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Mitofusin-2 enhances cervical cancer progression through Wnt/β-catenin signaling

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Overexpression of mitofusin-2 (MFN2), a mitochondrial fusion protein, is frequently associated with poor prognosis in cervical cancer patients. Here, I aimed to investigate the involvement of MFN2 in cervical cancer progression and determine the effect of MFN2 on prognosis in cervical cancer patients. After generating *MFN2*-knockdown SiHa cells derived from squamous cell carcinoma, I investigated the effect of MFN2 on SiHa cell proliferation using the Cell Counting Kit-8 assay and determined the mRNA levels of proliferation markers. Colony-forming ability and tumorigenesis were evaluated using a colony-formation assay and tumor xenograft mouse models. The migratory and invasive abilities associated with MFN2 were measured using wound-healing and invasion assays. Wnt/β-catenin-mediated epithelial-mesenchymal transition (EMT) markers related to MFN2 were assessed through quantitative RT-PCR. *MFN2*-knockdown SiHa cells exhibited reduced proliferation, colony formation, migration, invasion, and tumor formation *in vivo*. The motility of SiHa cells with *MFN2* knockdown was reduced through Wnt/β-catenin-mediated EMT inhibition. MFN2 promoted cancer progression and tumorigenesis in SiHa cells. Overall, MFN2 could serve as a therapeutic target and a novel biomarker for cervical cancer. [BMB Reports 2024; 57(4): 194-199]

INTRODUCTION

Cervical cancer, which is characterized by uncontrolled growth, migration, and infiltration of cells in the cervix, is the second most prevalent form of cancer in women globally (1). The World Health Organization (WHO) classifies cervical cancer as the most common type of gynecological cancer, accounting for 12% of all cancer cases worldwide (2). Various factors, such as high-risk human papilloma virus; sexual, reproductive, and genetic factors; and environmental or lifestyle risks, contribute to cervical cancer development (3). Although cervical cancer is a common type of cancer, its prognosis is poor and the underlying molecular mechanisms that contribute to its development are not well understood.

To identify new biomarkers, organelles involved in cancer development must be determined (4). Mitochondria are closely involved in cancer progression (5). Mitochondria function as stress sensors, enabling cells to adjust to harsh conditions and modify their metabolic environments (6). Thus, the function of mitochondria as stress sensors is crucial for cancer development (7).

Mitofusin-1/2, which is situated in the outer mitochondrial membrane, and optic nerve atrophy 1, which is located in the inner mitochondrial membrane, are essential for the process of mitochondrial fusion (8, 9). Dynamin-associated protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1) are essential factors that play crucial roles in mitochondrial fission (10). High expression or enhanced activation of DRP1 is associated with various cancer phenotypes, such as lung cancer, metastatic breast cancer, glioblastoma, neuroblastoma, colorectal cancer, pancreatic cancer, and melanoma. Moreover, FIS1 overexpression promotes gastric cancer metastasis. Mitochondrial fusion and fission imbalances occur in tumors, and an incomplete mitochondrial network influences cancer cell proliferation. Thus, mitochondrial interactions contribute substantially to cancer progression (11, 12).

MFN2 is associated with cancer progression; however, whether MFN2 is a promoter or suppressor of tumors remains controversial. This appears to be due to differences in cancer cell types and various signaling pathways involved in the progression of cancer. MFN2 serves as a tumor suppressor by utilizing the Ras-NF-κB signaling to regulate cell proliferation and prevent uncontrolled cell growth in HeLa cells (13). *MFN2* knockout in MCF7 and A549 cells markedly increases cancer cell proliferation, colony formation, and invasion (14). Overexpression of MFN2 in bladder cancer cells leads to inhibition of cell

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cycle progression and induction of apoptosis, thereby suppressing the proliferation of cancer cells (15). However, several studies have reported that MFN2 functions as a tumor promoter in cancers, such as gastric cancer (16). *MFN2* knockdown attenuates the migratory and invasive abilities of lung adenocarcinoma cells (17). The expression of MFN2 is substantially elevated in the cervical tissues of women with cervical cancer and has a poor prognosis compared to healthy women (18). MFN2 also functions as a tumor promoter in HeLa cells and cervical cancer patients. Thus, the intricate role of MFN2 in tumor progression suggests the need for additional exploration and clarification.

The role of MFN2 in cervical carcinogenesis has not been adequately investigated. The primary objective of this research was to assess the influence of MFN2 on the proliferation, migration, and invasion of SiHa cells, and to elucidate its mechanism of action in promoting epithelial-mesenchymal transition (EMT) through the Wnt/β-catenin pathway, with the ultimate goal of determining how MFN2 functions as a tumor promoter in the progression of cervical cancer.

RESULTS

Efficient knockdown of *MFN2* by transfection with short hairpin RNA (shRNA) in human cervical cancer cells

Immunocytochemistry confirmed MFN2 expression in SiHa cells (Fig. 1A). *MFN2* knockdown in SiHa cells was achieved through shRNA transfection. Western blotting and qRT-PCR analysis showed that MFN2-shRNA transfection inhibited MFN2 expression at the protein and mRNA levels in SiHa cells compared to that in non-transfected wild-type cells and scrambled shRNA control cells (Scr-shRNA) (Fig. 1B).

MFN2 knockdown attenuates cervical cancer cell proliferation

Compared to that of Scr-shRNA-transfected cells, the viability of MFN2-shRNA-transfected cells was significantly reduced at 48-72 h (MFN2-shRNA-1, $P < 0.05$, $P < 0.01$) and 24-72 h (MFN2-shRNA-2, $P < 0.001$) after transfection (Fig. 1C). Both types of MFN2-shRNA-transfected SiHa cells (MFN2-shRNA-1 and 2) showed significantly decreased mRNA expression of MKI67 (both $P < 0.001$) and PCNA (both $P < 0.001$) relative to the Scr-shRNA group 24 h after transfection (Fig. 1D).

Inhibition of *MFN2* leads to suppression of cervical cancer cell growth

Using a colony-formation assay, I investigated the effect of MFN2 on the ability of SiHa cells to form colonies. Transfection with MFN2-shRNA (MFN2-shRNA-1 and 2) markedly suppressed colony formation in SiHa cells, as measured by colony area (both $P < 0.001$) and colony intensity (both $P < 0.001$) (Fig. 1E). Furthermore, the effect of *MFN2* knockdown on tumorigenesis was examined in a tumor xenograft mouse model. *MFN2* knockdown significantly decreased the tumor volume ($P < 0.01$) and relative tumor weight ($P < 0.001$) of SiHa cells *in vivo*. However, body weight was not affected (Fig. 1F). These results suggest that MFN2 exerts a positive effect on the growth of cervical cancer cells *in vitro* and *in vivo*.

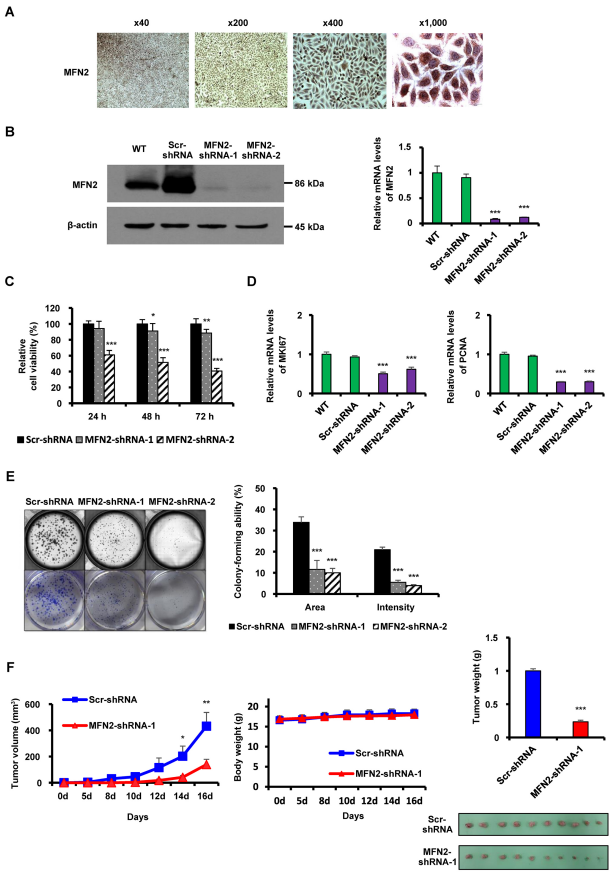

Fig. 1. *MFN2* knockdown inhibited SiHa cell growth. (A) MFN2 expression in SiHa cells was confirmed through immunocytochemistry (original magnification, 40×, 200×, 400×, and 1,000×; scale bar = 10 μm). (B) Protein and mRNA expression of MFN2 were compared between MFN2-shRNA-transfected cells and Scr-shRNA control cells using western blotting. (C) Viability of *MFN2*-knockdown cells was significantly lower than that of Scr-shRNA control cells at the indicated time points. (D) mRNA expression of MKI67 and PCNA was significantly decreased in *MFN2*-knockdown cells compared to Scr-shRNA control cells. The results were analyzed using ANOVA followed by Dunnett's *post-hoc* test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. Scr-shRNA). (E) Colony-forming ability (both area and intensity) was significantly decreased in *MFN2*-knockdown cells compared to that in Scr-shRNA SiHa cells. The results were analyzed using ANOVA followed by Dunnett's *post-hoc* test (***$P < 0.001$ vs. Scr-shRNA). (F) Tumorigenic ability was significantly reduced in MFN2-shRNA-1 SiHa cells compared to that in Scr-shRNA control cells. Results were analyzed using a two-tailed Student's *t*-test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. Scr-shRNA) (n = 10 mice). All experiments were conducted in triplicate.

MFN2 knockdown diminishes the migration and invasion abilities of cervical cancer cells

A wound-healing migration assay was conducted to investigate the effect of MFN2 on cervical cancer cell migration. Relative to the corresponding control group (Scr-shRNA), transfection with MFN2-shRNA (MFN2-shRNA-1 and 2) significantly suppressed the wound closure rate of SiHa cells, as measured at 24 h ($P < 0.001$) after scratch wounding (Fig. 2A). Additionally, a Matrigel invasion assay was conducted to investigate the influence of MFN2 on cervical cancer cell invasion. The invasive ability of MFN2-shRNA-transfected SiHa cells was significantly inhibited (MFN2-shRNA-1, $P < 0.01$; MFN2-shRNA-2, $P < 0.05$) compared with that of the control group (Fig. 2B).

MFN2 knockdown inhibits Wnt/β-catenin-mediated EMT in cervical cancer cells

I evaluated whether there were any differences in the mRNA levels of EMT markers in SiHa cells following MFN2 knockdown. The relative mRNA levels of epithelial markers, Claudin1 (MFN2-shRNA-1, $P < 0.01$; MFN2-shRNA-2, $P < 0.01$), Occludin (MFN2-shRNA-1, $P < 0.01$; MFN2-shRNA-2, $P < 0.01$), and E-cadherin (MFN2-shRNA-1, $P < 0.01$; MFN2-shRNA-2, $P < 0.01$), were significantly increased in MFN2-knockdown groups compared with those in the scrambled shRNA control group (Fig. 3A). Contrastingly, the relative mRNA levels of the mesenchymal markers, N-cadherin (MFN2-shRNA-1, $P < 0.001$; MFN2-shRNA-2, $P < 0.001$), Axin2 (MFN2-shRNA-1, $P < 0.01$; MFN2-shRNA-2, $P < 0.01$), and Snail1 (MFN2-shRNA-1, $P < 0.001$; MFN2-shRNA-2, $P < 0.001$), were significantly inhibited in the MFN2-knockdown groups (Fig. 3B). The relative mRNA levels of the Wnt signaling marker β-catenin (MFN2-shRNA-1, $P < 0.001$; MFN2-shRNA-2, $P < 0.001$) were significantly decreased in the MFN2-knockdown groups (Fig. 3C).

High levels of MFN2 expression are associated with negative prognosis in patients with cervical cancer

Western blotting and reporter assays showed that MFN2-shRNA transfection inhibited β-catenin protein levels and transcriptional activity of TCF/LEF in SiHa cells compared to that in non-transfected wild-type and scrambled shRNA control (Scr-shRNA)

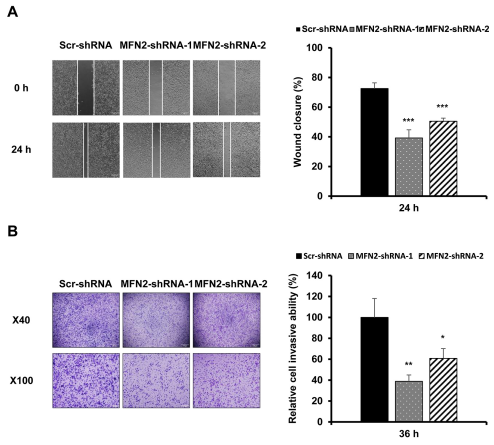

Fig. 2. *MFN2* knockdown attenuated SiHa cell migration and invasion. (A) Migratory ability was significantly attenuated in *MFN2*-knockdown SiHa cells compared to that in Scr-shRNA control cells at the indicated time points (original magnification, 100×; scale bar = 200 μm). Quantitative results indicate the mean ± SD of values from three independent experiments, which were performed in triplicate (n = 9). The results were analyzed using ANOVA followed by Dunnett's *post-hoc* test (***$P < 0.001$ vs. Scr-shRNA). (B) Invasive ability of SiHa cells was significantly attenuated in *MFN2*-knockdown cells compared with that in Scr-shRNA cells (original magnification, 40× or 100×; scale bar = 500 μm or 200 μm). Quantitative results indicate the mean ± SD of values from three independent experiments, which were performed in triplicate (n = 9). The results were analyzed using ANOVA, followed by Dunnett's *post-hoc* test (*$P < 0.05$, **$P < 0.01$, vs. Scr-shRNA).

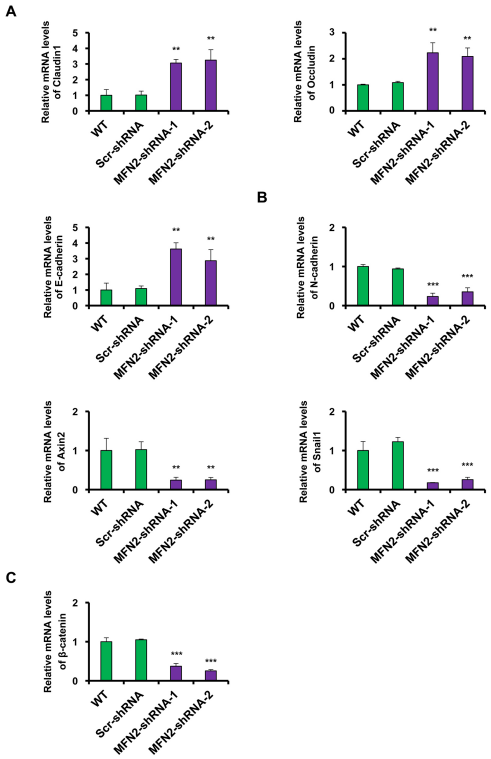

Fig. 3. *MFN2* knockdown suppressed Wnt/β-catenin-mediated EMT in SiHa cells. (A) mRNA expression of Claudin1, Occludin, and E-cadherin was significantly upregulated in MFN2-knockdown cells compared to that in Scr-shRNA SiHa cells. (B) mRNA expression of N-cadherin, Axin2, and Snail1 was significantly downregulated in MFN2-knockdown cells compared to that in Scr-shRNA SiHa cells. (C) mRNA expression of β-catenin was significantly downregulated in MFN2-knockdown cells compared to that in Scr-shRNA SiHa cells. Results were analyzed using one-way ANOVA followed by Dunnett's *post-hoc* test (**$P < 0.01$, ***$P < 0.001$ vs. Scr-shRNA). All experiments were conducted in triplicate.

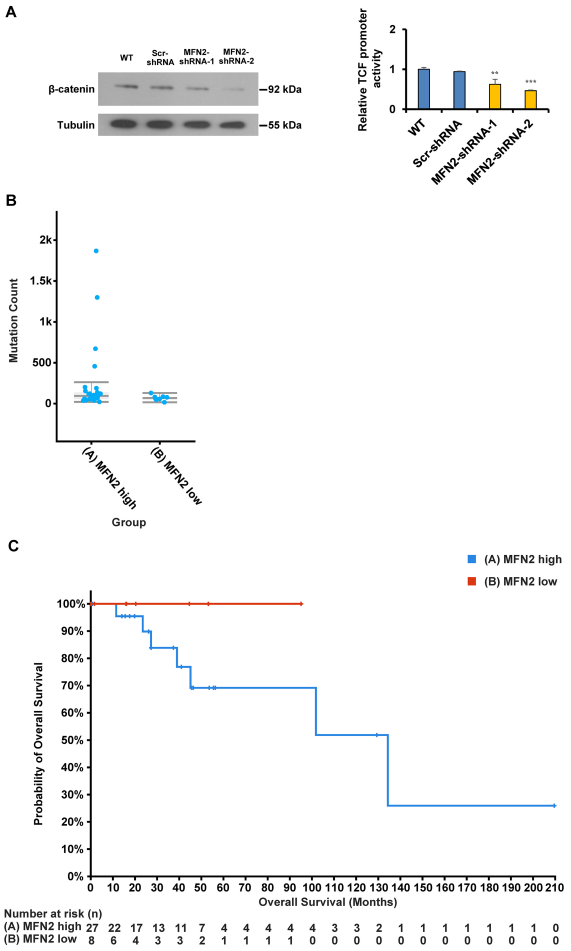

Fig. 4. Cervical cancer patients with high MFN2 expression showed a poor prognosis. (A) β-catenin abundance (left panel) and TCF/LEF reporter (TOPFlash) activity (right panel) were significantly lowered in SiHa cells with MFN2-knockdown compared to that in Scr-shRNA control cells. The results were analyzed using ANOVA followed by Dunnett's *post-hoc* test (**P < 0.01, ***P < 0.001 vs. Scr-shRNA). All experiments were performed in triplicate. In silico data from cervical cancer patients were correlated with MFN2 expression. (B) Comparison of mutation counts between groups with high and low MFN2 expression. (C) The overall survival curve for cervical cancer patients showing high and low MFN2 expression was obtained using the cBioPortal (https://www.cbioportal.org/).

groups (Fig. 4A). To determine the effects of high or low MFN2 expression in cervical cancer patients, a survival curve for these patients with high or low MFN2 mRNA expression was plotted using cBioPortal (https://www.cbioportal.org/). Human cervical cancer patient samples (n = 275) were obtained from the cervical squamous cell carcinoma dataset of TCGA and PanCancer Atlas. The mutation count was greater in the group with high MFN2 expression than that in the group with low MFN2 expression (Fig. 4B). As shown in Fig. 4C, patients with cervical cancer with elevated levels of MFN2 expression had a more adverse prognosis than those with lower MFN2 expression levels. These findings suggest that increased levels of MFN2, facilitated by β-catenin, are associated with negative outcomes in cervical cancer patients.

DISCUSSION

In this research, MFN2 demonstrated increased proliferation, colony formation, motility, and tumor growth in SiHa cells. Additionally, MFN2 stimulated Wnt/β-catenin-mediated EMT in cervical cancer cells. EMT is a critical biological process that plays a significant role in the initiation and progression of cancer, particularly metastasis (19). A hallmark of EMT is the loss of E-cadherin, typically an epithelial marker, and an increase in the expression of mesenchymal markers, such as N-cadherin and vimentin, accompanied by an invasive phenotype (20).

The proliferation assay demonstrated that cell viability of MFN2-shRNA SiHa cells gradually reduced over time compared to that of the scrambled shRNA group. RT-qPCR, which utilizes proliferation markers, such as *PCNA* and *MKi67*, revealed that MFN2 enhanced the proliferation of SiHa cells. Moreover, using a colony-formation assay and tumor xenograft model, I discovered that MFN2 promoted the proliferation of SiHa cells in both *in vitro* and *in vivo*. Similarly, I showed that MFN2 increased both motility and EMT in SiHa. Wnt signaling-related genes, such as *Snail1*, also showed reduced expression in MFN2-shRNA-transfected SiHa cells. Snail is an E-cadherin transcriptional repressor. Snail1 directly binds to E-box sequences in the E-cadherin promoter region, thereby suppressing its transcription (21).

Various signaling pathways have been implicated in MFN2 expression. The canonical Wnt signaling pathway is an important pathway dependent on β-catenin transcription (22). In breast and lung cancer cells, MFN2 suppresses tumor progression by inhibiting mTORC2/Akt signaling (14). Mitochondrial dynamics are highly dysregulated in cancer cells. microRNA-195 (miR-195) regulates the mitochondrial dynamics of breast cancer cells by targeting MFN2. This results in impaired mitochondrial function (23). Hippo-YAP, a tumor promoter in gastric cancer, is mediated by activation of the SIRT1/MFN2/mitophagy axis, could be applied in gastric cancer treatment, which is associated with cancer survival and migration (24). MFN2 has been shown to restrict the incidence of chronic rejection in the rat abdominal aorta by regulating the activity of the TGF-β/Smad pathway (25). The tumor suppressor p53 is responsible for controlling the transcription of several target genes that are involved in cell cycle arrest, apoptosis, DNA repair, and other important cellular responses. *MFN2* is a novel p53-induced target gene that provides insights into the regulation and related activities of MFN2 in the inhibition of cell proliferation, promotion of apoptosis, and tumor suppression (26).

In the canonical Wnt signaling pathway, Wnt activates

downstream signals by stabilizing β-catenin and enabling its binding to transcription factors such as T-cell factor (TCF) (27). Engagement in the canonical Wnt pathway results in generation of a β-catenin-TCF transcription complex, which triggers the EMT that characterizes the tissue invasion phenotype (28). The alteration of *MFN2* is linked to the Wnt signaling pathway, and among the genes implicated, the glycogen synthase kinase-3β/adenomatous polyposis coli (GSK-3β/APC) gene ranks first in cervical cancer. GSK-3β and APC play crucial roles in canonical Wnt signaling. These genes are involved in β-catenin destruction by forming a β-catenin destruction complex with casein kinase 1 and Axin upon inactivation of the Wnt signaling pathway (29).

Wnt proteins bind to the LRP co-receptor and the Frizzled receptor and act in concert with Dishevelled to suppress the activity of GSK-3β. This avoids degradation by preventing GSK-3β from phosphorylating β-catenin, among other substrates, which results in accumulation of β-catenin in the cytoplasm and nucleus. In the nucleus, β-catenin is implicated in the transcription of Wnt target genes through its interaction with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) (30). Therefore, the proliferation, growth, and metastasis of SiHa cells are regulated by MFN2, which activates the Wnt signaling pathway. This was determined to be the mechanism by which MFN2 promoted the growth and metastasis of cervical cancer cells. I demonstrated that MFN2 promotes Wnt/β-catenin-mediated EMT in cervical cancer cells. EMT enhances the metastasis, chemical resistance, and stemness of various cancers. Additionally, β-catenin-mediated increase in MFN2 expression is likely associated with negative prognosis in patients with cervical cancer.

In conclusion, MFN2 serves as a tumor promoter that enhances cervical cancer progression through Wnt/β-catenin signaling. This study proposed that MFN2 could potentially serve as a novel target for the development of therapeutic strategies for cervical cancer. In the future, I plan to conduct further research on MFN2 expression to uncover new mechanisms that contribute to the understanding and treatment of cervical cancer.

MATERIALS AND METHODS

Supplementary data

The online version contains materials and methods available.

ETHICAL STATEMENT

All animal experiments were approved by the Seoul Metropolitan Government, Seoul National University Boramae Medical Center Institutional Animal Care and Use Committee (IACUC No. 2023-0033).

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CONFLICTS OF INTEREST

The author has no conflicting interests.

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