Role of MFN2 gene as prognostic biomarker for cutaneous squamous cell carcinoma recurrence

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Directed by Professor Mi Ryung Roh

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<ABSTRACT>

Role of MFN2 gene as prognostic biomarker for cutaneous squamous cell carcinoma recurrence

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Although vast amount of research has been done on genes related to cancers, it remains unclear whether Mitofusin2 (MFN2) functions as a tumor suppressor or oncoprotein in cancer progression. The impact of MFN2 on carcinogenesis is likely to be more complicated than expected, so further research is needed. In this study, I aimed to investigate the effect of MFN2 on the pathogenesis of cutaneous squamous cell carcinoma (cSCC) and analyzed the expression of MFN2 in cSCC tissue and investigated the influence of MFN2 expression on the biological behavior of cSCC.

In this retrospective study, 111 cSCC patients treated with Mohs micrographic surgery (MMS) at the Department of Dermatology in Severance hospital from 2000 to 2017 were reviewed. MFN2 expression was examined by immunohistochemistry in 111 specimens, 93 from primary cSCC patients with no recurrence and 18 cSCC patients with recurrence. Correlation between various clinicopathologic factors including MFN2 expression and recurrence were analyzed. Moreover, the influence of MFN2 on biological behavior of cSCC cells was investigated *in vitro*.

In the 111 surgical samples, immunoreactivity against MFN2 was low in 65 (58.6%) cSCC tissues and high in 46 (41.4%) cSCC tissues. Recurrence-free survival was significantly related to tumor size, differentiation status, and expression of MFN2, according to the Kaplan-Meier analysis. In multivariate analysis using age, gender, lesion site, level of MFN2 expression as cofactors, MFN2 expression was an independent risk factor for recurrence of cSCC with a hazard ratio of 8.262 (95% CI, 2.070-32.974; p=0.003). The proliferative, migratory, and invasive abilities of the cells were significantly decreased after MFN2-knockdown than control cells (all p<0.001).

In conclusion, it was found that MFN2 expression is a significant indicator of poor prognosis among patients with cSCC and MFN2 has a strong influence on the behavior of cSCC cells *in vitro*. This study suggests that MFN2 may act as an oncogene by affecting the aggressiveness of cSCC and can be used as a biomarker to predict the prognosis of cSCC.

Key Words : MFN2, cutaneous squamous cell carcinoma, prognostic marker

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I. INTRODUCTION

Skin cancers can be divided into two major groups: melanoma, and non-melanoma skin cancer (NMSC). Nonmelanoma skin cancer is the most common malignancy worldwide. Cutaneous squamous cell carcinoma (cSCC) is the second most common non-melanoma skin cancer. cSCC has been thought to comprise about 20% of all nonmelanoma skin cancers, with a ratio of basal cell carcinoma (BCC) to squamous cell carcinoma (SCC) estimated to be 4:1[1,2]. However, several studies established a trend for an increasing incidence of cSCC compared with BCC, particularly in the aging population[3-6]. Distinguishing the minority of high-risk cSCC with a poor prognosis from the majority of low-risk tumor with a favor prognosis is clinically challenging[7]. A standard of care has not been defined for management of these patients and no prognostic algorithms exist to predict which individuals are at risk for local

recurrence or metastasis.

Over the recent years, several biomarkers have been suggested to be useful in the diagnosis and treatment of melanoma. Lactate dehydrogenase (LDH), S100 protein and tyrosinase are clinical serological biomarkers in melanoma. Also, cyclooxygenases and matrix metalloproteinases (MMP) are the well-known tissue specific biomarkers of melanoma[8]. In addition to protein biomarkers, epigenetic biomarkers can be detected in melanoma patients[9]. However, study for biomarker on BCC and SCC has been minimal. In BCC, methylation of the FHIT promoter has been demonstrated to be present in a significant portion of BCC specimen[10], but this finding did not lead to development of the biomarker. In cSCC, genes commonly mutated in patients include TP53, CDKN2A, Ras, and NOTCH1[11]. Telomerase reverse transcriptase gene (TERT) promoter (TERTp) mutations are associated with poor prognosis in cSCC[12]. cSCC with parotid metastasis specimens exhibited significantly higher average pS6 positivity than those from cSCC without metastasis[13]. However, due to the limited research result and scope, further research is needed to identify biomarkers of greater sensitivity in order to improve prognostic accuracy in cSCC.

Mitofusin2 (MFN2) is a mitochondrial outer membrane protein that plays an essential role in mitochondrial fusion and contributes to maintenance and action of the mitochondrial network[14-16]. MFN2 is a well-known hyperplasia-suppressor gene, and was initially identified in the vascular smooth muscle cells of hypertensive rats[17]. Genetic mutations in MFN2 gene interrupt mitochondrial fusion and cause the untreatable neurodegenerative condition Charcot-Marie-Tooth disease type 2A (CMT2A)[18]. Recent research indicated crucial roles for MFN2 in cancer progression; however, the question of whether it acts as a tumor suppressor or oncogene in this process remains controversial. Several

studies supported the hypothesis that MFN2 is a tumor suppressor; for example, its expression has been shown to be lower in hepatocellular carcinoma, bladder cancer, and gastric cancer tissues than in corresponding healthy tissue samples[19-21]. Similarly, its overexpression can restrict the proliferative, colony-forming, migratory, and invasive abilities of cells of gastric cancer lines[21]. In contrast, other studies have provided evidence that MFN2 functions as an oncogene in gastric cancer[22]. Moreover, *in vitro* experiments have demonstrated that MFN2 knockdown exerts inhibitory effects on lung cancer cell proliferation, migration, and invasion[23]. Thus, the impact of MFN2 on carcinogenesis and cancer progression is likely to be more complicated than expected and further research is needed.

The role of MFN2 in cSCC has not yet been examined as far as I know. Therefore, in the present work, I investigated the relevance of MFN2 expression to the clinicopathological characteristics of patients with cSCC and also assessed the influence of MFN2 knockdown on the behavior of cells of cell lines, with the aim of determining the implications of MFN2 expression in cSCC pathogenesis.

II. MATERIALS AND METHODS

1. Patient selection

Patients with cSCC treated by Mohs micrographic surgery (MMS) at the Department of Dermatology in Severance hospital, Seoul, South Korea from 2000 to 2017 were retrospectively reviewed. Medical records of included patients were reviewed following institutional review board (IRB) of Severance hospital, Yonsei Medical Center (approval no. 4-2018-0331).

2. Analysis of clinicopathological factor

The electronic medical records of included patients were reviewed for the information of baseline demographics. Photographs taken before surgery were also reviewed for the information of location and size of the initial tumor. Reviewed characteristics included age, gender, location of the tumor, size of the initial tumor. Recurrence of cSCC after MMS included local recurrence or distant metastasis. Recurrence of the tumor within 2cm from the primary tumor was referred as local recurrence and the others were referred as distant metastasis. Pathology of cSCC was classified into well differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) groups.

3. Immunohistochemistry for MFN2 expression

Specimens were deparaffinized with xylene and hydrated using a graded ethanol series. Endogenous peroxidase activity was inhibited using a 1:40 H_2O_2: methanol mixture before antigen retrieval was performed with an antigen retrieval solution (Dako, Carpinteria, CA, USA) and the pressure cooker method. The sections were then exposed to a mouse monoclonal IgG primary antibody to MFN2 (Abcam, Cambridge, MA, USA) diluted 1:200, and a REAL EnVision HRP Rabbit/Mouse Detection System (Dako) secondary antibody. Visualization of antibody binding was performed using the chromogen 3,3′-diaminobenzidine, and the tissues were then counterstained with haematoxylin. Mouse IgG (DakoCytomation Denmark A/S, Glostrup, Denmark) was used as a negative control. A cell block was constructed using HeLa cells (ATCC, Rockville, MD, USA), sections of which were then made for use as positive controls.

The weighted histoscore method was used to score MFN2 expression according to staining intensity and the percentage of positively stained cells. The intensity of staining was scored as 0 (negative), 1 (light brown), 2 (brown),

and 3 (dark brown). The histoscore was calculated as follows: final score = (0 × percentage of negative cells) + (1 × percentage of light-brown cells) + (2 × percentage of brown cells) + (3 × percentage of dark-brown cells). For analysis, the patients were subdivided into two groups, a low expression (histoscores 0 through 100) and a high expression (histoscores 101 through 300) group.

4. *In vitro* study

A. Cell culture

Two human cSCC cell lines, HSC-1 and HSC-5, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and the Korean Cell Line Bank (Seoul, Korea), respectively, for use in this study. Cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Biosciences, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA). MFN2 expression was down-regulated in these cells by transfection of small-interfering RNA (siRNA) (Bioneer, Seoul, South Korea) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Knockdown efficiency was evaluated by reverse-transcription real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting.

B. RT-qPCR

Total RNA was isolated from HSC-1 and HSC-5 cells following transfection with scrambled (Scr)-siRNA or MFN2-siRNA using TRIzol Reagent (Invitrogen), and complementary DNA synthesis was performed using oligo(dT) primer, recombinant RNasin ribonuclease inhibitor, and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Marker of proliferation Ki-67 (MKI67), proliferating cell nuclear antigen (PCNA), and

MFN2 mRNA expression was determined by qPCR using 2× SYBR Premix Ex Taq II (Tli RnaseH Plus) (RR82LR; Takara, Ann Arbor, MI, USA) on an Applied Biosystems (Foster City, CA, USA) instrument. The following primers were used: MFN2 forward: 5′-GACCCCGTTACCACAGAAGA-3′ and reverse: 5′-GCAGAACTTTGTCCCAGAGC-3′; MKI67 forward: 5′-AAGCCCTCCAGCTCCTAGTC-3′ and reverse: 5′-GCAGGTTGCCACTCTTTCTC-3′; PCNA forward: 5′-GGCGTGAACCTCACCAGTAT-3′ and reverse: 5′-TTCTCCTGGTTTGGTGCTTC-3′; β-actin forward: 5′-ATAGCACAGCCTGGATAGCAACGTAC-3′ and reverse: 5′-CACCTTCTACAATGAGCTGCGTGTG-3′. The relative expression of each gene was normalized to that of actin, and data analysis was performed using the ΔΔCq method.

C. Immunocytochemistry

For immunocytochemistry, 5×10^4 each group of cells were cultured on chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) and then fixed with 95% ethanol. Endogenous peroxidase activity was inhibited with a mixture of methanol and hydrogen peroxidase (40:1), and 5% bovine serum albumin was used for blocking prior to primary antibody incubation. Real EnVision HRP Rabbit/Mouse Detection System (Dako Products) was used as the secondary antibody in this study. Visualization was performed using 3,3'-diaminobenzidine chromogen, and counterstaining was performed with hematoxylin. Mouse IgG (DakoCytomation A/S, Copenhagen, Denmark) were used as negative controls.

D. Cell proliferation assay

To investigate the effect of MFN2 expression on cell proliferation, the

number of viable HSC-1 and HSC-5 cells was counted at different time points following transfection with Scr-siRNA or MFN2-siRNA. Cells were seeded in a 6-well plate at a density of $1x10^5$ and counted after trypan blue staining each day for 3 days.

E. Wound-healing and invasion assays

The impact of MFN2 expression on cell migration and invasion was assessed by subjecting cells transfected with Scr-siRNA or MFN2-siRNA to wound-healing and matrigel invasion assays. For the wound-healing assay, the cells were seeded in a 24-well plate at a density of $5x10^4$. After reaching about 90% confluence, the cells were scratched by a sterile 0.2mL pipette tip to create wounds, and relative wound closure was determined after 18 hours of scratch wounding.

The matrigel invasion assay was performed using transwell chambers with a pore size of 8 μm (BD Biosciences, Bedford, MA, USA) coated with 8 μg/μl matrigel (BD Biosciences, San Jose, CA, USA). The cells were seeded in the upper chamber of transwell at a density of $5x10^4$ with culture medium containing 3% BSA and culture medium containing 10% FBS was added in the bottom chamber. After 34 h of culture, the lower side of each membrane was stained with 0.25% crystal violet, and the number of invading cells was counted under a microscope.

5. Statistical analysis

The chi-squared test and Fisher's exact test were used to examine the association between MFN2 protein expression and clinicopathological parameters. The Kaplan-Meier estimate was used to analysis the survival over time with several clinicpathologic factors. To identify the independent risk

factors for recurrence-free survival in patients with cSCC, the Cox-regression analysis was used. For *in vitro* study, differences between groups of cells in the proliferation, migration, and invasion assays were analyzed using the Mann–Whitney U-test. Differences were considered to be statistically significant when $p<0.05$.

III. RESULTS

1. Baseline patient characteristics

A total of 111 cSCC patients treated by MMS at Severance Hospital were analyzed. The demographics of the 111 patients are described in Table 1. Median age of patients was 74 years, and there were 61 female patients and 50 male patients. The head and neck area was the most common site of the tumor, accounting for 83.3%, including the face, ears, scalp, and lips. Median size of the primary tumors was 1.7cm. Eight patients (7.2%) showed poorly differentiated histology while 41 patients (36.9%) showed well differentiated histology. Among 111 cSCC patients, 18 patients (16.2%) showed recurrence after MMS. Mean follow-up period was 11.0 months.

2. Risk factors for recurrence in cSCC patients treated with MMS

First, the level of expression for MFN2 was analyzed in cSCC tissue samples obtained from 111 patients. Cytoplasmic expression of MFN2 was found in 93 (83.8%) cSCC tissues, and the immunoreactivity against MFN2 was low in 65 (low-MFN2, 58.6%) and high in 46 (high-MFN2, 41.4%) cSCC. Representative stained pictures are shown in Figure 1. Following that, several baseline clinicopathological parameters were statistically analyzed to evaluate the association between MFN2 expression. The tumor size was significantly associated with MFN2 expression (p =0.048). No significant association was

Table 1. Baseline clinical characteristics.

Variable		No. of cases, (%)
Age	≤ 74 years	56 (50.5)
	> 74 years	55 (49.5)
Gender	Male	50 (45.0)
	Female	61 (55.0)
Site	Scalp	14 (12.6)
	Face	54 (48.6)
	Ear	10 (9.0)
	Lip	15 (13.5)
	Acral	18 (16.2)
Size	≤1.7	56 (50.5)
	>1.7	55 (49.5)
Differentiation*	WD	41 (36.9)
	MD	62 (55.9)
	PD	8 (7.2)
Recurence	Yes	18 (16.2)
	No	93 (83.8)

*WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

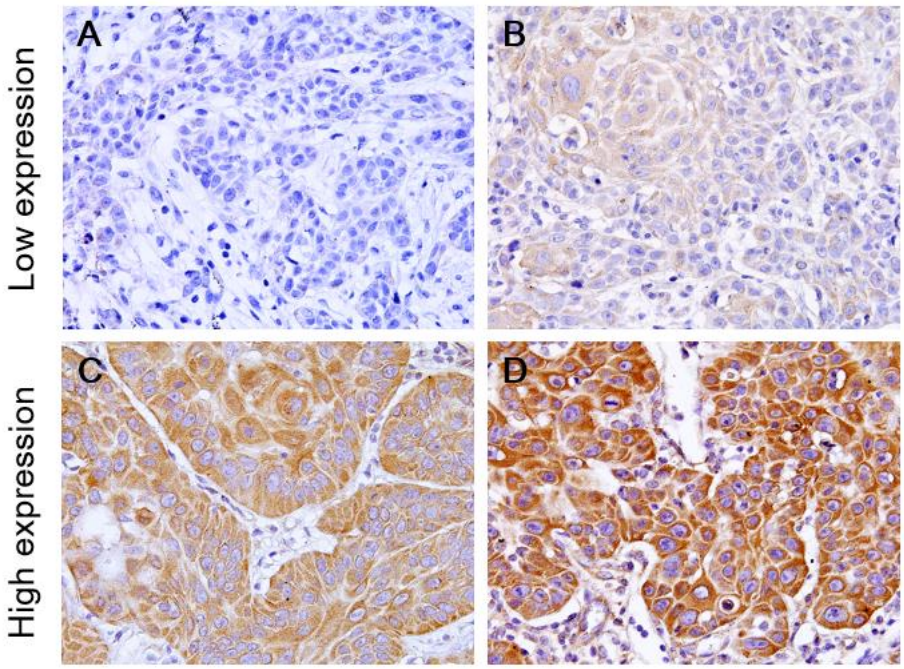

Figure 1. Expression level of MFN2 in cSCC tissues. Visualization using antibody to MFN2 was performed to 111 specimens of cSCC. The weighted histoscore method was used to score MFN2 expression according to staining intensity and the percentage of positively stained cells. Examples of specimens measured as histoscores 0 (A), 100 (B), 200 (C), 300 (D) were presented. The samples were subsequently divided into two groups according to the final histoscore into low expression (histoscores from 0 through 100) and high expression (histoscores from 101 through 300).

Table 2. MFN2 expression and baseline clinicopathological parameters.

Variables	No. of patients (%)	MFN2		*P* value
		Low	High	
Age				
≤74 years	56 (50.5)	36(64.3)	20(35.7)	0.251
>74 years	55 (49.5)	29(52.7)	26(47.3)	
Gender				
Male	50 (45.0)	29(58.0)	21(42.0)	1
Female	61 (55.0)	36(59.0)	25(41.0)	
Site				
Scalp	14 (12.6)	7(50.0)	7(50.0)	
Face	54 (48.6)	32(59.3)	22(40.7)	
Ear	10 (9.0)	5(50.0)	5(50.0)	0.379
Lip	15 (13.5)	12(80.0)	3(20.0)	
Acral	18 (16.2)	9(50.0)	9(50.0)	
Size (cm)				
≤1.5	46 (41.4)	32(69.6)	14(30.4)	0.048
>1.5	65 (58.6)	33(50.8)	32(49.2)	
Differentiation*				
WD	41 (36.9)	21(51.2)	20(48.8)	
MD	62 (55.9)	41(66.1)	21(33.9)	0.155
PD	8 (7.2)	3(37.5)	5(62.5)	
Recurrence				
Yes	18 (16.2)	8(44.4)	10(55.6)	0.202
No	93 (83.8)	57(61.3)	36(38.7)	

*WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

found between MFN2 expression and the other baseline clinicopathological parameters, such as age, gender, lesion site, differentiation (Table 2). According to the Kaplan-Meier analysis, it was found that recurrence-free survival was significantly related to tumor size ($p = 0.025$), differentiation status ($p < 0.001$), and expression of MFN2 ($p < 0.001$) (Figure 2). Poor recurrence-free survival was observed in patients with tumors of larger size (median survival duration: 13 months for tumor size ≤ 1.7 cm versus 8 months for tumor size > 1.7 cm) (Figure 2A), with histological grade of PD (median survival duration: 12.0 and 11.0 months for histological grade of WD and MD, respectively; 8.0 months for patients with histological grade of PD) (Figure 2B), with MFN2-high expression (median survival duration: 18.0 months for MFN2-low versus 6.0 months for MFN2-high) (Figure 2D). Moreover, Cox-regression analysis showed that MFN2-high expression was independent risk factors for recurrence-free survival in patients with cSCC (HR: 8.262, 95% CI = 2.070-32.974; $p = 0.003$) (Table 3).

3. The influence of MFN2 on biological behavior of cSCC cells *in vitro*

Based on clinical data analysis, I have attempted to analyze the effect of MFN2 on tumor cells. To confirm the function of MFN2, experiments were performed using MFN2 knockdown cell lines and wild-type cell lines. MFN2 knockdown in cSCC cell lines was achieved by siRNA transfection. Relative to those treated with a control scrambled siRNA, MFN2 mRNA expression in HSC-1, HSC-5 cells transfected with MFN2-siRNA was significantly reduced 24 h and 48h (all $p < 0.001$) after transfection. (Figure 3A, B). RT-qPCR confirmed the decrease of MFN2 mRNA expression in MFN2 knockout cell lines, and the decrease of MFN2 protein expression in MFN2 knockout cell lines was further confirmed by immunocytochemistry (Figure 3C, D).

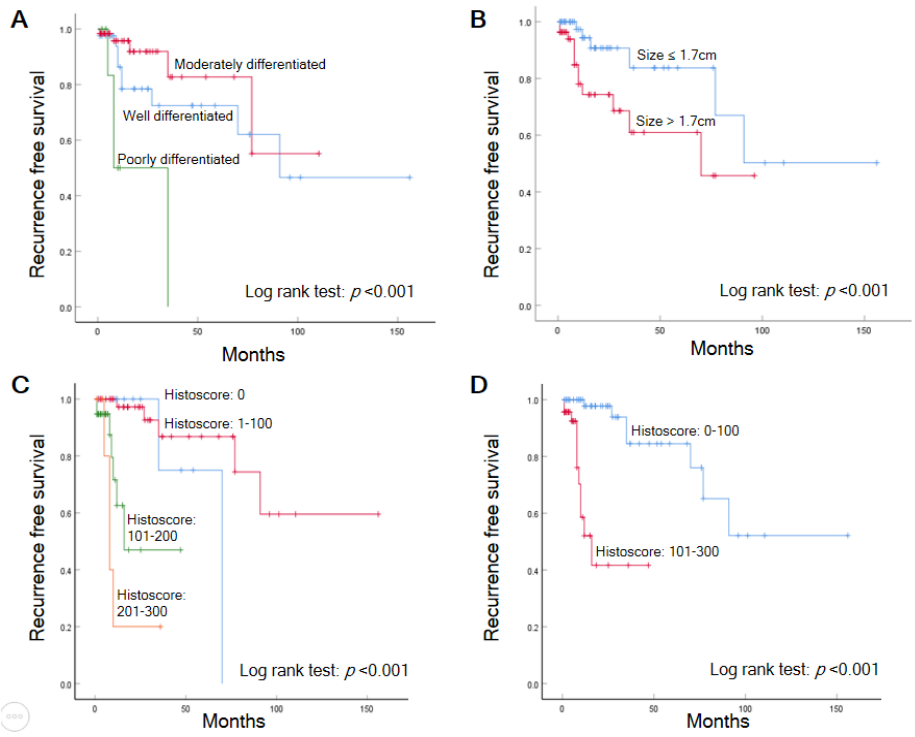

Figure 2. Recurrence-free survival was significantly related to tumor size, differentiation status, and expression of MFN2. A: Poor recurrence-free survival was observed in patients with tumors of larger size (median survival duration: 13 months for tumor size ≤ 1.7 cm versus 8 months for tumor size > 1.7 cm). B: Poor recurrence-free survival was observed in patients with histological grade of PD (median survival duration: 12.0 and 11.0 months for histological grade of WD and MD, respectively; 8.0 months for patients with histological grade of PD). C, D: Poor recurrence-free survival was observed in patients with MFN2-high expression (median survival duration: 18.0 months for MFN2-low (Histoscore 0~100) versus 6.0months for MFN2-high (Histoscore 101~300)).

Table 3. Cox-regression analysis for recurrence-free survival in patients with cSCC.

Variable	Hazard ratio (95% CI*)	*p*-value
Gender	1.270 (0.400-4.035)	0.685
Age	0.964(0.927-1.001)	0.058
Lesion site		
Scalp	1	0.419
Face	0.299 (0.070-1.276)	0.103
Ear	0.728 (0.103-5.128)	0.75
Lip	0.316 (0.048-2.088)	0.232
Acral	0.232 (0.040-1.343)	0.103
Size	1.187 (0.692-2.035)	0.533
Differentiation**		
WD	1	0.143
MD	0.600 (0.193-1.863)	0.377
PD	3.433 (0.684-17.238)	0.134
MFN2	8.262 (2.070-32.974)	0.003

*CI: Confidence interval

**WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

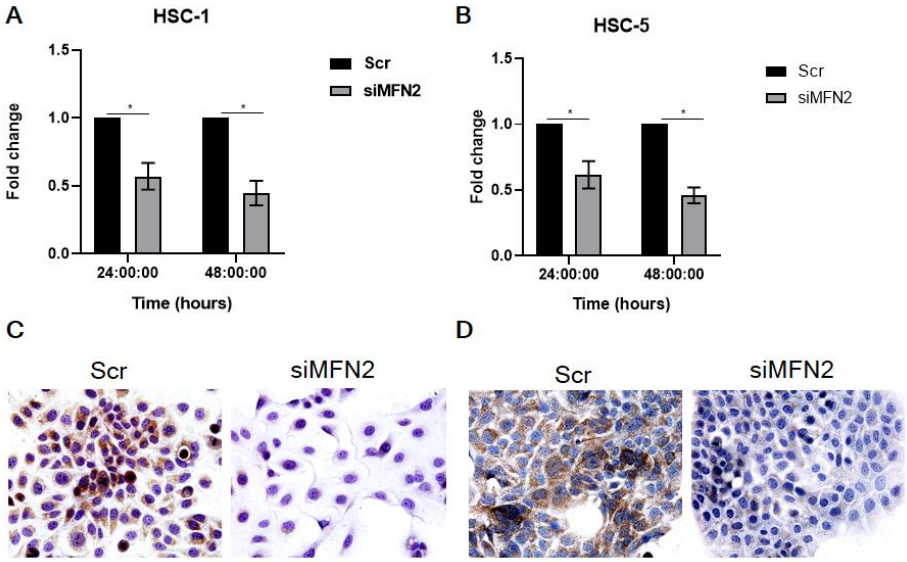

Figure 3. MFN2 mRNA and MFN2 protein expression were decreased in HSC-1, HSC-5 cells transfected with MFN2-siRNA, compared with those treated with the Scr-siRNA control. A: Relative to those treated with the Scr-siRNA control, MFN2 mRNA expression in HSC-1 cells transfected with MFN2-siRNA was 1.75- and 2.24-fold reduced each 24 hours and 48 hours after transfection (all $p < 0.001$). B: In HSC-5 cells transfected with MFN2-siRNA, MFN2 mRNA expression was 1.54- and 2.15-fold reduced each 24 h and 48h after transfection (all $p < 0.001$). C, D: Reduction in MFN2 protein expression in MFN2 knockout cell lines was further confirmed by immunocytochemistry.

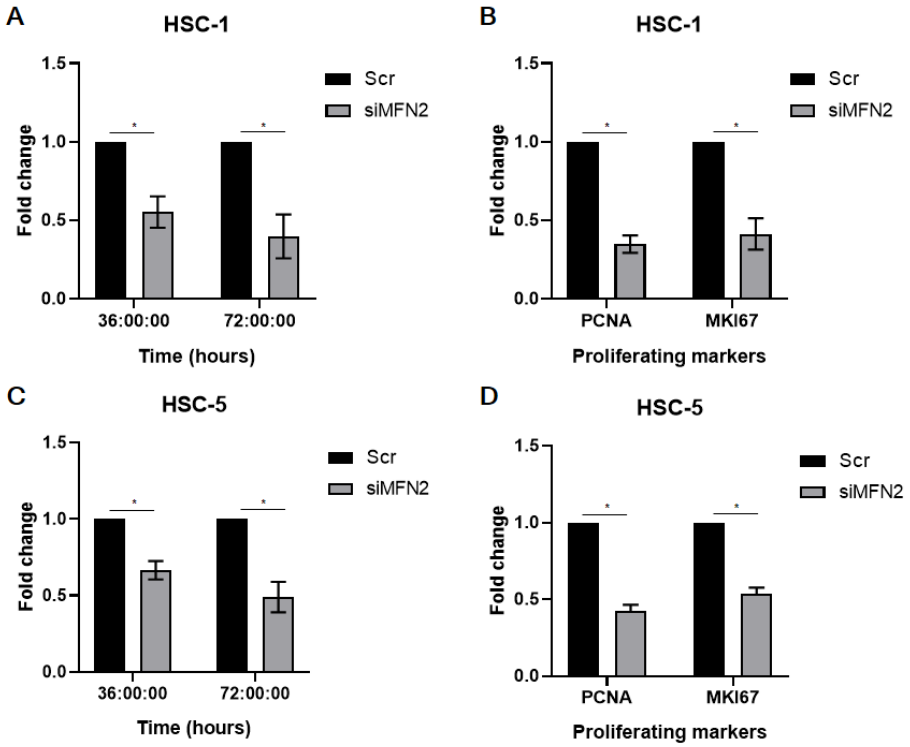

Figure 4. Cell proliferation assay for HSC-1 and HSC-5 cells at different time points following transfection with Scr-siRNA or MFN2-siRNA. A: The number of cell counts was 1.81- and 2.52-fold decreased after MFN2 knockdown in HSC-1 cells than related scr-control in each 36 hours and 72 hours (all $p < 0.001$). B: The proliferating markers, PCNA and MKI67 expression was also significantly decreased after MFN2 knockdown in HSC-1 (2.88- and 2.42-fold, all $p < 0.001$). C: The number of cell counts was 1.50- and 2.05-fold decreased after MFN2 knockdown in HSC-5 cells than related scr-control in each 36 hours and 72 hours (all $p < 0.001$). D: The proliferating markers, PCNA and MKI67 expression was also significantly decreased after MFN2 knockdown in HSC-5 (2.35- and 1.86-fold, all $p < 0.001$).

The proliferative ability was 1.81- and 2.52-fold decreased after MFN2 knockdown in HSC-1 cells than related the HSC-1 cells transfected with scrambled siRNA in each indicated time points (all $p < 0.001$) (Figure 4A). Similar results were also found in HSC-5. Compared to the control transfected with scrambled siRNA, MFN2 knockdown in HSC-5 cells showed 1.50- and 2.05-fold decreases in proliferating ability of the cells at each indicated time points (all $p < 0.001$) (Figure 4C). Supportively, proliferating markers, PCNA and MKI67 expression were also significantly decreased after MFN2 knockdown both in HSC-1 (2.88- and 2.42-fold, all $p < 0.001$) and HSC-5 cells (2.35- and 1.86-fold, all $p < 0.001$) (Figure 4B, 4D).

The motility of the cells was 2.14- and 3.12-fold decreased in HSC-1 and HSC-5 cells after MFN2 knockdown than the control cells transfected with scrambled siRNA (both $p < 0.001$) (Figure 5). Moreover, the invasion ability of the cells demonstrates 3.37- and 5.08-fold decrease after MFN2 knockdown in HSC-1 and HSC-5 cells, respectively (both $p < 0.001$) (Figure 6).

IV. DISCUSSION

Cutaneous squamous cell carcinoma is one of the most common skin malignancies of skin worldwide and there is a persistent trend for an increasing incidence of cSCC. Most of cSCC cases can be treated with surgical excision. However, in some cases, when malignancy recurs it shows aggressive behaviors. So, it is important to detect the high-risk factors predictive of an aggressive tumor behavior. A better comprehension of the molecular mechanisms of cSCC is important to identify new prognostic markers. As many other studies have identified, the recurrence-free survival was significantly related to tumor size, differentiation. Poor recurrence-free survival was observed in patients with tumors of larger size, with histological grade of PD

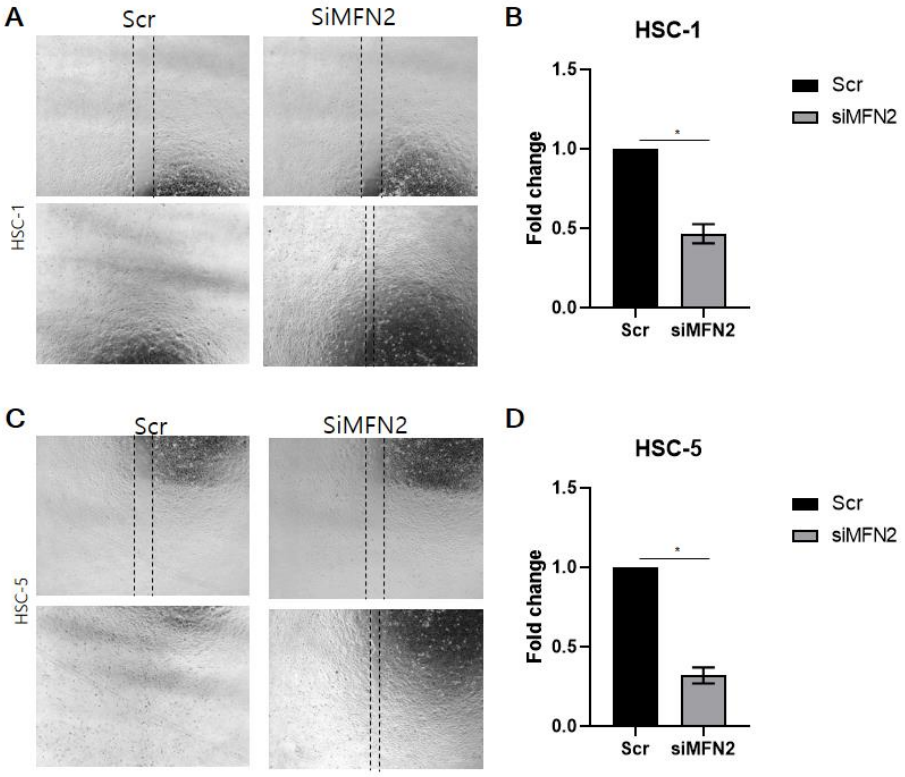

Figure 5. Wound-healing assay for HSC-1 and HSC-5 cells at different time points following transfection with Scr-siRNA or MFN2-siRNA. A, B: Cell motility decreased 2.14-fold in MFN2-knockdown cells compared to scrambled control in HSC-1 cell lines ($p < 0.001$). C, D: Cell motility decreased 3.12-fold in MFN2 knockdown cells compared to scrambled control in HSC-5 cell lines ($p < 0.001$).

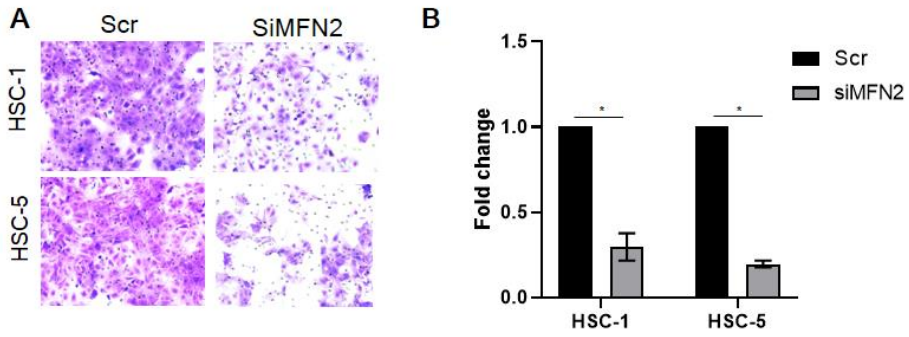

Figure 6. Invasion assay for HSC-1 and HSC-5 cells at different time points following transfection with Scr-siRNA or MFN2-siRNA. A: The picture of invading cells (lower chamber) stained with 0.25% crystal violet shows the decrease of the number of invading cells in MFN2 knockdown cells. B: Invasion ability of the cells was significantly reduced in MFN2 knockdown cells compared to the scr-control in HSC-1 and HSC-5 cell lines (3.37- and 5.08-fold, all $p < 0.001$).

in this cohort. Not only these clinicopathologic factors but also level of MFN2 expression were analyzed to identify the correlation of the recurrence of cSCC. In this study, MFN2 expression levels were compared and analyzed in cSCC tissue samples. It was found that patients with high MFN2 expression showed poor prognosis in this cohort. Moreover, MFN2 expression was an independent risk factor for recurrence of cSCC with a hazard ratio of 8.262. These results are consistent with previous studies of gastric cancer which showed significant correlation between increased MFN2 expression and poor prognosis. MFN2 expression has been positively correlated with depth of invasion, clinical stage, and vascular invasion in the gastric cancer[22]. However, MFN2 has also been reported to act as a tumor suppressor in various cancer types[19-21]. In addition to reports of its down-regulation in cancer tissues compared to corresponding healthy normal tissues, a reduced level of MFN2 expression has been identified as a poor prognostic indicator in several types of cancer, including hepatocellular carcinoma[19,24]. Moreover, MFN2 overexpression in cells of various malignancies significantly reduced their proliferative, migratory, and invasive abilities[19-21]. Such observations are clearly inconsistent with the notion that MFN2 functions as an oncogene.

In contrast to this concept, in the present study, the proliferation, migration, and invasion of cSCC cells were reduced in cells in which MFN2 gene had been knockdown. The proliferating markers, PCNA and MKI67 expression were also decreased after MFN2 knockdown. These findings are consistent with the concept that MFN2 acts as an oncogene. In line with this study, in previous investigation in A549 lung cancer cells and SiHa and HeLa cervical cancer cells, knockdown of MFN2 was found to exert similar significant decreasing effects on cell behavior including proliferation, migration, and invasion[23,25].

The role of MFN2 in carcinogenesis may largely depend on the cancer type or

microenvironmental conditions and remains a curiosity. Several mechanisms may justify the role played by MFN2 in the onset and development of tumors. It has various effects on intracellular Ca^{++} signaling, the generation of growth-promoting factors, the actions on the metabolic systems, on cell cycle, autophagy, cell death and apoptosis[26]. Several results have demonstrated the ability of tumor cells to influence intracellular Ca^{++} signaling, promoting their growth and survival[27]. In addition, as mitochondria play a central function in apoptosis by influencing caspase activation, any modification in the mitochondrial fusion system changes the function of mitochondria for apoptotic stimuli[26]. Although it is to be kept in mind that the action of MFN2 is strictly specific for each tissue and tumor and that totally different effects can be found in different tumors, the findings of this study consistently point towards a crucial role for this protein in cancer progression.

In summary, MFN2 expression is significantly associated factor with poor prognosis among patients with cSCC and shows a strong influence on the behavior of cSCC cells *in vitro*. Thus, it can be suggested that MFN2 expression would be involved in cSCC pathogenesis. These findings also provide evidence that MFN2 may serve as a molecular biomarker in cSCC. Due to the limitation of the number of samples, further investigation of the role of MFN2 in cSCC progression using a larger cohort of patients is needed. For molecular research, further studies are needed to clarify the mechanism and pathways by which MFN2 affects carcinogenesis.

V. CONCLUSION

In conclusion, it was found that MFN2 expression is a significant indicator of poor prognosis among patients with cSCC and MFN2 has a strong influence on

the behavior of cSCC cells *in vitro*. This study suggests that MFN2 may act as an oncogene by affecting the aggressiveness of cSCC and can be used as a biomarker to predict the prognosis of cSCC.

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ABSTRACT (IN KOREAN)

피부 편평세포암의 예후 예측 인자로써 MFN2 유전자 발현의 역할

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조미연

피부암과 연관된 유전자에 대한 연구는 많이 이루어졌지만, 아직 MFN2가 종양을 억제하는 역할을 하는지 암을 유발하는 역할을 하는지는 명확하게 알려져 있지 않다. MFN2가 암 발생에 미치는 영향은 생각보다 복잡할 것으로 사료되고 있어, 이에 대한 추가적인 연구가 필요한 실정이다. 따라서 본 논문에서는 피부 편평세포암의 발병 및 재발에 MFN2가 어떤 역할을 하는지 연구하고자 하였다.

본 연구는 후향적 분석 연구와 생체 외 실험으로 구성되어있다. 2000년부터 2017년 사이에 세브란스병원 피부과에서 피부 편평세포암을 모즈미세도식수술로 수술한 111명의 환자들을 대상으로 하였다. 93명의 비재발군 환자에서 얻은 초기 조직과 18명의 재발군 환자에서 얻은 초기 조직의 면역조직염색을 통해 MFN2의 발현을 평가하고 재발과의 연관성을 분석하였다. 다양한 임상 병리학적 인자들과 MFN2의 발현 정도가 재발과 연관이

있는지 또한 분석하였다. 생체 외 실험에서는 피부 편평세포암 세포주를 사용하여 MFN2의 발현에 따라 세포의 증식, 이동, 침투 능력이 어떻게 변화하는지 확인하였다.

면역화학염색 결과 111개의 조직 중 65개(58.6%)의 검체에서 MFN2가 낮은 발현을 보였고, 46개(41.4%)의 검체에서 높은 MFN2의 발현을 보였다. 환자들의 재발 여부를 추적 관찰한 결과 재발 없이 생존하는 기간은 종양의 크기, 분화도, MFN2의 발현 정도와 통계학적으로 유의미한 상관관계를 보였다. 또한 나이, 성별, 종양의 위치, MFN2의 발현 정도를 포함하여 다변량분석을 시행하였을 때, 피부 편평세포암의 재발에 MFN2의 발현만이 유의미한 위험 인자로 작용하였다.

생체 외 실험에서는 피부 편평세포암 세포주에 siRNA를 핵내주입하여 MFN2 유전자를 녹아웃시켰다. RT-qPCR과 Immunocytochemistry에서 각각 MFN2 mRNA와 MFN2 단백질이 감소하는 것을 확인하였다. 이와 같은 실험군 세포주와 대조군에서 세포 증식 실험을 하였고, 실험 결과 MFN2의 발현이 감소된 세포주에서 대조군보다 세포 증식이 감소한 것을 확인하였다. 세포 이동 실험에서도 MFN2의 발현이 감소된 세포주에서 대조군보다 이동 능력이 감소한 것을 확인하였고, 세포 침투 실험에서도 MFN2의 발현이 감소된 세포주에서 대조군보다 침투 능력이 감소한 것을 확인하였다.

요약하자면 본 연구는 임상적 데이터를 분석한 후향적 연구를 통해 MFN2의 발현이 피부 편평세포암의 나쁜 예후와 관련이 있음을 밝혔고, 실험실 연구 결과 MFN2가 피부 편평세포암 세포주의 행동에 영향을 미친다는 것을 밝혀내었다. 이에 MFN2

유전자가 피부 편평세포암의 재발 및 예후의 예측인자로서 사용될 수 있음을 제시하는 바이다.

핵심되는 말: MFN2, 피부 편평세포암, 예후 예측인자