Disabled homolog 2 interactive protein functions as a tumor suppressor in osteosarcoma cells

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Abstract. The disabled homolog 2 interactive protein (DAB2IP) gene is a member of the family of Ras GTPases and functions as a tumor suppressor in many types of carcinoma; however, its function in osteosarcoma remains unclear. The aim of the present study was to determine the function of DAB2IP in osteosarcoma and normal bone cells in vitro. The expression of DAB2IP protein was assessed in osteoblast and osteosarcoma cell lines by western blot analysis. The effects of DAB2IP expression on cell proliferation, colony formation, apoptosis, cell cycle, and cell migration and invasion were evaluated by in vitro studies. DAB2IP expression was lower in osteosarcoma cell lines than in normal osteoblast cell lines. DAB2IP expression affected cell proliferation, apoptosis and cell cycle distribution. In addition, DAB2IP inhibited the migration and invasion of osteosarcoma and normal osteoblast cells. Therefore, DAB2IP may function as a tumor suppressor in osteosarcoma cell lines by inhibiting cell proliferation and invasion.

Introduction

Osteosarcoma is the most common type of primary bone malignancy and is frequently diagnosed in children and adolescents (1). A large proportion of patients diagnosed with osteosarcoma will develop distant metastasis (2). Owing to the development of multidisciplinary therapy, the mortality rate for patients with osteosarcoma has been decreasing in recent years. Nevertheless, patients continue to exhibit a high risk of metastasis and/or recurrence (1,2). Previous studies have indicated that several genetic aberrations are associated with tumor initiation and osteosarcoma progression (3,4). Thus, identifying the underlying mechanisms that lead to the development and progression of osteosarcoma is crucial.

Disabled homolog 2-interacting protein (DAB2IP), located at chromosome location 9q33.1-q33.3, is a member of the Ras GTPase family (5-7). Downregulation of DAB2IP is frequently detected in various types of human cancer, including gastrointestinal cancer, breast tumor, prostate carcinoma, pulmonary tumor, pancreatic cancer, hepatocellular cancer and medulloblastoma (6,8-16). Loss of DAB2IP expression may facilitate cell proliferation and restrain cancer cell metastasis through several pathways, including Ras-extracellular-related kinase, apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase (JNK) and phosphoinositide-3 kinase (PI3K)-Akt (6,7,17-19). DAB2IP suppression may induce resistance to ionizing radiation and chemoresistance in prostate cancer and nonmuscle invasive bladder cancer (20-23). Furthermore, a role is hypothesized for DAB2IP in normal brain development and vascular inflammation (24-29), and the loss of DAB2IP may promote the epithelial-mesenchymal transition (EMT) and induce cancer stem cells to facilitate colorectal carcinoma progression and metastasis (16,30).

Although its role as a potent tumor suppressor in several carcinomas has been demonstrated (8-16), the expression and biological function of DAB2IP in osteosarcoma remain uncharacterized. In the present study, the endogenous expression of DAB2IP and its role in osteosarcoma cell lines were assessed, as was the effect of DAB2IP expression on cell progression and motility in osteosarcoma cells.

Materials and methods

Cell lines and cell culture. The osteosarcoma MG-63 and HOS cell lines an osteoblast hFOB 1.19 cell line and the
human embryonic kidney 293T cells were all purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MG-63, HOS and 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A DMEM/Ham's F12 (Thermo Fisher Scientific, Inc.) mix was used to culture hFOB 1.19 cells. Media contained 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and antibiotics, including 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37°C and 5% CO₂ in a humidified incubator.

Plasmid construction, lentivirus infection and establishment of stably transfected cell lines. The pLEX DAB2IP, pLEX Control, DAB2IP short hairpin (shRNA) pLKO.3G and scrambled shRNA pLKO.3 G plasmids were gifts from Professor Wang Min (Yale University, New Haven, CT, USA). The DAB2IP shRNA sequence was as follows: 5'-TAAATACGACTCACTATAGGG-3'. The scrambled shRNA sequence was as follows: 5'-GACGCTCTTCCGCTTCAAGG-3'. The target vectors (1.2 µg) were mixed with 1.2 µg lentivirus packaging helper plasmids pCMV-dR8.2 (Addgene, Cambridge, MA, USA) and 0.6 µg pCMV-SVG plasmids (Addgene), and the mixed vectors were diluted in 120 µl Opti-MEM (Thermo Fisher Scientific, Inc.). Following this, 5 µl P3000 Reagent was added (Invitrogen; Thermo Fisher Scientific, Inc.) to the final volume of 125 µl. Meantime, 7.5 µl Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted into Opti-MEM medium to a final volume 125 µl. Then, the diluted vectors and diluted Lipofectamine 3000 reagent were mixed, and incubated for 15 min at room temperature. The complex was added into ~70% confluence 293T cells in the 6-wells plate. Viruses were harvested 48 h after transfection and subsequently used to infect HOS and hFOB 1.19 cells, with puromycin (Thermo Fisher Scientific, Inc.) used to select the positive clones. The stable cell lines obtained were correspondingly designated as HOS pLEX DAB2IP, HOS pLEX Control; hFOB 1.19 DAB2IP shRNA cells (1x10⁵ cells/100-mm dishes) were plated in the appropriate FBS-free medium and incubated for 24 h at 37°C. Following a further 24 h of incubation at 37°C in medium containing FBS, cells were collected and resuspended in 200 µl of 1X binding buffer at a concentration of 1x10⁶ cells and stained with 5 µl annexin V-fluorescein isothiocitrate or APC dye, followed by staining with 5 µl PI. The proportion of cells undergoing apoptosis was analyzed by a flow cytometer (eBioscience; Thermo Fisher Scientific, Inc.), and the results were analyzed using FlowJo 7.6.2 software (Tree Star, Inc., Ashland, OR, USA).

Three independent experiments were performed.

Cell cycle assay. HOS, hFOB 1.19 and hFOB 1.19 DAB2IP shRNA cells (1x10⁶ cells/100-mm dishes) were plated in the appropriate FBS-free medium and incubated for 24 h at 37°C. Following a further 24 h of incubation at 37°C in medium containing FBS, cells were collected and resuspended in 500 µl of cold PBS, then stained with 25 µl of PI kit. Cells were analyzed for cell cycle distribution using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle distribution was calculated and the result was analyzed using FlowJo 7.6.2 software (Tree Star, Inc., Ashland, OR, USA). The results were analyzed using ModFit LT3.1 software (Verity Software House, Inc., Topsham, ME, USA). The experiment was repeated three times.

Migration and invasion assays. Cell migration and invasion rates were determined as described in previously published methods (31-33) in HOS, hFOB 1.19 and hFOB 1.19 DAB2IP shRNA cells. To study migration, 2x10⁴ cells were seeded into each well of a 96-well plate. When cells had reached 80-90% confluence, a wound was generated using the Cellplayer 96-well Woundmaker (Essen BioScience, Inc., Ann Arbor, MI, USA). The images of cells were automatically acquired every 2 h for 24 h using the Incucyte LiveCell Imaging system (Essen BioScience, Inc.).
To study invasion, a scratch was made by using the Cellplayer 96-well Woundmaker, when cells had reached 80-90% confluence. Next, the wound was covered with 50 µl of Matrigel solution (1 mg/ml Matrigel in PBS) that was allowed to set for 1 h at 37˚C. Appropriate growth medium (100 µl) was added and representative images were recorded at 2 h intervals for the duration of the experiment (24 h). All images were processed using the IncuCyte™ software package version 2015.1 (Essen BioScience, Inc.) to measure cell migration and invasion by obtaining the relative wound density.

**Statistical analysis.** Data are presented as the mean ± standard deviation. Comparisons between two groups were performed using unpaired Student's t-tests. Multiple group comparison was performed using one-way analysis of variance. And the post hoc test was Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using the commercially available packages PASW statistics 18.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

**Results**

**DAB2IP is downregulated in osteosarcoma cell lines.** Western blotting was performed to assess endogenous DAB2IP expression levels in the normal human osteoblast cell line, hFOB 1.19, and the human osteosarcoma MG63 and HOS cell lines. DAB2IP expression was downregulated in the osteosarcoma cell lines compared with the normal osteoblast cell line (Fig. 1A).

To examine the effects of DAB2IP expression in osteosarcoma, DAB2IP was overexpressed or silenced in HOS cells. Marked differences in DAPB2IP expression were observed between the pLEX control and pLEX DAB2IP cells (Fig. 1B). The shRNA-mediated interference of DAB2IP (Fig. 1C) and its expression (Fig. 1D) through lentivirus infection were confirmed.

**DAB2IP inhibits cell proliferation and colony formation.** MTS and colony formation assays were performed to determine the effects of ectopic DAB2IP expression on cell growth in HOS pLEX DAB2IP cells and controls (Fig. 2). As demonstrated in Fig. 2A, the proliferation of HOS cells transfected with pLEX DAB2IP was significantly reduced compared to HOS cells transfected with a vector control. By contrast, it was observed that the proliferation was significantly increased when DAB2IP was knocked down in HFOB cells (Fig. 2B), and the restoration of DAB2IP expression in DAB2IP-shRNA-treated hFOB cells suppresses osteoblast proliferation (Fig. 2C). The number of colonies formed by HOS cells transfected with pLEX DAB2IP was decreased compared with the control group (Fig. 2D). Similar to the proliferation results, the colony formation abilities of the DAB2IP-shRNA-treated cells was significantly increased compared with those treated with scrambled shRNA (Fig. 2E), and restoring DAB2IP expression suppressed the colony formation ability of the osteoblast cells (Fig. 2F).

**DAB2IP promotes bone cell apoptosis and increases G0/G1 phase distribution.** To determine whether DAB2IP could induce apoptosis in osteosarcoma cells, the cell cycle distribution of HOS pLEX and HOS pLEX DAB2IP cells was evaluated by flow cytometry. As presented in Fig. 3A, the rate of apoptosis in HOS pLEX DAB2IP cells was significantly higher than control HOS pLEX cells (6.804±1.051% vs. 1.717±0.208%, respectively). Similarly, upregulation of DAB2IP induced apoptosis in pLEX-DAB2IP cells compared with control cells (5.291±0.808% vs. 1.038±0.277%, respectively; Fig. 3B). These findings suggested that DAB2IP induces cell apoptosis in osteosarcoma cells and osteoblasts.

In order to further assess the mechanism by which DAB2IP affects cell proliferation, cell cycle progression was determined using flow cytometry. As shown in Fig. 3B, a significantly greater proportion of HOS pLEX DAB2IP cells were in the G0/G1 phase compared with the control cells (47.543±1.810% vs. 39.940±1.628%, respectively; Fig. 4A). By contrast, downregulation of DAB2IP expression in hFOB 1.19 cells resulted in a greater proportion of cells in S-phase than the hFOB 1.19 Scrambled shRNA cells (47.893±2.409% vs. 37.277±2.247%, respectively; Fig. 4B). However, the G0/G1 phase distribution of pLEX DAB2IP cells was higher than pLEX control cells (43.097±5.997% vs. 31.450±3.922%), with fewer cells in S phase (39.613±3.964%; Fig. 4C).

**DAB2IP suppresses cell migration and invasiveness.** Given that DAB2IP can inhibit the proliferation of osteosarcoma and normal osteoblasts, its function on cell motility was assessed. The effect of DAB2IP on cell migration was examined using a scratch wound-healing assay, with cell migration measured by determining the relative wound density. The migration rate of HOS pLEX DAB2IP cells was significantly lower than that observed for HOS pLEX control cells (Fig. 5A). However, downregulation of DAB2IP expression in hFOB 1.19 cells resulted in a greater proportion of cells in S-phase than the hFOB 1.19 Scrambled shRNA cells (47.893±2.409% vs. 37.277±2.247%, respectively; Fig. 4B). However, the G0/G1 phase distribution of pLEX DAB2IP cells was higher than pLEX control cells (43.097±5.997% vs. 31.450±3.922%), with fewer cells in S phase (39.613±3.964%; Fig. 4C).

The effect of DAB2IP on the invasive potential of osteosarcoma and normal osteoblasts was then investigated. As presented in Fig. 6A, the upregulation of DAB2IP could inhibit the osteosarcoma HOS cell invasion into Matrigel, whereas the downregulation of DAB2IP in hFOB 1.19 cells promoted cell invasion through the Matrigel barrier, with restoration of DAB2IP expression suppressing cell invasion when compared with hFOB 1.19 DAB2IP shRNA cells (Fig. 6B and C, respectively). Collectively, these results demonstrated that DAB2IP inhibits cell migration and invasion in osteosarcoma cell lines.

**Discussion**

In the present study, a lentivirus transfection-mediated approach was used to investigate the role of DAB2IP in osteosarcoma cells and osteoblasts. Overexpression of DAB2IP led to a significant change in proliferation, apoptosis, cell cycle distribution, migration and invasion in the HOS cell line. Downregulation of DAB2IP in hFOB 1.19 cells confirmed that it promoted proliferation, with restoration of its expression resulting in the suppression of cell growth.

DAB2IP is a member of the Ras GTPase family that was originally identified in prostate cancer (5). To date, its downregulation has been observed in a range of types of...
Figure 1. Expression of DAB2IP in osteoblasts and osteosarcoma cells. (A) DAB2IP expression in the immortalized normal human osteoblast hFOB 1.19 cell line, and the human osteosarcoma MG63 and HOS cell lines. (B) DAB2IP expression in HOS cells following transfection with pLEX DAB2IP. (C) DAB2IP expression in hFOB 1.19 cells following shRNA silencing. (D) DAB2IP expression in hFOB 1.19 DAB2IP shRNA cells following transfection with pLEX DAB2IP. GAPDH was used as a loading control. DAB2IP, disabled homolog 2 interactive protein; shRNA, short hairpin RNA; pLEX DAB2IP, plasmid containing DAB2IP.

Figure 2. Effect of DAB2IP overexpression and silencing on the in vitro cell proliferation and colony formation of osteosarcoma and osteoblast cells. Effect of DAB2IP on the proliferation of (A) HOS, (B) hFOB 1.19 and (C) hFOB 1.19 pLEX DAB2IP cells, as determined by an MTS cell proliferation assay. Effect of DAB2IP on colony formation by (D) HOS, (E) hFOB 1.19 and (F) hFOB 1.19 pLEX DAB2IP cells. *P<0.05, comparing HOS PLEX control cells with HOS PLEX DAB2IP cells in A and D. #P<0.05, comparing hFOB Scrambled shRNA cells with hFOB DAB2IP shRNA cells in B and E. *P<0.05, comparing hFOB DAB2IP shRNA PLEX control cells with hFOB DAB2IP shRNA PLEX DAB2IP cells in C and F. DAB2IP, disabled homolog 2 interactive protein; shRNA, short hairpin RNA; OD, optical density; pLEX DAB2IP, plasmid containing DAB2IP.
human malignancy, including in cancer of the prostate, breast, liver, pancreas, gastrointestinal tract and bladder (6,8-16). In addition, downregulation of DAB2IP gene expression, predominantly due to epigenetic modification, was associated with unfavorable tumor characteristics and outcomes in cancer of the prostate, bladder, gastrointestinal tract and liver (8-11,34,35). Two genome-wide association studies of aggressive prostate cancer suggested that DAB2IP is a putative prostate tumor-suppressor gene (36). In the present study, differential DAB2IP expression was confirmed through western blot analysis, suggesting that DAB2IP may function as a tumor suppressor gene.

DAB2IP can facilitate the dissociation of 14-3-3 proteins from ASK1, leading to enhanced ASK1-JNK activation in response to tumor necrosis factor (TNF) (7). DAB2IP also associates with TNF receptor-associated factor 2 (TRAF2) and mediates TNF/TRAF2-induced ASK1-JNK activation while inhibiting IκB kinase/nuclear factor κB (NF-κB) signaling (24). Furthermore, DAB2IP induces G0/G1 cell cycle arrest and promotes apoptosis through suppression of the PI3K-Akt pathway accompanied with activating ASK-JNK signaling in prostate cancer cells (18). In the present study, DAB2IP expression was downregulated in osteosarcoma cell lines compared with normal human osteoblasts. Gain- and loss-of-function approaches were employed to investigate the effect of DAB2IP expression on cell-cycle distribution and apoptosis. Overexpression of DAB2IP led to an increased rate of apoptosis and induced G0/G1 cell cycle arrest, as reported in a previous study (18). Therefore, DAB2IP may inhibit cell growth by inducing apoptosis and inhibiting cell cycle progression.

The majority of osteosarcoma patients develop metastasis, and outcomes for these patients remain poor (37-39). Previous studies suggest that DAB2IP may regulate cancer cell metastasis (13,15,16,19,40). A previous study revealed that loss of DAB2IP facilitated EMT, leading to prostate cancer metastasis via the GSK-3β-β-catenin signaling pathway (19). Min et al (41) identified that the epigenetic silencing of DAB2IP by EZH2 promoted tumorigenesis and distant metastasis by Ras and NF-κB activation. Further previous studies

Figure 3. Effects of DAB2IP expression on apoptosis and cell cycle progression in osteosarcoma cells and normal osteoblasts. (A) Effect of DAB2IP expression on the apoptosis of HOS cells. Representative images are on the left and quantification on the right. (B) Effect of DAB2IP expression on apoptosis of human osteoblast hFOB 1.19 cell line as determined by an apoptosis assay. Representative images are shown on the left, and quantification is shown on the right. All results are representative of three independent experiments. *P<0.05, comparing HOS PLEX control cells with HOS PLEX DAB2IP cells. **P<0.05, comparing hFOB DAB2IP shRNA PLEX control cells with hFOB DAB2IP shRNA PLEX DAB2IP cells, DAB2IP, disabled homolog 2 interactive protein; pLEX DAB2IP, plasmid containing DAB2IP; FITC, fluorescein isothiocyanate; shRNA, short hairpin RNA.
revealed that DAB2IP could restrain tumor growth and metastasis by suppressing tumor angiogenesis, pre-metastatic niche formation and tumor EMT initiation via inhibition of vascular endothelial growth factor receptor 2-dependent signaling in the tumor niche (42). In the present study, an increase in DAB2IP expression in osteosarcoma cells significantly reduced cancer cell migration and invasion. The decreased expression of DAB2IP in osteoblasts enhanced their migration and invasion. Furthermore, the restoration of DAB2IP expression may suppress cell migration and invasion.

DAB2IP acts as a tumor suppressor in osteosarcoma, inhibiting cell proliferation and survival in osteosarcoma.
cells and osteoblasts by inducing apoptosis and a greater G0/G1-phase distribution. Additionally, the expression of DAB2IP may inhibit the migration and invasion of osteosarcoma cells and normal osteoblasts. The identification of these roles for DAB2IP in osteosarcoma may be beneficial, enriching the understanding of the mechanism of osteosarcoma progression, and possibly providing an approach for the early detection of osteosarcoma and novel therapeutic modalities.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH conducted the experiments and participated in analyzing the experiment data, and also was a major contributor in writing the manuscript. SH participated in analyzing the experiment data particularly the cell migration and invasion assay, and also participated in writing the manuscript. ZL, JZ and JS participated in performing the experiment and analyzing the experiment data. WJ and XL designed the experiment and...
participated in analyzing the experiment data particularly the cell cycle assay and the cell apoptosis assay, and also participated in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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