Microfibril-Associated Protein 2 (MFAP2) Potentiates Invasion and Migration of Melanoma by EMT and Wnt/β-Catenin Pathway

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Background: Growing evidence indicates an association between microfibril-associated protein 2 (MFAP2) and a number of physiological and pathological mechanisms. The potential role of MFAP2 in cancer requires further elucidation. The present study investigated the biological behavior of MFAP2 in melanoma patients.

Material/Methods: MFAP2 inhibition was established in the B16 melanoma cell line through the use of RNA interference and was assessed by quantitative real-time PCR (qRT-PCR) and Western blot analysis. Wound-healing analysis, transwell assay, and in vivo imaging were performed to investigate the roles of MFAP2 reducing cell mobility, migration, and invasion abilities in vitro and in vivo.

Results: We found substantially higher MFAP2 expression in B16 melanoma cells. The knockdown of MFAP2 inhibited B16 melanoma cells migration and invasion. Western blot analysis was used to assess changes in biomarkers of EMT, indicating the function of MFAP2 in EMT. We found that downregulation of MFAP2 altered the expression of Wnt/β-catenin-linked protein.

Conclusions: Our results suggest that MFAP2 has potential as a molecular target to treat melanoma and suppress metastasis of melanoma cells.

MeSH Keywords: Epithelial-Mesenchymal Transition • Melanoma, Amelanotic • Neoplasm Invasiveness • Transcellular Cell Migration

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Background

Melanoma is the most aggressive malignant tumor and is prone to lymphatic and hematogenous metastasis in the early stage [1,2]. Despite substantial advances in surgery and chemotherapy, melanoma continues to have a high recurrence rate and poor prognosis [3,4]. The metastasis of melanoma is an important cause of treatment failure [5,6]. Understanding the molecular mechanisms underlying metastasis is necessary for improving treatment strategies for this fatal disease.

Microfibril-associated protein 2 (MFAP2), also referred to as microfibril-associated glycoprotein 1 (MAGP1), is a protein component of extracellular matrix microfibrils [7–9]. MFAP2 has been demonstrated to be involved in physiological and pathological mechanisms like elastic fiber formation, vascular and bone integrity, lung disease, and metabolic disease [10–12]. Recent research revealed that MFAP2 significantly contributes to tumorigenesis [13]; for instance, upregulated MFAP2 expression is found in gastric cancer and it is significantly associated with the prognosis of GC patients [14]. Elevated levels of MFAP2 are also observed in head and neck squamous cell carcinoma [15]. However, the underlying function of MFAP2 in the pathology of malignant melanoma is unclear.

In the present study we investigated the role of MFAP2 in melanoma by assessing the biological behavior of MFAP2 silencing in the melanoma cell line and by exploring the underlying mechanisms. We discovered that MFAP2 enhances melanoma cell metastasis potential by EMT via activating the Wnt/β-catenin pathway.

Material and Methods

Cell lines and culture

The mouse B16 melanoma cell line was obtained from the Shanghai Institutes for Biological Sciences (CAS; Shanghai, China), followed by incubation in DMEM (WelGene, Daegu, Korea) that contained 10% fetal calf serum and 100 U/ml penicillin-streptomycin solution (Gibco, North Andover, MA, USA). All cells were cultured in an incubator with 5% CO₂ at 37°C.

Construction of stably transfected melanoma cell line

The shRNA and sh-NC of MFAP2 were synthesized by Sigma Company (Shanghai, China). For construction of the β-catenin vector, the β-catenin cDNA was cloned into Flag tagged-pDNA3.1 (GenePharma, China). With regard to transfecting the MFAP2-shRNA, the transfection of control sh-NC or β-catenin-vector in B16 cells was carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) in accordance with the instructions of the manufacturer.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cell samples using TRizol reagent in accordance with the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). We used the cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to synthesize first-strand cDNA. Moreover, RT-PCR was carried out with the use of SYBR Green PCR Master Mix (Applied Biosystems) to detect the transcript abundance of MFAP2. The following primer pairs were used for MFAP2:

- forward 5’-ATGTCGTAACACCACATGGC-3’ and reverse 5’-GTCGAGGACTCATTGCGT-3’.
- GAPDH: forward 5’-AGGTCCTGTTGAAACGGATTGG-3’ and reverse 5’-GGGTCGCGTTAGGCCAAC-3’.

The calculation of the relative quantification was performed according to the method of 2^-DDCt.

Western blot analysis

Immunohistochemistry (IHC)

Immunohistochemistry was performed using a standard streptavidin-biotin-peroxidase complex methodology (Boster Biological Technology, SA2010). The tissue slides were incubated overnight at 4°C in a moist chamber with MFAP2 (1: 3000, Cell Technology, #13987) antibody. We assessed the MFAP2 expression level through integration of the percent-age of positive tumor cells and intensity of positive staining, scored as: negative=0, borderline=1, weak=2, moderate=3, and strong =4. Staining was scored according to the percentage of positively stained tumor cells: negative=0, 0–25%=1, 26%–50%=2, 51%–75%=3, and 76%–100%=4. We considered the product of the severity and the level score as the final IHC score (values: 0–16). The staining was also evaluated independently by 2 pathologists.

Scratch wound assay

The cells were cultured in 24-well plates at 80% density the night before. After overnight incubation, we scratched the area in the middle of the well with a pipette tip and washed the floating cells with PBS. At 0 and 24 h after wounding, photos
were taken with a light microscope (Nikon, Japan) to assess the wound-healing mechanism.

Cell migration assay

To assess the impact of MFAP2 on the metastasis of cancer, the transwell assay (8 µm pore size, Millipore, MA, USA) was carried out as described earlier. In brief, the cells were added in the upper chambers coated with matrix gel. The bottom culture chambers were filled with DMEM containing 10% FBS. After 48-h incubation, the invaded cells were stained with 0.5% crystal violet and observed using a light microscope (Nikon, Japan).

Tumor metastasis assay

We purchased male BALB/c nude mice (aged 6 weeks) from the Institute for Experimental Animals of the Chinese Academy of Medical Sciences (Beijing, China). Mice were maintained in pathogen-free condition and treated after getting approval from the Animal Care and Use Committee of Anhui Medical University. We intravenously injected 2×10^6 B16 cells transfect-ed with sh-MFAP2 or sh-NC into the tail veins of mice (n=6). After 50 days, mice were sacrificed, followed by excising their lungs. The observation of the lung metastatic nodules was done using a fluorescent imaging system.

Statistical analysis

SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA) software was used for the analysis of statistical data. Differences between the cohorts were assessed using the paired 2-tailed t test. P<0.05 was considered to be statistically significant.

Results

Establishment of MFAP2 silencing B16 melanoma cell line

To investigate the function of MFAP2 in malignant melanoma, the specific knockdown of the expression of MFAP2 was carried out in melanoma cell line B16 using shRNA interference, as assessed by qRT-PCR and Western blotting. As illustrated in Figure 1A, there was a significant decrease in the mRNA (p<0.001) and protein levels of MFAP2 in B16 cells (Figure 1B). The MFAP2 silencing B16 melanoma cell model was successfully established.

MFAP2 modulates the migration and invasion of B16 melanoma cells

To explore the function of MFAP2 in melanoma development, we first carried out the MFAP2 downregulation expression experiments with the use of RNA interfering in the B16 melanoma cell line, as assessed by RT-PCR and Western blot analysis. As evident from Figure 2, the inhibition of MFAP2 in B16 melanoma cell line substantially slowed the closure of the wound region in comparison with their controls in accordance with the wound-healing assay. Additionally, the impact of MFAP2 expression on the cellular invasion potential of B16 melanoma cell (Figure 3). For the validation of the role of MFAP2 in tumorigenesis, we established a lung metastasis model in nude mice through the use of the B16 melanoma cell lines for evaluating the impact of endogenous MFAP2 in vivo metastasis. The living image suggested that the downregulated MFAP2 cells had a significantly decreased capability of forming tumors in lungs as compared with the controls (Figure 4A). Similar results were

![Figure 1. The mRNA and protein levels of MFAP2 in B16 cells with shRNA interference. The mRNA (A) and protein (B) levels of MFAP2 in B16 cells with or without shRNA interference. Data are presented in the form of mean±standard error. GAPDH serve as an internal reference. All experiments were carried out 3 times. ** P<0.01.](image-url)
Figure 2. Representative images of scratch wound assays for B16 cells transfected sh-NC or sh-MFAP2 at 0 and 24 h after transfection (A). Wound healing was quantified by measurement of the average linear speed of movement of the wound edges (B). All experiments were performed 3 times. Data are presented in the form of mean±standard error. ** P<0.01.

Figure 3. Representative images and bar graphs depicting the invasion ability with B16 cells transfected with sh-NC or sh-MFAP2. Data are presented in the form of mean±standard error. Bar=100 um, ** P<0.01.
observed by HE staining (Figure 4B). Taken together, these findings indicated that the downregulated expression of MFAP2 substantially limited the cell migration and invasion abilities in vitro and in vivo.

**MFAP2 improves the EMT in B16 melanoma cells**

Accumulating evidence shows that the migration and invasion of melanoma cells is regulated by the epithelial-mesenchymal transition (EMT). To investigate whether MFAP2 is linked to EMT, the levels of epithelial (E-cadherin) and mesenchymal markers (Vimentin and Snail) in B16 melanoma cells with or without MFAP2 knockdown were assessed. Western blotting analysis indicated that there was an increase in epithelial markers (E-cadherin) in comparison with the controls, whereas there was a significant decline in the mesenchymal marker (Vimentin) in B16 melanoma cells with reduced MFAP2, as shown by Western blot analysis (Figure 5). These results suggest that MFAP2 promotes the development of EMT in B16 melanoma cells.

**Figure 4.** (A) Representative living images of tumors from NOD/SCID mice at 20 days after injection with B16 cells transfected with sh-NC or sh-MFAP2. Data are presented in the form of mean±standard error. **P<0.01.** (B) Representative HE staining images of lung metastatic tumor.
MFAP2 regulates EMT by activating Wnt/β-catenin signaling pathway

Wnt/β-catenin is among the main signaling pathways that contribute to EMT in growth and progression of various cancers. To explore the association of MFAP2 with the Wnt/β-catenin pathway, we first examined the activation of GSK3β and β-catenin. As indicated by the Western blot analysis, MFAP2 interference affected Wnt/β-catenin-linked protein levels. As evident from Figure 5, the level of phosphorylated GSK3β was substantially downregulated by MFAP2 inhibition and β-catenin expression in comparison with the control. To assess the role of Wnt/β-catenin as a crucial pathway that links MFAP2 and EMT, we studied the overexpression of β-catenin in B16 melanoma cells. As verified by Western blot analyses, β-catenin neutralized the effect of MFAP2 on EMT, as suggested by the
 survives this process, which induces the re-expression of epithelial markers [26]. Knowledge of the association between EMT and melanoma is likely to contribute to the identification of new therapeutic targets for melanoma.

MFAP2 is regarded as a constituent of microfibrils and was originally identified in nuchal ligaments [9]. MFAP2 has also been observed to interact with a number of elastic fiber components other than fibrillar proteins, including tropoelastin and decorin, and is thus regarded as an integral part of elastin [28]. MFAP2 is involved in obesity, diabetes, and osteopenia, in addition to head and neck squamous cell carcinoma [10–12]. Wang et al. reported that MFAP2 promotes epithelial-mesenchymal transition in gastric cancer cells by activating the TGF-β/SMAD2/3 signaling pathway [14]. In addition, MFAP2 was reported to be a novel oncogene in GC and it was found that miR-29/MFAP2/ integrin αβ1/FAK/ERK1/2 is an important oncogenic pathway in GC progression [29]. Nonetheless, the molecular role and the prognostic value of MFAP2 have never been reported in melanoma. Studies with large clinical samples are needed and the molecular mechanism remains to be elucidated.

Conclusions

We found that MFAP2 promotes the process of EMT through activation of the Wnt/β-catenin signaling in melanoma cells. Subsequent to the downregulation of MFAP2 expression, not just the metastasis, but also the invasion of melanoma, was inhibited in vitro and in vivo. MFAP2 interference upregulated the expression of E-cadherin and downregulated the expression of vimentin, as determined by Western blot assay. Overexpression of β-catenin neutralized the effect of MFAP2 on EMT, as shown by the changed protein levels of EMT molecular markers in melanoma cells. Collectively, these findings show that MFAP2, which acts as an EMT-inducer, has promise as a target for the anti-metastasis treatment in melanoma.

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