MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e923808
DOI: 10.12659/MSM.923808

Received: 2020.02.25
Accepted: 2020.05.07
Available online: 2020.05.20
Published: 2020.05.28

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

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF Zenghong Chen*
ABCDEF Yang Lv*
BCD Dongsheng Cao
BCD Xiaocan Li
BCD Yuanyi Li

Department of Plastic Surgery, The Second Hospital of Anhui Medical University, Hefei, Anhui, P.R. China

* Zenghong Chen and Yang Lv contributed equally to this work

Corresponding Author: Zenghong Chen, e-mail: chenzenghong0609@126.com
Source of support: Departmental sources

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

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/923808

2015 — 6 29

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

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% CO_2 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-pcDNA3.1 (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'-ATGTCCAGTACAACCACTATGGC-3' and
reverse 5'-GTCCGAGGACTCACTTCTTGG-3'.
GAPDH: forward 5'-AGGTCGGTGTGAACGGATTTG-3' and
reverse 5'-GGGGTCGTTGATGGCAACA-3').
The calculation of the relative quantification was performed according to the method of $2^{-\Delta\Delta Ct}$.

Western blot analysis. For the detection of the protein expression, Western blot analysis was carried out as previously described. The antibodies used were: MFAP2 (Abcam, UK), E-cadherin (Sigma-Aldrich, Chicago, IL, USA), vimentin (Sigma-Aldrich, Chicago, IL, USA), Snail (Sigma-Aldrich, Chicago, IL, USA), β-catenin (Sigma-Aldrich, Chicago, IL, USA), and GAPDH (Vazyme, Piscataway, NJ, USA), using standard chemical luminescence methodology.

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 Signaling Technology, #13987) antibody. We assessed the MFAP2 expression level through integration of the percentage 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

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

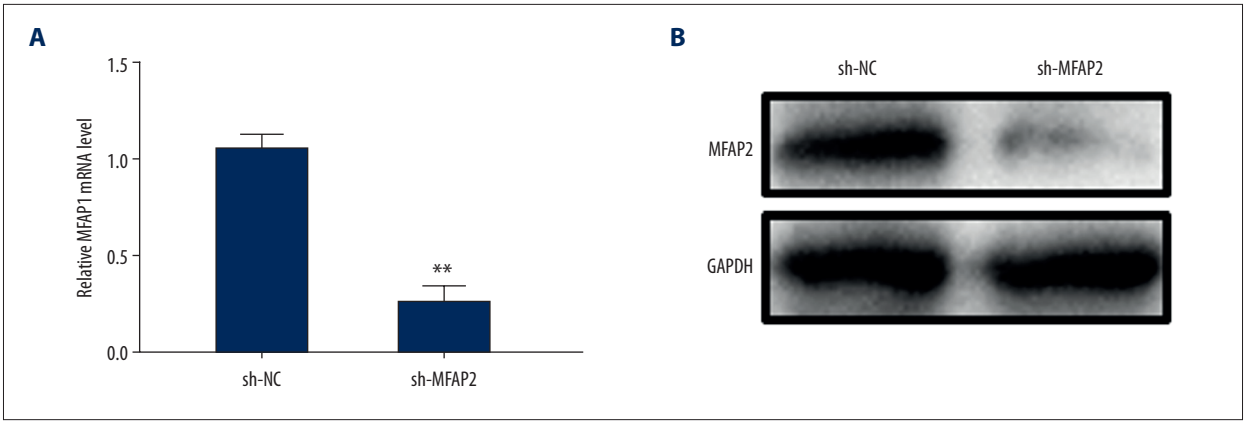

Figure 1. The mRNA and protein levels of MFAP2 in B16 cells with shRNA interference. The mRNA (**A**) and protein (**B**) levels of MFAP2 in B16 cells with or without shRNA interference. Data are presented in the form of mean±standard error. GAPDH serve as an internal reference. All experiments were carried out 3 times. ** P<0.01.

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 transfected 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 line was analyzed as well. In the transwell invasion assay, the cells with the inhibition of MFAP2 manifested an inhibition in the invasion and migration capability in comparison with the control cohort in 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 cells 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

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

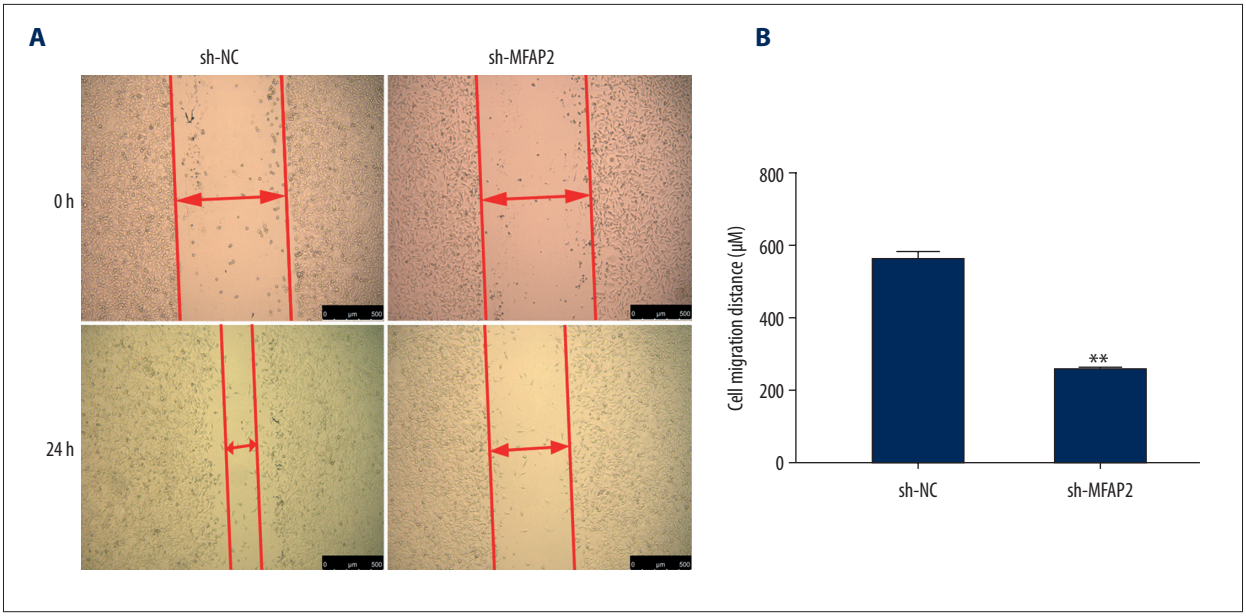

Figure 2. Representative images of scratch wound assays for B16 cells transfected sh-NC or sh-MFAP2 at 0 and 24 h after transfection (**A**). Wound healing was quantified by measurement of the average linear speed of movement of the wound edges (**B**). All experiments were performed 3 times. Data are presented in the form of mean±standard error. ** P<0.01.

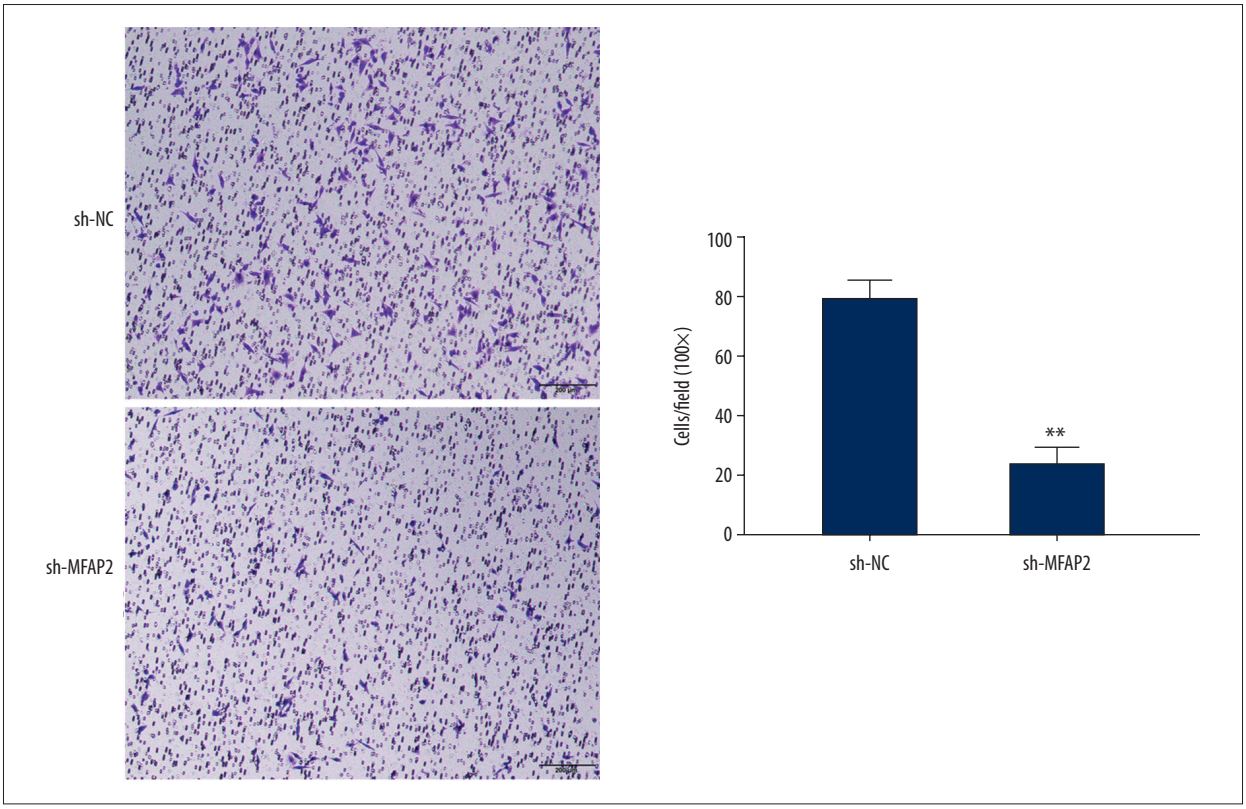

Figure 3. Representative images and bar graphs depicting the invasion ability with B16 cells transfected with sh-NC or sh-MFAP2. Data are presented in the form of mean±standard error. Bar=100 um, ** P<0.01.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

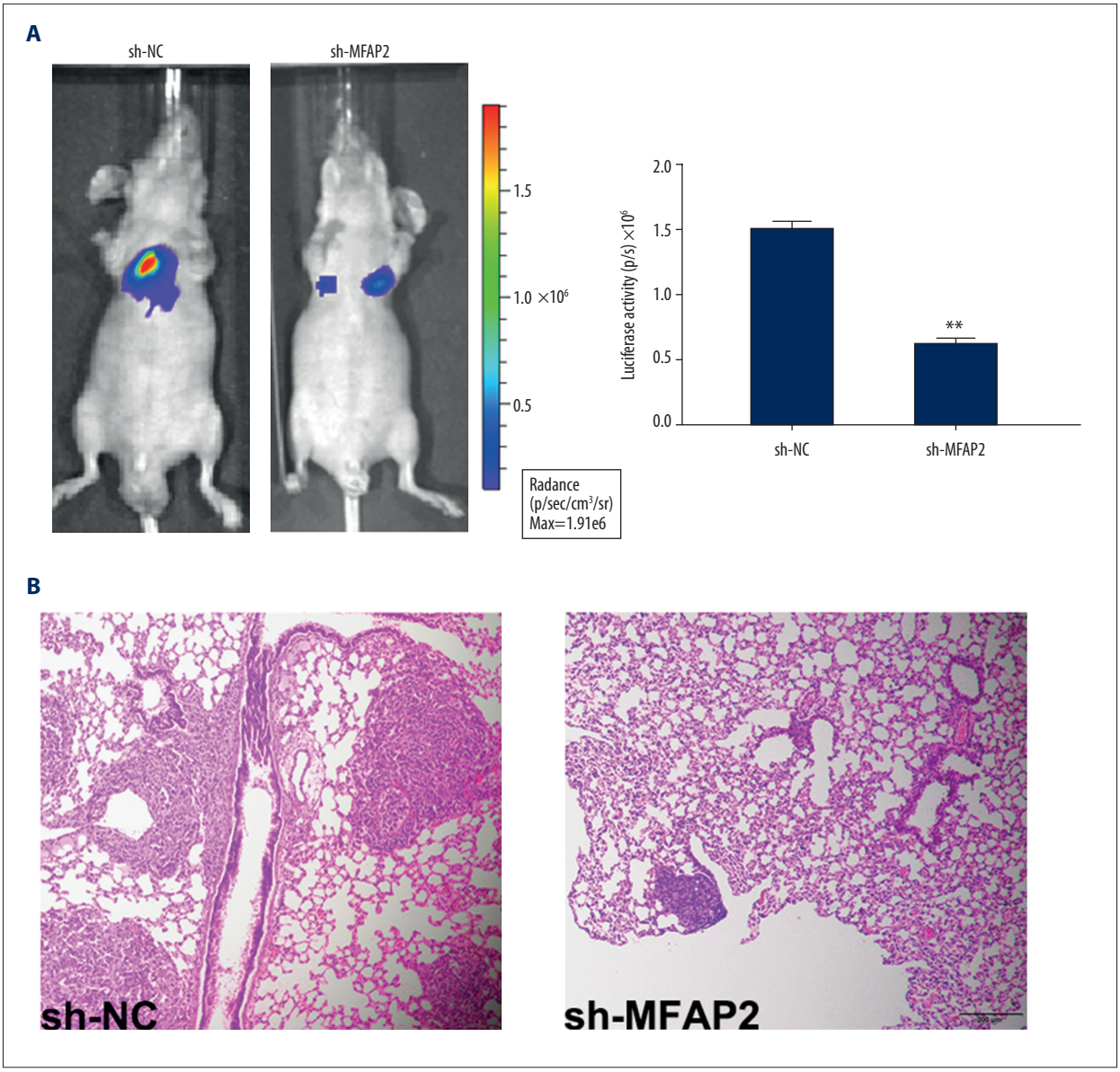

Figure 4. (**A**) Representative living images of tumors from NOD/SCID mice at 20 days after injection with B16 cells transfected with sh-NC or sh-MFAP2. Data are presented in the form of mean±standard error. ** P<0.01. (**B**) Representative HE staining images of lung metastatic tumor.

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.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

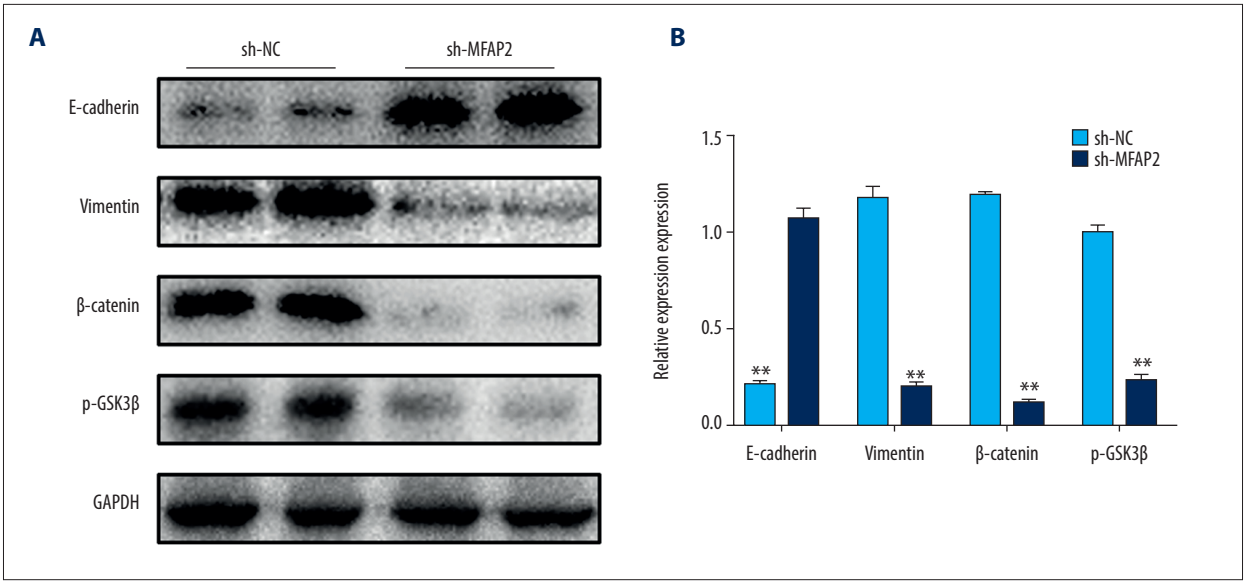

Figure 5. (**A, B**) Representative Western blot and summarized data showing the expression levels of epithelial and mesenchymal related markers, β-catenin and phosphorylated GSK3β with B16 cells transfected with sh-NC or sh-MFAP2. ** P<0.01, versus sh-NC group.

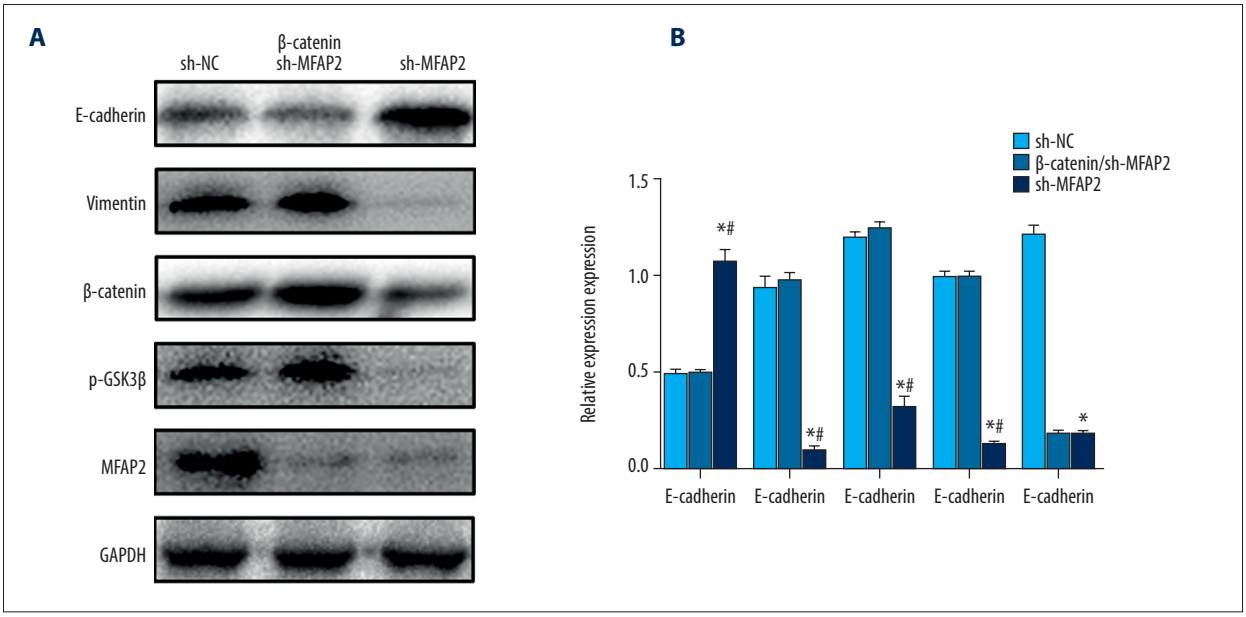

Figure 6. (**A, B**) Overexpression of β-catenin counteracted the effects of MFAP2 on EMT. Representative Western blots and summarized data showing the protein expression levels of E-cadherin, vimentin, β-catenin, and phosphorylated GSK3β and MFAP2 protein in B16 cells. * P<0.05, versus sh-NC groups, # P<0.05 versus β-catenin overexpression groups.

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

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

changed protein levels of EMT molecular markers (Figure 6). Collectively, our study convincingly suggests MFAP2 potentiated EMT through activation of Wnt/β-catenin signaling in the metastasis of melanoma.

Discussion

Melanoma is a skin tumor that has immense potential for invasion and metastasis, especially in the absence of early and effective treatment [16]. Melanoma is the deadliest skin malignancy [17]. The prevalence of malignant melanoma is higher in white people [18]; in South Africa, the prevalence rate is 2.7/100 000 [19], and the rate in China is slightly lower [20]. Nevertheless, the incidence rate in China has doubled in recent years, with about 20 000 new cases every year [21]. Liver metastasis occurs in nearly 90% of patients with advanced malignant melanoma, and brain metastases occur in up to 43% of patients [22,23].

Assessment of EMT shows the transdifferentiation of epithelial cells to mesenchymal cells subjected to particular pathological and physiological situations, which accompany the variations in cell morphology, as well as the associated genes [24,25]. The collective evidence indicates that EMT is involvement in the development and metastasis of melanoma [26]. EMT is to the mechanism by which cells lose their epithelial attributes and acquire a mesenchymal phenotype. The mesenchymal protein expression augments motility, in addition to the invasiveness and metastatic ability of melanoma cells. Furthermore, a number of pathways crucially contribute to promotion of mesenchymal protein expression, which include RAS/RAF/MEK/ERK, together with PI3K/AKT/mTOR, and Wnt/β-catenin [25]. The downstream effectors of these pathways stimulate the expression of EMT transcription determinants, which include Snail, Slug, Twist, and Zeb, promoting epithelial inhibition and induction of mesenchymal characteristics [27]. A growing body of evidence shows that many small-molecule inhibitors and phytochemicals affect EMT progression and may even be able to reverse 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 α5β1/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.

References:

1. Luke JJ, Flaherty KT, Ribas A, Long GV: Targeted agents and immunotherapies: Optimizing outcomes in melanoma. Nat Rev Clin Oncol, 2017; 14(8): 463–82
2. Shain AH, Bastian BC: From melanocytes to melanomas. Nat Rev Cancer, 2016; 16(6): 345–58
3. Suciu S, Eggermont AMM, Lorigan P et al: Relapse-free survival as a surrogate for overall survival in the evaluation of stage II–III melanoma adjuvant therapy. J Natl Cancer Inst, 2018; 110(1)
4. Spain L, Walls G, Julve M et al: Neurotoxicity from immune-checkpoint inhibition in the treatment of melanoma: A single centre experience and review of the literature. Ann Oncol, 2017; 28(2): 377–85
5. Carvajal RD, Schwartz GK, Tezel T et al: Metastatic disease from uveal melanoma: Treatment options and future prospects. Br J Ophthalmol, 2017; 101(1): 38–44
6. Maverakis E, Cornelius LA, Bowen GM et al: Metastatic melanoma – a review of current and future treatment options. Acta Derm Venereol, 2015; 95(5): 516–24
7. Mecham RP, Gibson MA: The microfibril-associated glycoproteins (MAGPs) and the microfibrillar niche. Matrix Biol, 2015; 47: 13–33
8. Craft CS, Broekelmann TJ, Mecham RP: Microfibril-associated glycoproteins MAGP-1 and MAGP-2 in disease. Matrix Biol, 2018; 71–72: 100–11
9. Craft CS: MAGP1, the extracellular matrix, and metabolism. Adipocyte, 2015; 4(1): 60–64
10. Penner AS, Rock MJ, Kielty CM, Shipley JM: Microfibril-associated glycoprotein-2 interacts with fibrillin-1 and fibrillin-2 suggesting a role for MAGP-2 in elastic fiber assembly. J Biol Chem, 2002; 277(38): 35044–49
11. Combs MD, Knutsen RH, Broekelmann TJ et al: Microfibril-associated glycoprotein 2 (MAGP2) loss of function has pleiotropic effects *in vivo*. J Biol Chem, 2013; 288(40): 28869–80

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

12. Turecamo SE, Walji TA, Broekelmann TJ et al: Contribution of metabolic disease to bone fragility in MAGP1-deficient mice. Matrix Biol, 2018; 67: 1–14
13. Villain G, Lelievre E, Broekelmann T et al: MAGP-1 and fibronectin control EGFL7 functions by driving its deposition into distinct endothelial extracellular matrix locations. FEBS J, 2018; 285(23): 4394–412
14. Wang JK, Wang WJ, Cai HY et al: MFAP2 promotes epithelial-mesenchymal transition in gastric cancer cells by activating TGF-beta/SMAD2/3 signaling pathway. Onco Targets Ther, 2018; 11: 4001–17
15. Silveira NJ, Varuzza L, Machado-Lima A et al: Searching for molecular markers in head and neck squamous cell carcinomas (HNSCC) by statistical and bioinformatic analysis of larynx-derived SAGE libraries. BMC Med Genomics, 2008; 1: 56
16. Testori A, Ribero S, Bataille V: Diagnosis and treatment of in-transit melanoma metastases. Eur J Surg Oncol, 2017; 43(3): 544–60
17. Teulings HE, Limpens J, Jansen SN et al: Vitiligo-like depigmentation in patients with stage III-IV melanoma receiving immunotherapy and its association with survival: A systematic review and meta-analysis. J Clin Oncol, 2015; 33(7): 773–81
18. Lee HY, Chay WY, Tang MB et al: Melanoma: Differences between Asian and Caucasian patients. Ann Acad Med Singapore, 2012; 41(1): 17–20
19. Tod BM, Kellett PE, Singh E et al: The incidence of melanoma in South Africa: An exploratory analysis of National Cancer Registry data from 2005 to 2013 with a specific focus on melanoma in black Africans. S Afr Med J, 2019; 109(4): 246–53
20. Lv J, Dai B, Kong Y et al: Acral melanoma in Chinese: A clinicopathological and prognostic study of 142 cases. Sci Rep, 2016; 6: 31432
21. Guo J, Qin S, Liang J et al: Chinese guidelines on the diagnosis and treatment of melanoma (2015 edition). Ann Transl Med, 2015; 3(21): 322
22. Bakalian S, Marshall JC, Logan P et al: Molecular pathways mediating liver metastasis in patients with uveal melanoma. Clin Cancer Res, 2008; 14(4): 951–56
23. Glitza Oliva I, Tawbi H, Davies MA: Melanoma brain metastases: Current areas of investigation and future directions. Cancer J, 2017; 23(1): 68–74
24. Nieto MA, Huang RY, Jackson RA, Thiery JP: Emt: 2016. Cell, 2016; 166(1): 21–45
25. Singh M, Yelle N, Venugopal C, Singh SK: EMT: Mechanisms and therapeutic implications. Pharmacol Ther, 2018; 182: 80–94
26. Pearlman RL, Montes de Oca MK et al: Potential therapeutic targets of epithelial-mesenchymal transition in melanoma. Cancer Lett, 2017; 391: 125–40
27. Serrano-Gomez SJ, Maziveyi M, Alahari SK: Regulation of epithelial-mesenchymal transition through epigenetic and post-translational modifications. Mol Cancer, 2016; 15: 18
28. Gibson MA, Hatzinikolas G, Kumaratilake JS et al: Further characterization of proteins associated with elastic fiber microfibrils including the molecular cloning of MAGP-2 (MP25). J Biol Chem, 1996; 271(2): 1096–103
29. Yao LW, Wu LL, Zhang LH et al: MFAP2 is overexpressed in gastric cancer and promotes motility via the MFAP2/integrin alpha5beta1/FAK/ERK pathway. Oncogenesis, 2020; 9(2): 17

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]