Mitofusin-2 facilitates EMT-induced cervical cancer development

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Directed by Professor Young-Min Hyun

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송지원 드림

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Mitofusin-2 facilitates EMT-induced cervical cancer development

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High expression of Mitofusin-2 (MFN2), a mitochondrial fusion protein, has been frequently associated with poor prognosis of patients with cervical cancer. We aimed to identify the function of MFN2 to investigate how MFN2 is involved in the carcinogenesis of cervical cancer to understand its influence on the prognosis of the disease.

After generating MFN2 knockdowned HeLa cells which are originated from cervical adenocarcinoma, we conducted MTT assay and quantitative reverse transcription PCR (RT-qPCR) to investigate how MFN2 influences the proliferation of HeLa cells. The ability of colony-formation and tumorigenesis was evaluated through colony formation assay and tumor xenograft mouse model. The migration ability related to MFN2 was measured by wound-healing assay. Then, epithelial-mesenchymal transition (EMT) markers related to MFN2 were assessed by quantitative reverse transcription PCR (RT-qPCR). Finally, clinical data were analyzed using cBioPortal and the human genome ATLAS.

Here, we showed that MFN2 knockdowned HeLa cells reduced proliferation, colony formation, migration, and in vivo tumorigenesis. Especially, migration of MFN2 knockdowned HeLa cells decreased through suppression of EMT. We concluded that MFN2 facilitates cancer

progression and in vivo tumorigenesis in HeLa cells. Moreover, clinical data were analyzed using cBioPortal and the human genome ATLAS. This analysis showed that high MFN2 expression induces high mutation counts and low survival rate in patients with cervical cancer, and MFN2 was highly involved in APC/β-catenin related to the Wnt signaling pathway.

In summary of this study, we suppose that MFN2 might act as a therapeutic target for patients with cervical cancer, but also it is expected to exhibit as a new biomarker for cervical cancer.

Keywords: Mitofusin-2, oncogene, cervical cancer development, therapeutic target, epithelial-mesenchymal transition (EMT)

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I. INTRODUCTION

Cervical cancer is involved in unregulated cell proliferation, migration, and invasion of the uterine cervix[1]. Globally, it is the second most common cancer among women[1]. According to the World Health Organization (WHO), cervical cancer comprises 12% of all cancers worldwide and is the most common gynecological malignancy in the world[2]. The human papilloma virus (HPV) poses a high risk for the disease, as do various sexual and reproductive factors, environmental and lifestyle factors, as well as genetic factors[3]. The incidence rate is remarkably high in low income and developing countries[4]. Despite its high prevalence, the prognosis of cervical cancer remains poor, and its molecular mechanisms of progression remain obscure.

It is essential to search for related organelles that are deeply involved in cancer development to discover target genes for a new biomarker[8]. It has been reported that mitochondria are examined deeply relate to cancer development[9]. Mitochondria have an important function in the production of adenosine triphosphate (ATP) which is necessary for the normal metabolic activity of cells. Mitochondria have several other functions including the generation of reactive oxygen species (ROS) and redox molecules, metabolites, and the regulation of cell signaling, cell death, and biosynthetic metabolism. When these various functions are combined, the mitochondria can serve as a stress sensor, helping cells adapt to harsh environments, and altering the metabolic environments by nutrient depletion, hypoxia, and chemotherapy[10] to suppression cancer-cell development and proliferation. Therefore, the function of mitochondria as a stress sensor is very important in tumoigenesis[11,12].

The processes by which mitochondria alters the various environmental conditions of a cell is quite dynamic, allowing the organelle to change its shape[14]. When there is insufficient nutrients or a cell is under mild stress, the damaged part of one mitochondrion and the normal part of another merge through ‘fusion’, to form a single enlarged mitochondria[12]. Fusion is beneficial for the production of higher ATP and for exchanging metabolites with mitochondrial DNA (mtDNA). When mitochondria are injured under extreme stress, such as stress caused by tumors with reduced cellular respiration, they divide into tiny fractions through a process known as ‘fission’ and are removed by mitophagy to maintain homeostasis[13]. With more persistent stress, mitochondria disappear through apoptosis[14]. Mitofusin-1/2 (MFN1/2), which is located in the outer mitochondrial membrane (OMM), and the optic atrophy1 (OPA1), which is located in the inner mitochondrial membrane (IMM), are both essential for mitochondrial fusion[15,16]; and dynamin-related protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1) are important for mitochondrial fission[13]. An imbalance of mitochondrial fusion and fission has been reported to occur in tumors, and an incomplete network of mitochondria has been shown to influence cancer cell proliferation. Taken together, this indicates that mitochondrial interaction substantially contributes to tumorigenesis (Figure 1)[17,18].

MFN2 has a vital function in mitochondrial fusion and maintains the activity of the mitochondria[19-21]. OMM GTPase MFN2 was initially recognized in the vascular smooth muscle cells of spontaneously hypertensive rats[22]. Several MFN2 mutations are associated with Charcot-Marie-Tooth disease type 2A[23]. Altered MFN2 expression is also related to various pathological conditions, including diabetes[24], obesity[25], atherosclerosis[26], Alzheimer's disease[27], Parkinson's disease[28], cardiomyopathy[29], and cancer[30]. Although recent large studies have reported that MFN2 is associated with cancer progression, its specific function remains unclear. Several reports indicated that MFN2 is a tumor suppressor; declined MFN2 expression levels have been reported in lung[31], colorectal[30], gastric[32], and liver[33]. In a recent study, MFN2 knockout from MCF7 and A549 cells remarkably elevated the proliferation, colony formation, and invasion of cancer cells[34]. It has also reported that the overexpression of MFN2 in urinary bladder carcinoma cell lines inhibits the proliferation of cancer cells by inducing cell-cycle arrest and apoptosis[35]. One recent study reported that MFN2 functions as an oncogene in gastric cancer[36], and another report indicated that MFN2 knock down attenuates the migration and invasion ability of lung adenocarcinoma cells[37]. Therefore, further research is required to investigate the influence of MFN2 on carcinogenesis and cancer progression.

It has been reported that the expression of MFN2 is higher in the cervical tissue of patients with cervical cancer with poor prognosis than in the cervical tissue of healthy women[38]. *In vitro* experiments showed that proliferation, migration, and invasion of cervical cancer cells were decreased when MFN2 was knockdowned by siRNA in HeLa cells relative to that of wild type[38]. HeLa cells have a demonstrated association with MFN2 expression in cervical cancer pathogenesis.

We recently reported that MFN2 may be involved in cervical cancer pathogenesis as an oncogene. We employed different methods using small-interfering RNA (siRNA) to knock down MFN2 in HeLa cells[38]. However, the MFN2 knockdown system using siRNA was not suitable for long-term experiments because it induces a transient decrease in MFN2 expression. For this reason, in the current study, we performed long-term experiments such as the tumor xenograft model through the MFN2 knockdown system using short-hairpin

RNA (shRNA). The secondary aim of the current study is to investigate and well demonstrate the correlation between MFN2 expression and cervical cancer development via a diverse series of assessment methodologies.

Whether MFN2 is a cervical cancer oncogene or a tumor suppressor gene is still under contention. MFN2 functions as a tumor suppressor through Ras-NF-kappaB signal pathway and apoptosis in HeLa cells[39,40]. On the other hand, MFN2 functions as an oncogene in HeLa cells and cervical cancer patients[38]. Here, we investigated the effects of MFN2 on the proliferation and migration of cervical cancer cells and examined its mechanisms of action within the cancer signaling pathway to determine how the MFN2 acts as an oncogene in cancer progression.

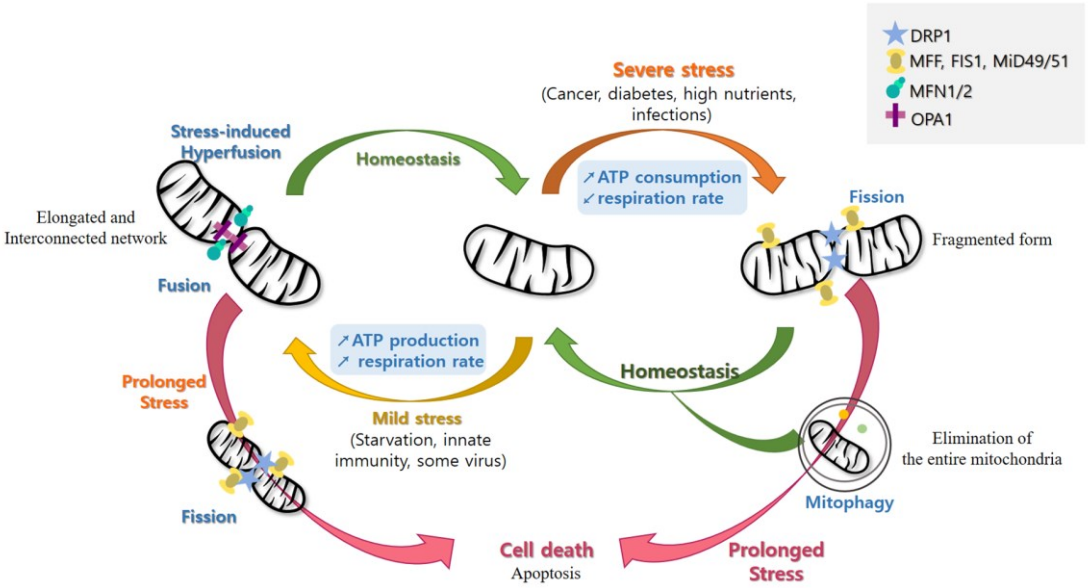

Figure 1. Morphodynamics of the mitochondria.

II. MATERIALS AND METHODS

1. Cell culture of MFN2 knockdown HeLa cells

HeLa cell lines (ATCC® CCL-2™) which originated from adenocarcinoma were cultured in RPMI-1640 medium (Cytiva, MA, USA) containing 10% fetal bovine serum (Atlas Biologicals, CO, USA), penicillin (100 U/ml), and streptomycin-amphotericin B (0.1 mg/ml) (Lonza, Switzerland)[41]. Puromycin (2 μg/ml) (Sigma-Aldrich, MO, USA) was added to the medium to maintain RNA interference of the HeLa cell lines. The cells were incubated at 37℃ with 5% CO_2.

2. shRNA treatment

For shRNA treatment, HeLa cells were transduced with two MFN2-human shRNA lentiviral particles (TL311490VA_ GAGGTTCTTGACTCACTTCAGAGCAAAGC and TL311490VB_ TCAAGTGAGGATGTTTGAGTTTCAGAATT), and a scrambleed lentiviral shRNA was used as a negative control (TR30021V; OriGene, South Korea). MFN2 knockdown efficiency was examined by western blotting and RT-qPCR.

3. Proliferation assay

Proliferation assay was conducted to investigate whether the expression of MFN2 influenced the viability of HeLa cells[42]. The experiment was conducted at three time points from seeding HeLa cells: 24 hours, 48 hours, and 72 hours. HeLa cells were seeded 3×10^3 cells per well in 96-well plates. Thiazolyl Blue Tetrazolium Bromide (MTT) solution (Amresco, OH, USA) was diluted with serum-free RPMI-1640 and put in the wells. The plates were incubated at 37℃ with 5% CO_2 for 3 hours under

light-blocking conditions. When water-insoluble formazans were formed, they were solubilized with DMSO (VWR, PA, USA) for 15 minutes on a shaker. Then absorbance was measured at 540 nm by ELISA Microplate Reader with SoftMax® Pro Software (Molecular Devices, CA, USA).

4. Colony formation assay

Colony formation assay was conducted to determine whether MFN2 expression affected the colony forming ability of HeLa cells[43]. HeLa cells were seeded at 800 cells per well in 6-well plates and RPMI-1640 media containing puromycin (2 μg/ml) was changed every other day. The plates were incubated at 37℃ with 5% CO_2 for 10 days. Formed colonies were fixed with 95% ethanol (Sigma-Aldrich, MO, USA) at 4℃ overnight, then stained with 0.25% crystal violet (Sigma-Aldrich, MO, USA) diluted with 20% methanol (Merck, Germany) at RT overnight. Microscopy was performed using an inverted research microscope ECLIPSE Ti2 Series (Nikon, Japan). Quantification was automatically performed by ImageJ-plugin, ColonyArea [44].

5. Migration assay

Migration assay was conducted to determine whether MFN2 expression affects the migration ability of HeLa cells[45]. Because the proliferating rate of HeLa cells in which MFN2 was knocked down was slower than the control group, the number of cells seeded was different in each group to make a 80~90% confluent monolayer: HeLa cells were seeded in 24-well plates at 2×10^5 cells/well for the Scr-shRNA group, 3×10^5 cells/well for the MFN2-shRNA-1 group, and 4×10^5 cells/well for MFN2-shRNA-2 group. The plates were incubated at 37℃ with 5% CO_2 overnight. After the HeLa cells reached an 80~90% monolayer, wounds were made by 200p tips sterilized with an

alcohol lamp. Photos were taken at the 0 hour, 24 hours, and 30 hours time points using Olympus CellSens Software (Olympus, Japan).

6. Western blotting

Western blotting was conducted to determine whether MFN2 was knocked down in HeLa cells at the protein level[46]. HeLa cell proteins were extracted using RIPA lysis buffer (Bio-Rad, CA, USA) then mixed with 4X Laemmli sample buffer (Bio-Rad, CA, USA) and boiled for 5 minutes at 95℃. SDS-PAGE was used to separate protein samples by molecular weight. Protein samples were transferred onto nitrocellulose membranes (Bio-Rad, CA, USA). After blocking for 1 hour at RT, membranes were incubated at 4℃ overnight with the MFN2 primary antibodies (Abcam, MA, USA) and β-actin (Cell Signaling, MA, USA) at a 1:1000 dilution in 5% blocking solution. Each sample was incubated in the corresponding secondary antibodies, and then protein bands were visualized using an enhanced chemiluminescence detection system (GW Vitek, South Korea).

7. Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was used to confirm the mRNA levels of various markers in HeLa cells[47]. mRNA from HeLa cells was prepared using TRIzol™ reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using AccuPower RT PreMix (Bioneer, South Korea) according to the manufacturer's protocol. qPCR was performed using the StepOnePlus Real-Time PCR system with TB Green Premix Ex Taq II (Takara, Japan).

Table 1. Sequences of primers

	Forward primer (5'-3') Reverse primer (5'-3')	Class
MFN2	GACCTTTGCTCATCTGTGTC TCCAACCAACCGGCTTTATTC	Target gene
β-actin	ATTGATTCGAAACCTTGCCC AGCTCCAGTACACCCTTCTA	House-keeping
PCNA	GGCGTGAACCTCACCAGTAT TTCTCCTGGTTTGGTGCTTC	Proliferation
MKi67	AAGCCCTCCAGCTCCTAGTC GCAGGTTGCCACTCTTTCTC	
Claudin	GGCTGCTTTGCTGCAACTGTC GAGCCGTGGCACCTTACACG	Epithelial
Occludin	CGGTCTAGGACGCAGCAGAT AAGAGGCCTGGATGACATGG	
E-cadherin	GGTTTTCTACAGCATCACCG GCTTCCCCATTTGATGACAC	
N-cadherin	TGAAACGGCGGGATAAAGAG GGCTCCACAGTATCTGGTTG	Mesenchymal
Axin2	AAGGGCCAGGTCACCAAAC CCCCCAACCCATCTTCGT	
Snail	TCTCTGAGGCCAAGGATCTC CTTCGGATGTGCATCTTGAG	Promotes mesenchymal

8. Tumor xenograft model

All experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (IACUC 2017-0353). Female BALB/c nude five-week-old mice were purchased (Orient Bio, South Korea). All mice were maintained under specific-pathogen-free systems in the animal establishment at Avison Biomedical Research Center of Yonsei University College of Medicine. Tumor models were generated in the flank of the mice by subcutaneous injection of 3×10^6 cells. Tumor volumes were measured by caliper once every 2 days and calculated with the formula: V (mm^3) = (L x W^2)/2 where V is tumor volume, W is tumor width, and L is tumor length. For puromycin treatment, 10 mg/kg puromycin was subcutaneously injected every 2 days, for 18 days. Mice were sacrificed by inhalation of CO_2 at the end of the experiment.

9. Bioinformatics

The effect of MFN2 expression on the prognosis of cervical carcinoma was analyzed using the cBioPortal and the Human Genome Atlas for cancer genomics. Information about cervical squamous cell carcinoma and endocervical adenocarcinoma was obtained from The Cancer Genome Atlas (TCGA) and Firehose Legacy. Genomic profile, mutations and putative copy-number alterations were selected from genomic identification of significant targets in cancer (GISTIC), mRNA expression z-scores relative to diploid samples were also selected. Mutation count, related signaling pathways, and overall survival rates of patients with cervical cancer were analyzed by cBioPortal according to MFN2 expression level.

10. Statistical analysis

Statistical analyses were performed using SPSS Statistics for Windows, Version 26.0 (IBM, NY, USA). Statistical significance of differences among groups was determined by a one-way ANOVA with Dunnett's post hoc test (for proliferation, migration, and colony formation assays). Data are expressed as means and standard deviation (SD) of at least three independent experiments. The statistical significance of animal experiments was determined using the two-tailed Student's t-tests. Data are expressed as mean and standard error of the mean (SEM) for tumor volume.

III. RESULTS

1. Expression level of MFN2 in HeLa cells

We confirmed that the expression level of MFN2 significantly decreased in MFN2-shRNA HeLa cells compared to scrambled-shRNA both in the protein level and the mRNA level (Figure 2).

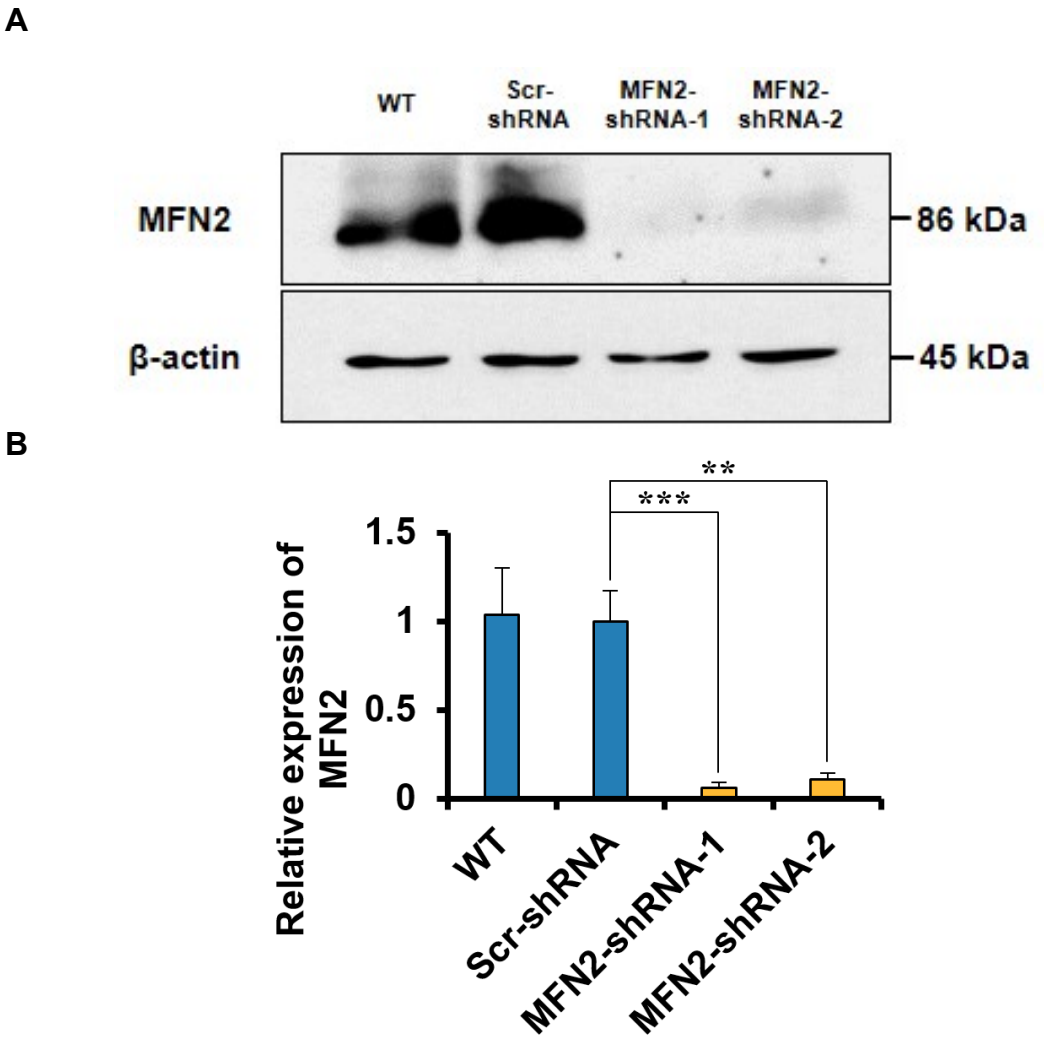

Figure 2. Expression level of MFN2 in HeLa cells. (A) MFN2 expression decreased in MFN2-shRNA groups compared to WT and Scr-shRNA in the protein level. (B) MFN2 expression decreased in MFN2-shRNA groups compared to WT and Scr-shRNA in the mRNA levels. $^{**}p<0.01$, $^{***}p<0.001$, one-way ANOVA analysis.

2. Proliferation ability reduced in MFN2 knockdown HeLa cells

The viability reduced significantly in MFN2-shRNA HeLa cells compared to the Scr-shRNA. We estimated the average measuring the three time-points from seeding HeLa cells (24 hours, 48 hours, and 72 hours), and performed in triplicate (Figure 3). In addition, the colony-forming ability of MFN2 knockdown HeLa cells meaningfully decreased than that of Scr-shRNA (Figure 4).

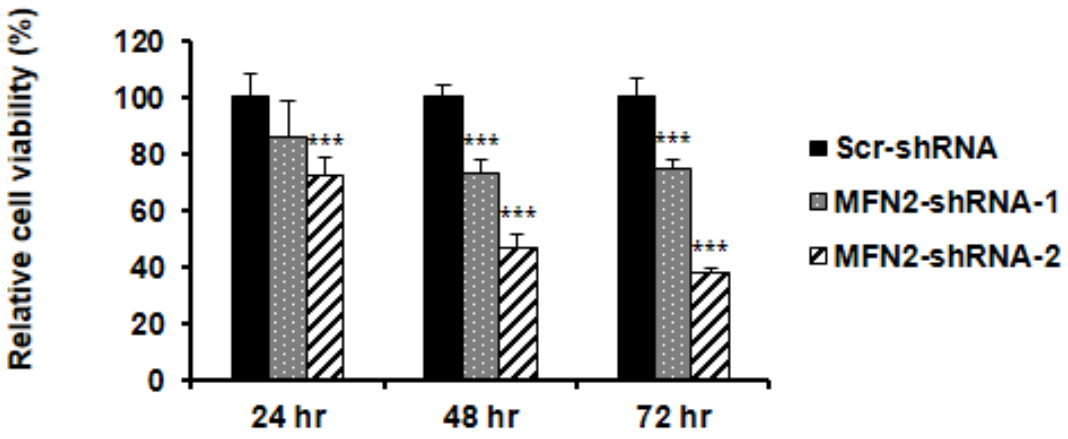

Figure 3. Proliferation ability reduced in MFN2 knockdown HeLa cells *in vitro*. The overall viability of MFN2-shRNA reduced compared to the Scr-shRNA. ***p<0.001, One-way ANOVA analysis.

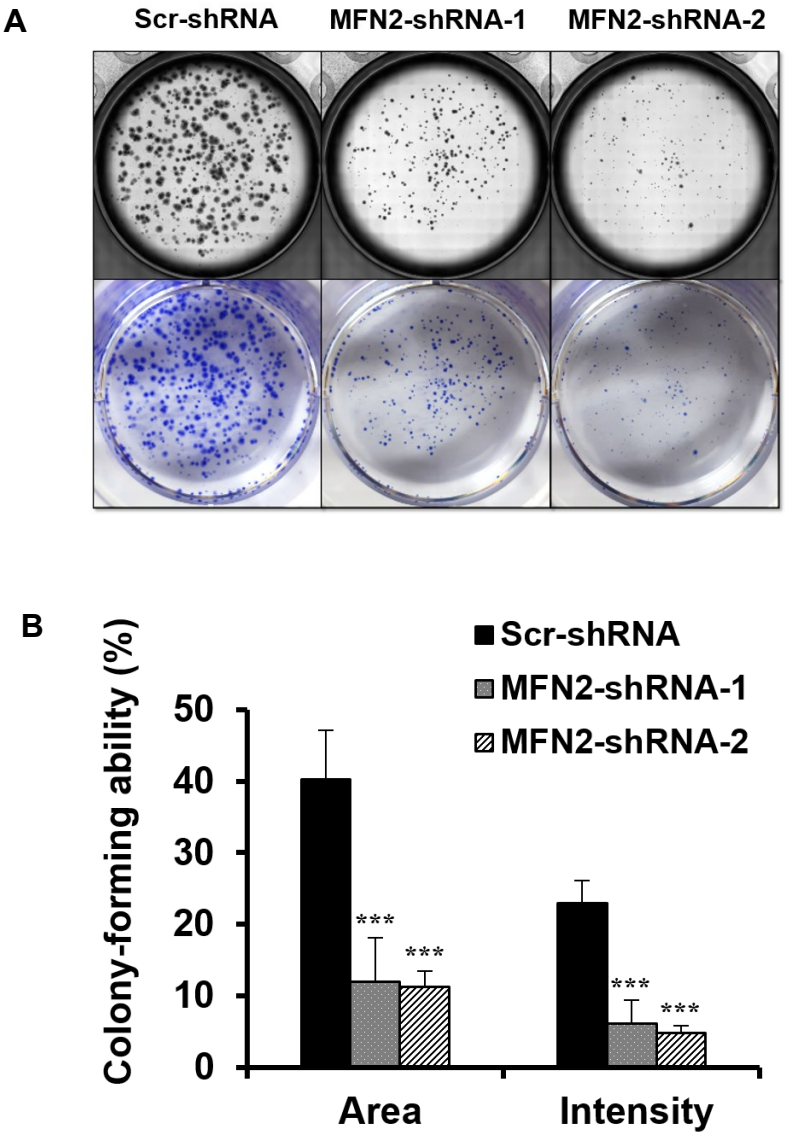

Figure 4. Colony-forming ability decreased in MFN2 knockdown HeLa cells *in vitro*. (A) The colony-forming ability of MFN2-shRNA groups diminished significantly compared to that of Scr-shRNA. The upper image is differential interference contrast (DIC), and the bottom image is a digital camera. (B) We analyzed results through ImageJ: ColonyArea plugin. Both colony area and intensity reduced meaningfully in MFN2-shRNA groups than that of Scr-shRNA. ***$p<0.001$, One-way ANOVA analysis.

After confirming the effect of MFN2 on proliferation ability by phenotype, we decided to confirm that MFN2 influences the mRNA levels through RT-qPCR. The mRNA expression of Ki67 and the proliferating cell nuclear antigen (PCNA) significantly decreased in MFN2-shRNA groups than the Scr-shRNA HeLa cells (Figure 5). Accordingly, we discovered that MFN2 promotes the cell viability, proliferation, and colony-forming ability of HeLa cells *in vitro*.

Moreover, we generated xenograft mouse models using HeLa Scr-shRNA and MFN2-shRNA-1 to unveil the correlation between MFN2 and the tumorigenesis *in vivo*. Tumor volume reduced significantly in MFN2-shRNA-1 than that of Scr-shRNA from 8 days after injection of HeLa cells to the mouse xenograft models (Figure 6A). Tumor weight also decreased meaningfully in MFN2-shRNA-1 compared to that of Scr-shRNA at 18 days after injection of HeLa cells to the mouse xenograft models (Figure 6B). There was no difference in the body weight between the two groups during 18 days from the injection of HeLa cells to the mouse (Figure 6C). Through the above results, we determined that MFN2 facilitates tumorigenesis of HeLa cells *in vivo*.

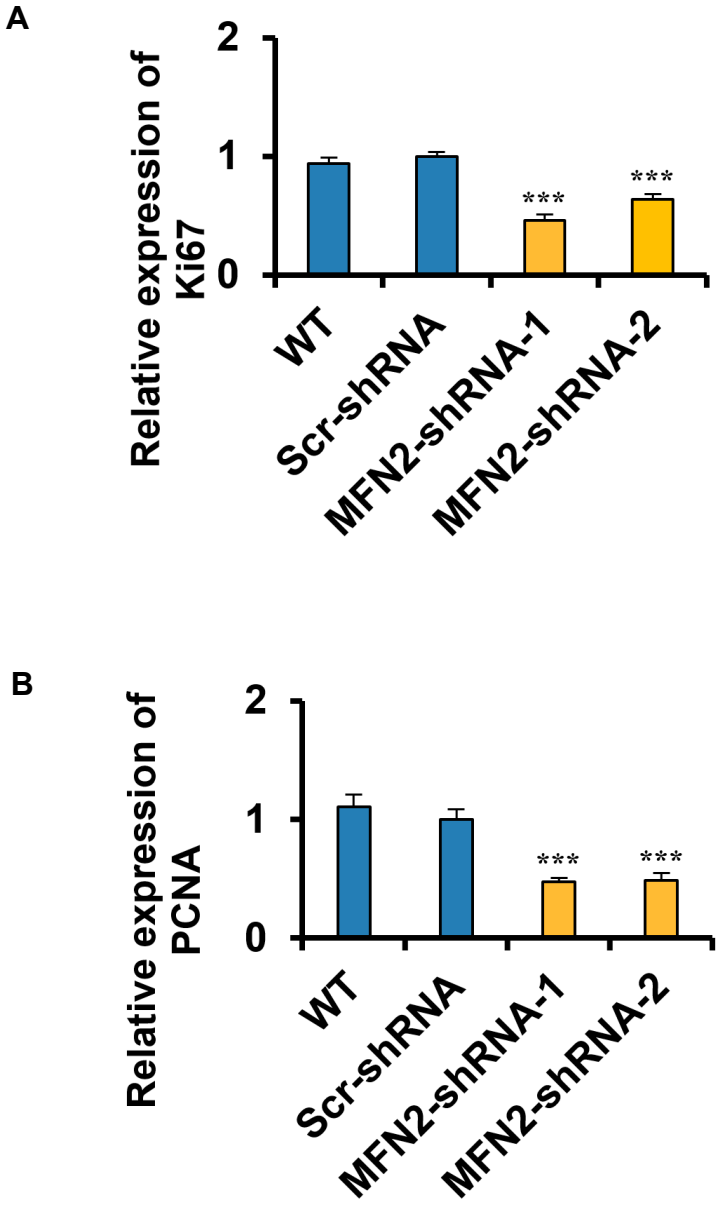

Figure 5. The mRNA expression of proliferation markers decreased in MFN2 knockdown HeLa cells. (A) Relative mRNA levels of Ki67 decreased in the MFN2-shRNA group compared to the Scr-shRNA group. (B) Relative mRNA levels of PCNA decreased in the MFN2-shRNA group compared to the Scr-shRNA group. ***p<0.001, one-way ANOVA analysis.

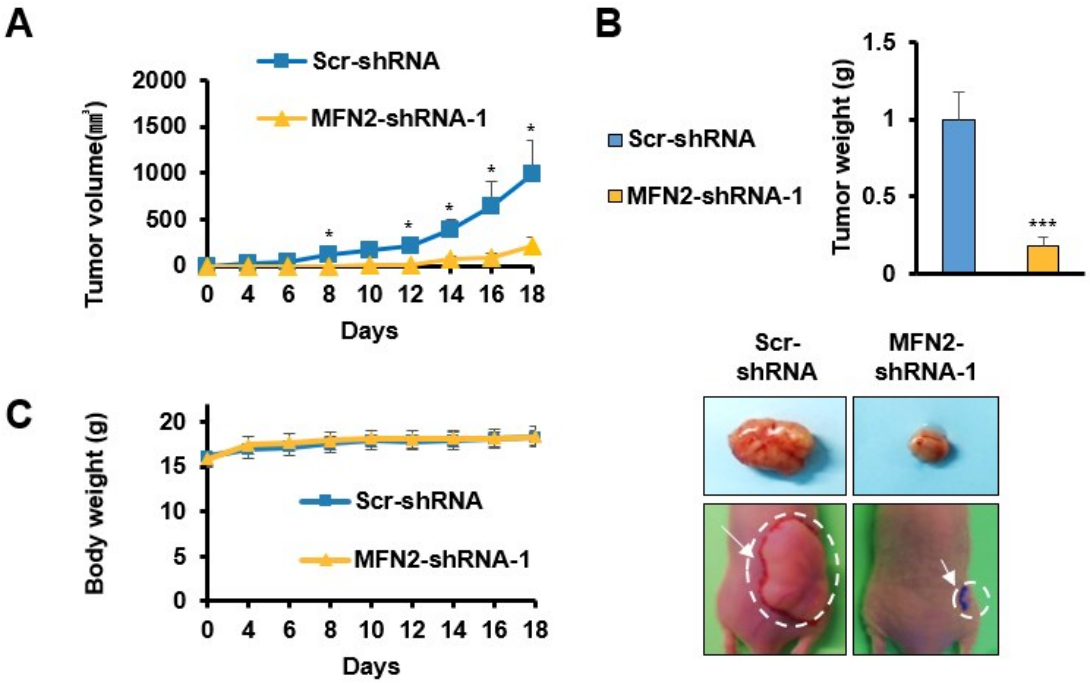

Figure 6. The tumorigenic ability decreased in MFN2 knockdown HeLa cells using xenograft mouse models *in vivo*. (A) The tumorigenic ability decreased significantly in MFN2-shRNA than the Scr-shRNA (*p<0.05, **p<0.01). (B) Tumor weight 18 days after injection of stable HeLa cell lines reduced in MFN2-shRNA compared to the Scr-shRNA (***p<0.001). The picture at the bottom right is the tumor of xenograft mouse models 18 days after injection of HeLa cells. (C) There was no difference in body weight of BALB/c nude mice between Scr-shRNA and MFN2-shRNA groups during tumorigenesis.

3. Mobility decreased in MFN2 knockdown HeLa cells

We investigated whether MFN2 affects the mobility of HeLa cells using migration assay, and we measured the distance HeLa cells moved by CellSens software. Thirty hours after scraping, the wound of the MFN2-shRNA was less closed compared to the Scr-shRNA, which was almost closed (Figure 7A). We measured the distance between wounds in seven areas for more exact comparison and analyzed the mean and the standard deviation. The wound closure of the MFN2-shRNA group was significantly less than the Scr-shRNA group 24 hr and 30 hr after wounding (Figure 7B). Together, this data indicates that MFN2 promotes the migration ability of HeLa cells.

To better understand, we compared relative mRNA levels of epithelial-mesenchymal transition (EMT) markers between MFN2-shRNA and Scr-shRNA to examine the effect of MFN2 on the invasion, cancer cell stemness, and metastasis in HeLa cells. The relative mRNA levels of claudin, occludin, and E-cadherin, which are epithelial markers, were significantly increased in MFN2 knocked down HeLa cells compared to that of Scr-shRNA (Figure 8A). In addition, the mRNA levels of Axin-2 and N-cadherin, which are mesenchymal markers, were meaningfully decreased in MFN2-shRNA compared to the Scr-shRNA group (Figure 8B). Correspondingly, we found that Snail's relative mRNA levels related to Wnt signaling and known to promote mesenchymal markers were also significantly reduced in MFN2-shRNA HeLa cells compared to that of Scr-shRNA (Figure 8C). In this study, we finally elucidated that MFN2-shRNA induced the reduction of malignancy in HeLa cells because MFN2 promotes migration, invasion, and metastasis through EMT.

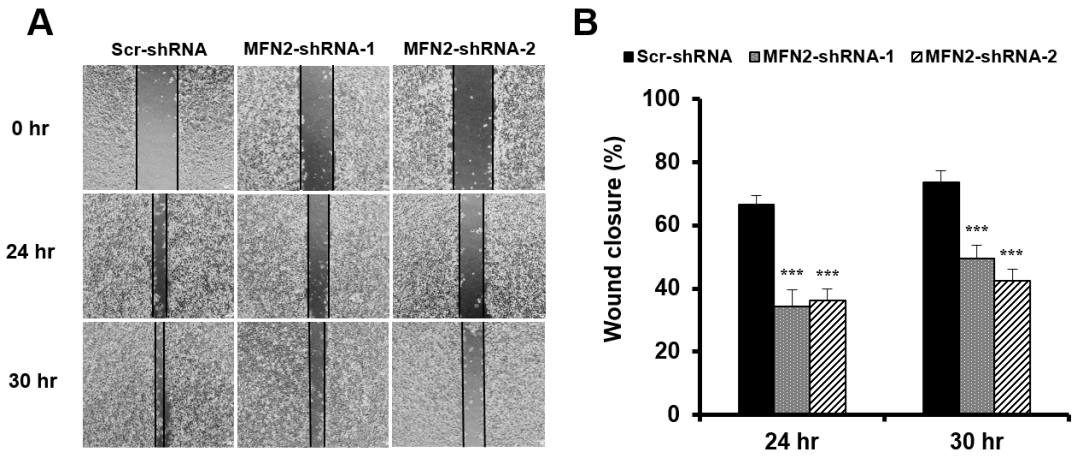

Figure 7. Mobility reduced in MFN2 knockdown HeLa cells *in vitro*. (A) Image of wound closure of HeLa cell lines at 0 hours, 24 hours, and 30 hours. (B) Wound closure of MFN2-shRNA was significantly less than that of Scr-shRNA at 24 hours and 30 hours after scratching the wound. ***p<0.001, one-way ANOVA analysis.

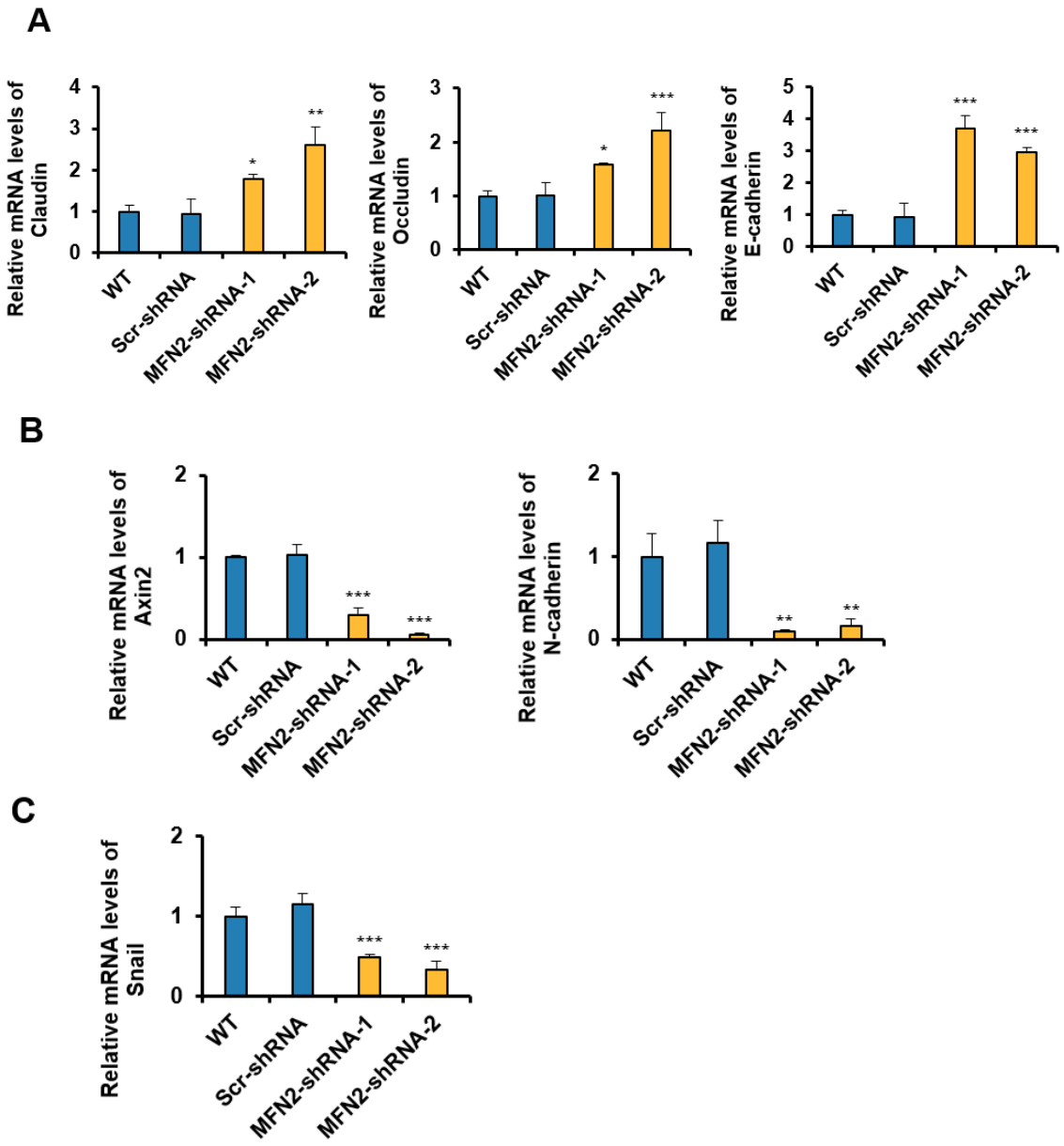

Figure 8. Epithelial to mesenchymal transition (EMT) decreased in MFN2 knockdown HeLa cells. (A) Relative mRNA levels of epithelial markers (Claudin, Occludin, E-cadherin) increased in MFN2-shRNA HeLa cells compared to that of Scr-shRNA (*p<0.05, **p<0.01, ***p<0.001). (B) Relative mRNA levels of mesenchymal markers (Axin2, N-cadherin) reduced in MFN2-shRNA HeLa cells compared to that of Scr-shRNA (**p<0.01, ***p<0.001). (C) Relative mRNA levels of Snail decreased in MFN2-shRNA HeLa cells compared to that of Scr-shRNA (***p<0.001).

4. Analysis of the effect of MFN2 patients with cervical cancer using cBioPortal

A. The overexpression of MFN2 induced high mutation counts and low survival rate in patients with cervical cancer

We next investigated the clinical effects of MFN2 on patients with cervical cancer. The genetic information of 190 patient samples from cervical squamous cell carcinoma and endocervical adenocarcinoma of The Cancer Genome Atlas (TCGA) was analyzed using cBioPortal. The mutation count was high in cervical cancer patients with high MFN2 expression than low MFN2 expression (Figure 9A). The overall survival rate of patients with low MFN2 was 100%, whereas patients with high MFN2 decreased over time. Specifically, the overall survival rate reduced to 25% after 210 months in cervical cancer patients with high MFN2 expression. Specifically, the overall survival rate of cervical cancer patients with high MFN2 expression reduced to 25% after 210 months, but that of cervical cancer patients with low MFN2 expression maintained 100% (Figure 9B).

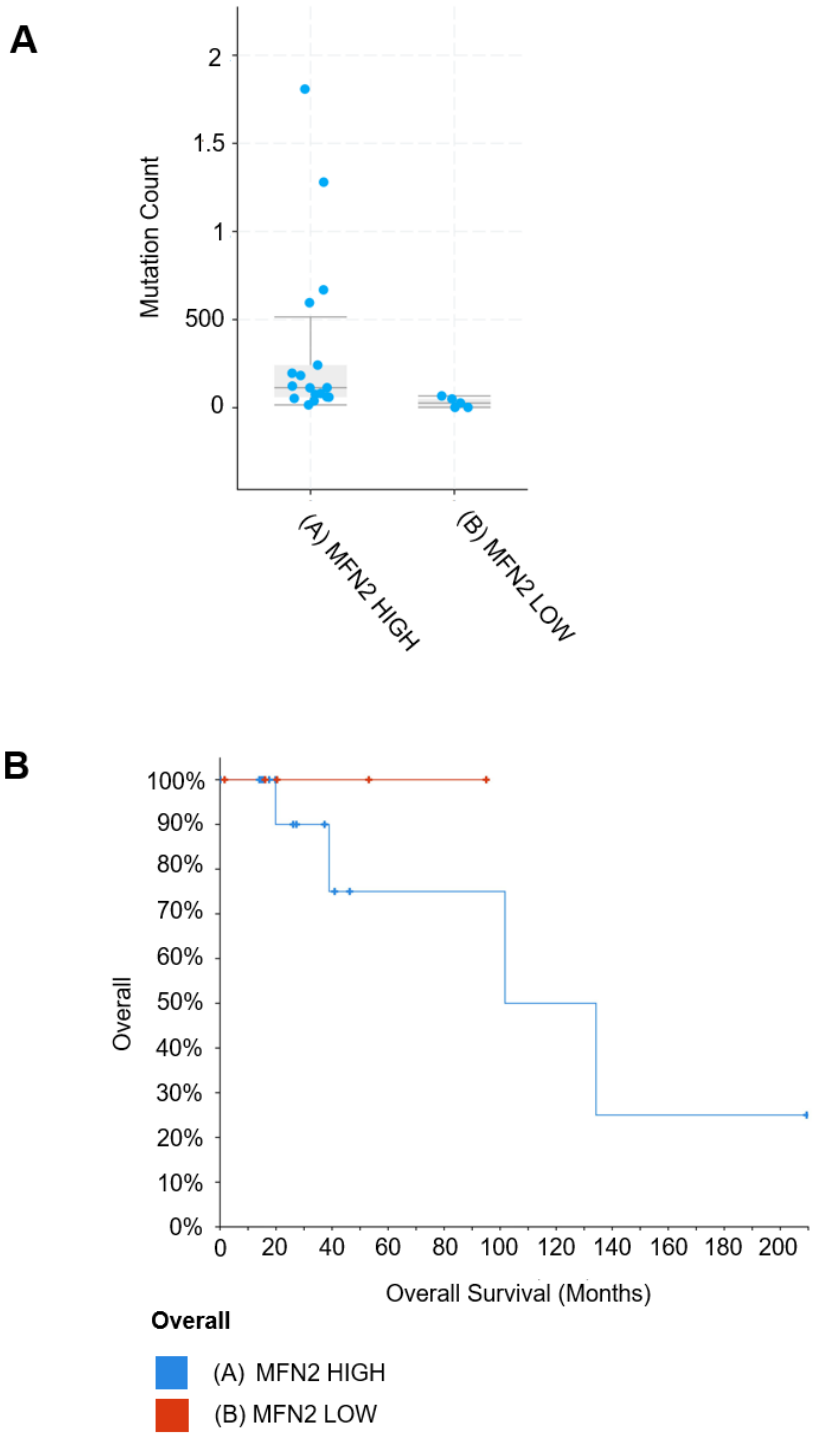

Figure 9. The overexpression of MFN2 induced a high mutation count and low survival rate in patients with cervical cancer *in silico.* (A) The overexpression of MFN2 induced a high mutation count in patients with cervical cancer. (B) The overall survival rate of cervical

cancer patients with high MFN2 expression decreased to 25% after 210 months, but cervical cancer patients with low MFN2 expression maintained 100%. We analyzed mutation count and overall survival curve for cervical cancer patients with high and low MFN2 expression using cBioPortal (http://www.cbioportal.org).

B. Wnt/β-catenin signaling pathway is associated with MFN2 alteration in patients with cervical cancer

We investigated the mechanism of MFN2 in patients with cervical cancer using cBioPortal's PathwayMapper. It shows that Wnt/β-catenin signaling pathway is associated with MFN2 alteration in patients with cervical cancer. Especially, GSK3β was most correlated with MFN2 at 19.5%, followed by APC (adenomatous polyposis coli) at 9.5% (Figure 10).

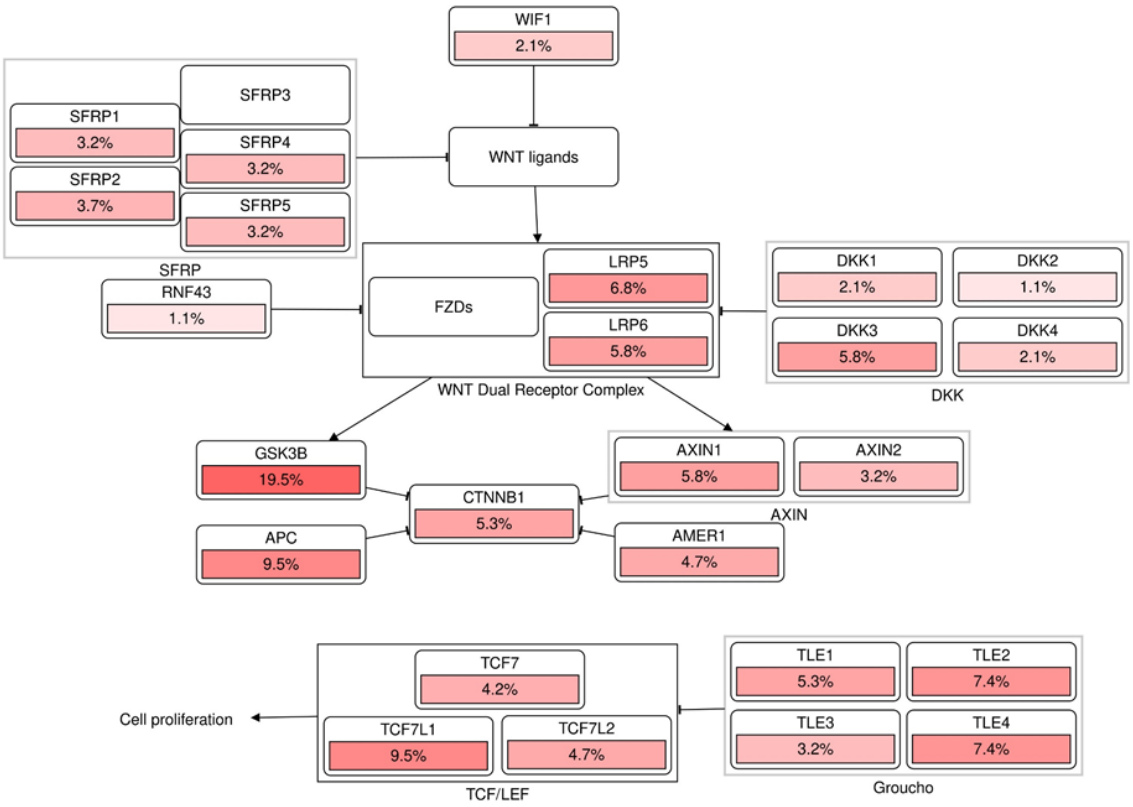

Figure 10. Wnt/β-catenin signaling pathway is associated with MFN2 alteration in patients with cervical cancer *in silico*. GSK3β was most correlated with MFN2 at 19.5%, followed by APC (adenomatous polyposis coli) at 9.5% according to cBioPortal.

IV. DISCUSSION

In the current study, we showed that MFN2 promotes the proliferation, colony formation, and migration of HeLa cells. More mutations existed in cervical cancer tissues with high MFN2 expression than those with low MFN2 expression, and the survival rate was low in patients with MFN2 overexpression based on the data analysis from the cBioPortal and the human genome ATLAS.

MTT assay showed that the viability of MFN2-shRNA HeLa gradually decreased over time compared to that of the Scr-shRNA group. RT-qPCR using PCNA and Ki67 proliferation markers showed the MFN2 promotes the proliferation of HeLa cells. Moreover, we unveiled that MFN2 facilitates the colony formation of HeLa cells using colony formation assay and tumor xenograft *in vitro* and *in vivo*. In the same context, we proved that MFN2 increases both mobility and EMT through migration assay and RT-qPCR in HeLa cells. Wnt signal-related genes, such as Snail, were also decreased in MFN2-shRNA HeLa cells. Snail is known as a transcriptional repressor of E-cadherin, and Axin2 is a regulator of Snail[48,49].

The genetic alteration of MFN2 was associated with the Wnt signaling pathway, and among them, the glycogen synthase kinase-3β/adenomatous polyposis coli (GSK-3β/APC) gene was ranked first in cervical cancer. Glycogen synthase kinase-3β (GSK-3β) and adenomatous polyposis coli (APC) have an essential function in the canonical Wnt signaling pathway. Because they are involved in the destruction of β-catenin, these genes determine the on and off-state of Wnt signaling by regulating transcription of the target genes. In the absence of Wnt signaling, β-catenin is hyper-phosphorylated by the destruction complex, composed of CKIα, GSK3β, APC, and Axin; and it becomes a target of decomposition and ubiquitination by the proteasome[50]. In the presence of Wnt signaling, Frizzled/LRP-5/6 receptors bind to the Wnt ligand and induce the stabilization of hypo-phosphorylated β-catenin to activate the transcription of Wnt-related genes[50]. Therefore, we determined that MFN2 promotes the proliferation, invasion, and metastasis of

HeLa cells by regulating Wnt signaling and influencing patients' overall survival rate with cervical cancer.

In this context, we investigated that MFN2 promotes cancer progression in cervical cancer, which means that this mitochondrial gene functions as an oncogene in the cervix. The worse the prognosis of patients with cervical cancer, the greater the overexpression of MFN2. Proliferation ability decreased when MFN2 was knockdowned in HeLa cells (cervical adenocarcinoma) and SiHa (squamous cell carcinoma)[38]. Recently, various journals inform that most tumors originate from mitochondrial metabolic dysfunction, which results in excess fusion or deficient fission, under the condition with high MFN2 overexpression[51,52]. The emerging findings revealed that cancer cell progression results in an ability to produce ATP by mitochondrial OXPHOS in the same manner as normal cells, although this overturns the Warburg effect[53,54]. Li T et al. reports that MFN2 knock-down in non-small cell lung cancer (NSCLC) reduced oxygen consumption, inhibiting malignant characteristics such as proliferation, migration, and tumorigenesis[51,53]. We discovered that MFN2 promotes EMT, which causes tumor initiation, cancer progression, stemness, migration, metastasis, and resistance to cancer therapies[55,56]. EMT decreases epithelial markers, such as E-cadherin, and increases levels of mesenchymal markers like N-cadherin[57]. Especially in stem cells, EMT induces mitochondrial fusion via upregulation of MFN1, which enhances GSH synthesis through PKCζ-mediated NUMB phosphorylation to promote the self-renewal of stem cells. The miR200c-PGC1α-MFN1 pathway also regulates malignant transformation in stem cells[56]. Moreover, Silibinin, an anticancer drug, represses the EMT of MDA-MB-231 breast cancer cells by increasing mitochondrial fusion[58].

There are various signaling pathways to regulate MFN2 expression. The canonical Wnt signaling is well established as a critical pathway that depends on the transcription of β-catenin[59,60]. In bladder cancer cells, MFN2 inhibits cell proliferation and invasion via the Wnt/β-catenin pathway[61]. In neuronal disease, MFN2 is activated under stress to regulate the Wnt signaling pathway to act in a neuroprotective capacity through synaptic remodeling[62]. In addition, Ras signaling regulates the proliferation, cell cycle, and morphology by controlling mitochondria and endoplasmic reticulum morphology and tethering[63]. In the fibroblasts,

MFN2 over-expression inhibits proliferation and expression of procollagen through the Ras/Raf/ERK axis underlying pelvic organ prolapse development[64]. Several reports indicated that regulation of MFN2 could be a treatment for lung injury caused by endotoxin and breast cancer through the PI3K/Akt pathway[65,66]. In hypoxic pulmonary hypertension, MFN2 controls pulmonary artery smooth muscle cell proliferation via regulation of the PI3K/Akt pathway[67]. Another mitochondrial fusion protein, MFN1, is an essential component for developing oocyte and interaction with follicular somatic cells[68]. Mitochondrial fusion also directs cardiomyocyte differentiation through calcineurin and Notch signaling[69] influencing the mitochondrial morpho-dynamics regulating cell viability[70]. Notch signaling maintains mitochondrial biogenesis by protecting the myocardium[71].

Mitochondria-related genes influence STAT3 signaling in the neuron. Specifically, microRNA-326-5p represses neuronal apoptosis and mitigates mitochondrial defects through inhibiting STAT3 in cerebral ischemia[72]. Indicated by Czapski et al, mitochondria-related genes contribute to the maintenance of homeostasis in neurodegenerative disorders by suppressing poly(ADP-ribose) polymerase-1[73]. The Hippo signaling pathway, a cancer-signaling pathway, is involved in gastric cancer progression through SIRT1/Mfn2/mitophagy[74] and attenuates reperfusion-mediated cerebral injury activating mitochondrial fusion by melatonin therapy[75]. MFN2 also regulates the TGF-β pathway, the principal director of carcinogenesis[76,77], linked with chronic rejection treatment and inhibitor of cellular senescence[76]. Another report suggests that dysregulation of Smad, a nuclear-shuttling transcriptional mediator of changing growth factor-β (TGF-β) signaling, and MFN2 causes malignant tumor growth and neurodegenerative disorders[76,77]. Finally, MFN2 is indicated as a p53-inducible target gene suppressing cell proliferation, facilitating apoptosis, and regulating tumor suppression, according to Wang et al[78].

The limitation of this study is that it does not confirm the relationship of MFN2 with EMT and cancer signaling at the protein level. Therefore, the mechanism of how MFN2 affects EMT in cervical cancer using claudin, occludin, E-cadherin, and N-cadherin at the protein level remains obscure. Also, it needs to discover related cancer signaling pathways regulating the expression of MFN2 in cervical cancer. We investigated related signaling

pathways using cBioPortal, but it needs to confirm the mRNA and protein level expression to elucidate more exact cancer signaling pathways related to MFN2.In the current study, we did not examine how drugs that inhibit MFN2 affect cervical cancer development. Therefore, further research on drug treatment regulating mitochondrial dynamics may help determine target drugs for cervical cancer therapy.

V. CONCLUSION

To investigate whether MFN2 functions as an oncogene in cervical cancer, we performed *in vitro* and *in vivo* experiments using MFN2-shRNA HeLa cells, and our study demonstrated that,

1. MFN2 promotes the viability and proliferation ability of HeLa cells.
2. MFN2 helps facilitate colony formation in HeLa cells.
3. MFN2 facilitates tumorigenesis of HeLa cells in a xenograft mouse model.
4. MFN2 stimulates the migration of HeLa cells through upregulating EMT.
5. In cervical cancer patients with high MFN2 expression, the mutation count increased, and the survival rate decreased.
6. MFN2 is involved in invasion and metastasis controlling Wnt/β-catenin signaling pathways through glycogen synthase kinase-3β/adenomatous polyposis coli (GSK-3β/APC) in cervical cancer.

Therefore, our findings suggest that MFN2 may be a new target gene for the development of cervical cancer treatment. We propose that further investigation into the relationship between MFN2 expression and the incidence of cervical cancer may contribute to the discovery of a novel biomarker for cervical cancer.

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ABSTRACT (IN KOREA)

EMT 를 유도하여 자궁 경부암의 진행을 촉진시키는 Mitofusin-2 에 관한 연구

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MFN2 과발현은 예후가 좋지 않은 자궁 경부암 환자들에게서 흔히 발견되었다. 본 연구의 목적은 MFN2 가 자궁 경부암의 진행에 어떻게 관여하지는 알아보기 위하여 자궁 경부암의 발암에서 MFN2 의 기능을 확인하는 것이다.

자궁 경부암 선암 유래인 HeLa 세포에서 MFN2 를 녹다운 한 뒤, 증식 분석을 통하여 MFN2 가 HeLa 세포의 성장에 주는 영향을 시험하였다. 콜로니 형성 능력과 종양 형성 능력은 콜로니 형성 분석과 종양 이종이식 생쥐 모델을 통하여 분석되었다. 또한, 상처-회복 실험을 통하여 MFN2 가 자궁 경부암의 이동에 주는 영향을 확인한 뒤, RT-qPCR 을 통하여 상피간엽이행성 마커와의 관련성을 조사하였다. 마지막으로, cBioPortal 와 The Human Genome Atlas 를 통하여 임상 데이터를 분석하였다.

MFN2 가 HeLa 세포에서 녹다운 되었을 때, 세포 생존력이 감소하였습니다. 또한, MFN2 는 HeLa 세포의 콜로니 형성 능력과 종양 형성을 촉진하였으며, HeLa 세포의 이동성 및 상피간엽이행성도 촉진하였다. cBioPortal 를 통하여

자궁경부암에서 MFN2 발현이 높을수록 돌연변이 수가 증가하였고, 환자 생존률은 감소하였으며 Wnt/β-catenin signaling 과 관련이 깊은 것을 확인하였다.

이 연구를 통하여 자궁 경부암 발병에서 MFN2 가 치료 표적이 될 수 있음을 알 수 있었다. MFN2 가 자궁 경부암 환자의 치료 및 바이오마커로 기여할 것으로 기대된다.

핵심되는 말 : Mitofusin-2 (MFN2), 종양 유전자, 자궁 경부암 발생, 치료 표적, 상피간엽이행