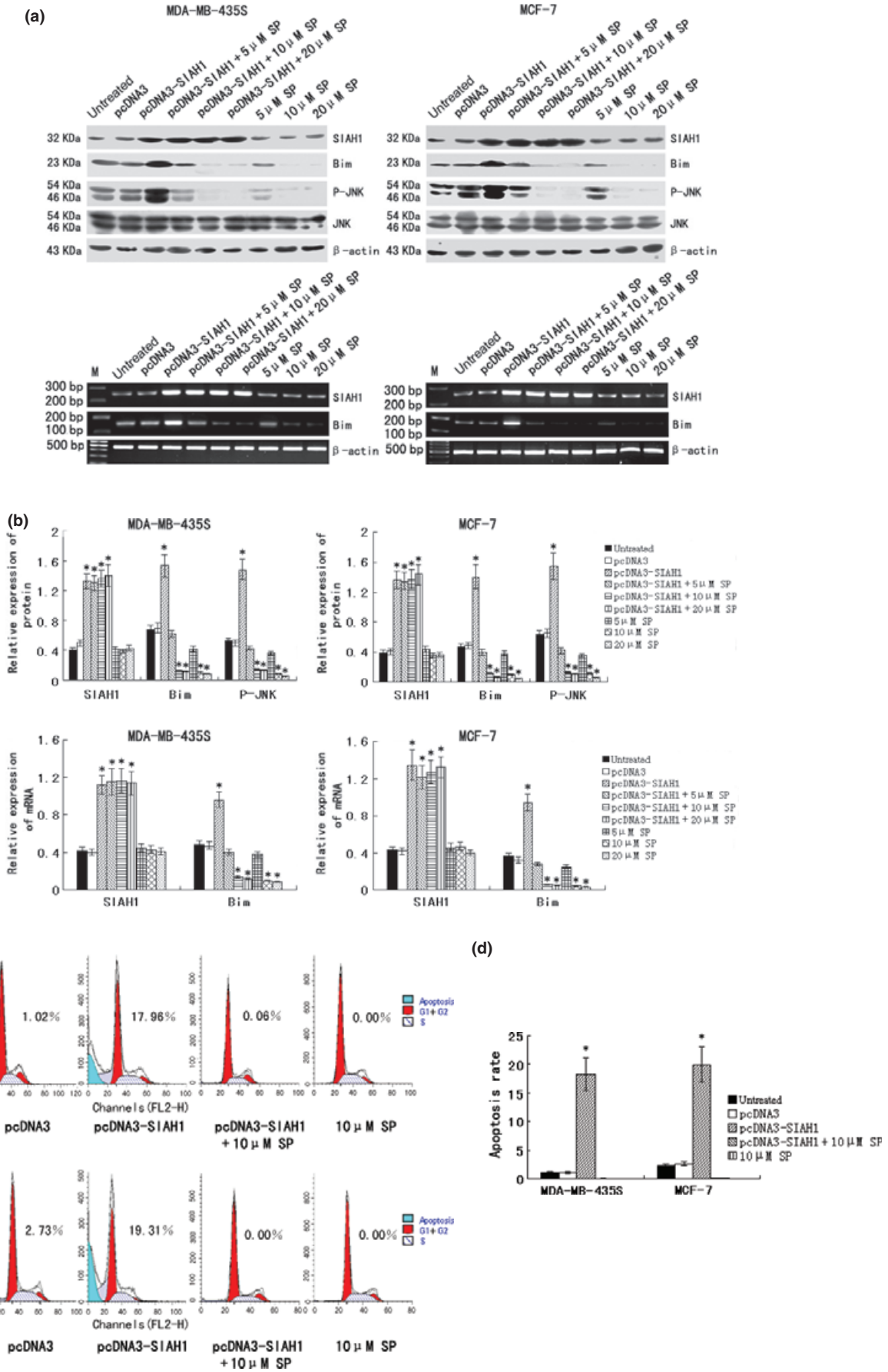


Fig. 2. The expression of MAPKs in MDA-MB-435S and MCF-7 cell lines with introduced- or knocked-down seven in absentia homolog 1 (SIAH1). (a) The overexpression of SIAH1 increased the level of P-JNK and decreased the level of P-ERK. Reduction of SIAH1 decreased the expression of P-JNK and increased the expression of P-ERK. Total JNK, ERK, P38, and P-P38 had no change. (b) The expression of P-JNK, P-ERK and P-P38 were statistically analyzed. Data represent the mean ± SD of three independent experiments. Columns, mean ($n = 3$); bar, SD; $^*P < 0.05$.



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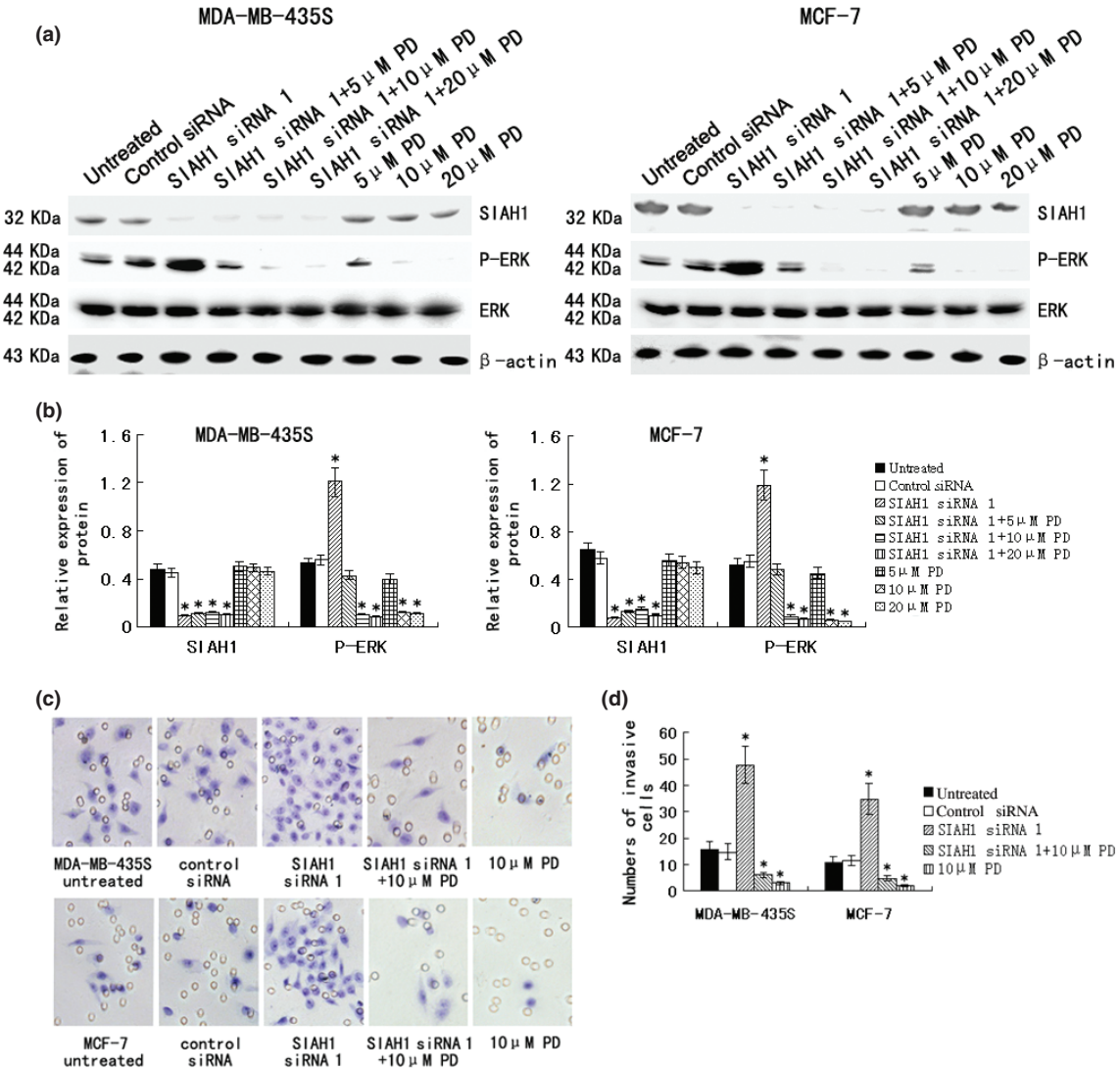

Fig. 4. The expression of P-ERK and invasive ability in MDA-MB-435S and MCF-7 cancer cells with introduced- or knocked-down seven in absentia homolog 1 (SIAH1). (a) The SIAH1 siRNA 1-increased expression of P-ERK was depressed by ERK inhibitor PD98059 at 10 and 20 μmol/L. Total ERK had no change. (b) The expression of P-ERK was statistically analyzed. (c) The SIAH1 siRNA 1-induced invasion of cancer cells was depressed by PD98059 at 10 μmol/L. (d) The number of invasive cells was statistically analyzed. Data represent the mean ± SD of three independent experiments. Columns, mean (n = 3); bar, SD; *P < 0.05 (b,d).

surfaces of the Transwell filters than cells transfected with SIAH1 siRNA 1 and meanwhile incubated with PD98059 at 10 μmol/L (6.32 ± 1.01 and 7.14 ± 1.12, respectively, P < 0.05, n = 3), control siRNA (14.96 ± 3.05 and 9.39 ± 1.09, respectively, P < 0.05, n = 3), or untreated cells (15.47 ± 3.36 and 10.13 ± 1.87, respectively, P < 0.05, n = 3) (Fig. 4c,d).

Both the JNK and ERK pathway affected SIAH1-associated cell growth inhibition. MTT assay results showed that the suppressed proliferation of the cancer cells transfected with pcDNA3-myc-SIAH1 was significantly reversed by SP600125 at 10 μmol/L (P > 0.05 [day 1]; P < 0.01 [days 2–4], n = 3) (Fig. 5a). We also observed that the level of proliferation in cells transfected with SIAH1 siRNA 1 was significantly higher than those cells transfected with SIAH1 siRNA 1 and incubated with PD98059 at 10 μmol/L, control siRNA, or untreated cells (P > 0.05 [day 1]; P < 0.01 [days 2–4], n = 3) (Fig. 5b).

Discussion

Some reports have shown that the expression of Bim was up-regulated by the JNK pathway[21,28,29] and SIAH1 could activate the JNK pathway in neuronal cells,[12] so we hypothesized that SIAH1 might regulate the expression of Bim through activating the JNK pathway. In the present study, we found that

Fig. 3. The expression of Bim (Bcl-2-interacting mediator of cell death) and P-JNK, and apoptosis in MDA-MB-435S and MCF-7 cell lines with introduced- or knocked-down seven in absentia homolog 1 (SIAH1). (a) The overexpression of SIAH1 up-regulated the expression of Bim and P-JNK. The overexpression of SIAH1-induced expression of Bim and P-JNK was depressed by JNK inhibitor SP600125 at 10 and 20 μmol/L. Total JNK protein did not change. (b) The expression of Bim and P-JNK was statistically analyzed. (c) The overexpression of SIAH1-induced apoptosis was significantly depressed by SP600125 at 10 μmol/L. (d) The apoptosis was statistically analyzed. Data represent the mean ± SD of three independent experiments. Columns, mean (n = 3); bar, SD; *P < 0.05 (b,d).

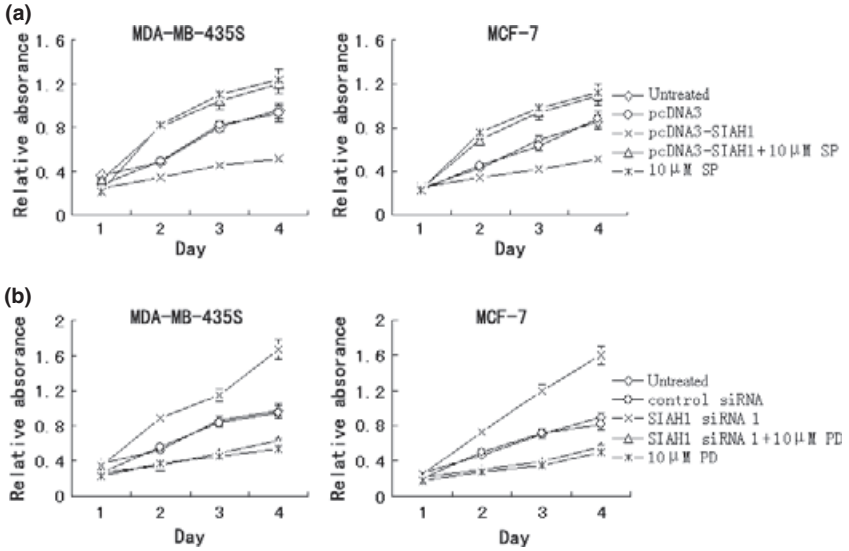

Fig. 5. Seven in absentia homolog 1 (SIAH1)-associated growth inhibition was dependent on the JNK and ERK pathways. (a) The depressed proliferation associated with SIAH1 was reversed by SP600125 at 10 μmol/L. (b) The level of proliferation was significantly lower in cells subjected to SIAH1 knockdown and incubation with PD98059 at 10 μmol/L than in SIAH1-knockdown cells alone. Data represent the mean ± SD of three independent experiments.

SIAH1-associated Bim expression was suppressed by the JNK inhibitor SP600125, indicating that SIAH1 might up-regulate the expression of Bim by the JNK pathway. This is the first evidence that the JNK pathway is involved in the positive regulation of SIAH1-induced Bim expression.

Recent study has suggested that the JNK pathway played key roles in SIAH1-associated apoptosis,[12] and that Bim was involved in JNK-dependent apoptosis.[19–21] Thus, we supposed that SIAH1 might induce apoptosis through the JNK/Bim pathway. In our present study, we observed that cell apoptosis induced by overexpression of SIAH1 was suppressed by JNK inhibitor SP600125, suggesting that SIAH1 might induce apoptosis by up-regulating the expression of Bim via the JNK pathway.

The ERK pathway can participate in the cellular proliferation, differentiation, angiogenesis, and tumor invasion.[30] Many reports have shown that the ERK pathway is responsible for cell invasion and migration.[31,32] In the present study, we observed that the reduction of SIAH1 in breast cancer cells increased the expression level of P-ERK. Conversely, the overexpression of SIAH1 decreased the expression of P-ERK. Therefore, we supposed that SIAH1 might inhibit the invasive ability of breast cancer cells through the ERK pathway. To further investigate the role of ERK activation in SIAH1-inhibited invasion of breast cancer cells, we applied the inhibitor of ERK activity PD98059 after cells were transfected with SIAH1 siRNA. Our results showed that the SIAH1 siRNA-induced invasive ability of breast cancer cells was inhibited by ERK inhibitor PD98059. So we confirmed that SIAH1 suppressed the malignant phenotype of breast cancer cells at least in part by affecting the activity of ERK.

Additionally, our work demonstrated that both the JNK and ERK pathways participated in SIAH1-associated cell growth inhibition, and the ERK pathway played a more important role than the JNK pathway in cell growth.

In summary, our results showed that SIAH1 could induce apoptosis and suppress cell growth by up-regulating the expression of Bim through the activation of the JNK pathway, and inhibit the invasion and growth of cells through the inhibition of the ERK pathway in human breast cancer. Because the expression of SIAH1 decreased or was lost in human breast cancer tissue and cells (Wen, Yang, Zhi-Qiang *et al*, unpublished data), it may be a good molecular therapeutic strategy to increase the expression level of SIAH1 to inhibit the ability of cell invasion and proliferation in human breast cancer.

Acknowledgment

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