Implications of MFN2 expression in pathogenesis of oral squamous cell carcinoma

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Implications of MFN2 expression in pathogenesis of oral squamous cell carcinoma

Directed by Professor In Ho Cha

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지금까지 헌신적인 사랑과 믿음으로 키워주신 부모님과 물심양면으로 최선을 다해주고 끝까지 믿고 따라주면서 고국에서 가족을 위해 모든 헌신을 한 사랑하는 아내에게 감사의 마음을 전합니다. 그리고 아직 어리지만 4년동안 씩씩하게 엄마 곁을 지켜주면서 훌륭하게 자란 아들 장정훈 그리고 항상 용기와 힘을 준 사랑하는 누나와 매형, 조카, 장인, 장모에게도 깊은 감사의 마음을 드립니다.

마지막으로 일일이 언급을 하지 못했지만 저를 아껴주신 모든 분들께 감사의

인사를 드립니다. 그 동안 배운 가르침과 은혜를 마음에 새기고 항상 초심을 잃지 않고 노력하는 자세로 모든 일에 임하도록 최선을 다 하겠습니다.

2017년 12월 장 평

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Implications of MFN2 expression in pathogenesis of oral squamous cell carcinoma

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I. INTRODUTION

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer. It is common cancer in South Asia, including India and Sri Lanka (Ferlay, et al., 2010). The rate of morbidity of OSCC showed gradually increasing in recent years (Matsuda, et al., 2012). OSCC frequently worsen a patient's quality of life via dysfunction of mastication, speech, and deglutition. Furthermore, even with

improvements in diagnostic and therapeutic techniques, OSCC still showed poor prognosis: the overall 5-year survival rate was about 50% over the past 2 decades (Kademani, et al., 2005).

As a well known dynamic organelles, mitochondria control many cellular functions, which are continuously undergoing fission and fusion (de Brito and Scorrano, 2008). Morphology of mitochondria was controlled by the balance of two opposing forces, fission and fusion of mitochondria. The balance can change the size, shape and position of mitochondria in a few seconds (Bereiter-Hahn and Voth, 1994). During fission, mitochondria can create a new one and also can removing the damaged portions. By contrast, fusion can mix the contents of damaged portion of mitochondira as a form of complementation (Youle and van der Bliek, 2012).

As a mitochondrial fusion protein, mitofusin-2 (MFN2) can promote mitochondrial elongation and activity (White, et al., 2011). As a major player of the fusion process, MFN2 is integral to the outer membrane of mitochondrial (Shen, et al., 2007). Some investigators showed that MFN2 play an important role in the neuronal differentiation and embryonic development via involvement in fusion process of mitochondrial (Song, et al., 2009).

Mutation locus of MFNs has been related to various human diseases, such as Charcot-Marie-Tooth type 2A neuropathy, Alzheimer's disease, and diabetes (Ranieri, et al., 2013). MFN2 is frequently expressed in muscle cells and its insufficiency has been related to the fragmentation of the mitochondrial network, which is essential for the normal

mitochondrial function (Ranieri, et al., 2013). Inhibition of mitochondrial fission can attenuates muscle loss via fasting (Kluge, et al., 2013). Moreover, MFN2 downregulation has been found in the muscle of obese and non-obese type 2 diabetic subjects (Bach, et al., 2005; Hernandez-Alvarez, et al., 2010). Some investigators also found that MFN2 mRNA expression decreased in the skeletal muscle of tumor-bearing mice with sever cachexia (White, et al., 2011). These results implied that MFN2 is involved in muscle wasting related to diseases such as cancer cachexia.

The various functions of MFN2 in cancer development and progression have recently been investigated. Downregulation of MFN2 expression in various human cancers was reported by many groups (Jin, et al., 2011; White, et al., 2011; Zhang, et al., 2013). Some investigators found that ectopic MFN2 expression can induce apoptosis via increasing Ca2+ influx into mitochondria from the endoplasmic reticulum in HepG2 cell lines. Similar results were also provided by other investigators. In bladder cancer cell lines, ectopic MFN2 expression can induce caspase-3-mediated apoptosis and cell cycle arrest in G1/S phases, there by inhibit the cell proliferation. Another investigators demonstrated that MFN2 overexpression in gastric cancer cell line attenuated cell migration and invasion ability via decrease MMP-2 and MMP-9 expression. All of these findings implied that an tumor suppressor role of MFN2.

However, in contrast to these studies, some investigators showed that MFN2 was overexpressed in lung cancer tissues than that corresponding normal tissue samples (Lou, et al., 2015). It was also shown that knockdown of MFN2 attenuates cell proliferation and

invasion abilities.

The clinicopathological significance of MFN2 expression in various cancer patients were also investigated by some investigators. Wang and Feng have showed that MFN2 expression was significantly related to poor prognostic indicators such as tumor size, clinical stage, and poor overall survival in liver cancer patients (Wang, et al., 2015; Wang, et al., 2010). However, in gastric cancer, MFN2 expression was not showed significant association with various prognostic factors in gastric cancer (Zhang, et al., 2013). Moreover, both oncogenic and tumor suppressor activities were reported in various cancers such as bladder, stomach, and lung (Zhang, et al., 2013). The role of MFN2 in cancer progression seems to be more complicated than expected and further studies were needed for investigate the details.

The crucial role of MFN2 in OSCC has not been clarified. The present study determined MFN2 expression in primary OSCC cell lines and tissues, and further investigated the clinicopathological significance of MFN2 expression in patients with OSCC.

Ⅱ. MATRIALS AND METHODS

1. Patients and samples

This study included OSCC tissue samples obtained from 103 patients with OSCC who received surgical treatment at Yonsei University Dental Hospital, Korea, between 1995 and 2010. The following clinical parameters were recorded: age, gender, lesion site, T stage, lymph node metastasis, histological grade, perineural invasion, and vascular invasion (Table 1). Follow-up for the cohort of patients was performed for at least 5 years. The study was approved by the Institutional Review Board of the Dental Hospital, Yonsei University Health system (IRB No.2-2011-0044).

Table 1. Clinicopathological characteristics of 103 patients with OSCC

Clinicopathological variables	No. of patients (%)
Total cases	103
Age, years	
Median age (range)	57(32-79)
≤60	57(55.3)
>60	46(44.7)
Gender	
Male	72(69.9)
Female	31(30.1)
Site	
Tongue	37(35.9)
Retromolar trigone	26(25.2)
Gingiva	40(38.8)
T stage	
T1-T2	43(41.7)
T3-T4	60(58.3)
N stage	
Negative	53(51.5)
Positive	50(48.5)
Vascular invasion	
Absent	57(55.3)
Present	46(44.7)
Perineural invasion	
Absent	173(87.4)
Present	25(12.6)

2. Immunohistochemistry

Formalin-fixed, paraffin-embedded archival OSCC tissue sections were used for immunohistochemistry. Mouse monoclonal antibody against MFN2 (1:200; Abcam, Cambridge, MA, USA) was used as the primary antibody for the staining. All sections were deparaffinized with xylene and rehydrated with graded alcohol. Following antigen retrieval with antigen retrieval buffer (Dako, Carpinteria, CA, USA), endogenous peroxidase activity was blocked with endogenous enzyme block solution (Dako). The sections were incubated with primary antibody at room temperature for 1h. Real Envision^{TM} HRP Rabbit/Mouse detection system (Dako) was used as the secondary antibody. Slides were visualized with 3,3'-diaminobenzidine (DAB) and then counterstained with hematoxylin. For a negative control, the primary antibody was replaced by phosphate buffered saline. The scoring of protein expression was performed using the weighted histoscore method, as described previously.(Witton, et al., 2004) The intensity of tumor cell staining was scored as 0 (negative), 1 (light brown), 2 (brown), and 3 (dark brown). The final score was calculated as follows: total score = (0x percentage of negative cells) + (1x percentage of light brown staining cells) + (2x percentage of brown staining cells) + (3x percentage of dark brown staining cells). Patients were subdivided into two groups based on the total score: low (total score 0–100) and high (total score 101–300) expression groups.

3. Cell lines and cell culture

OSCC cell lines, HSC-2, HSC-3, and CA9-22 were supplied by the Korean Cell Line Bank (Seoul, Korea), and YD-10B, YD-32, and YD-38 were previously established by the Oral Cancer Research Institute, Yonsei University, Korea.(Lee, et al., 2005) All OSCC cell lines were cultured in mixed medium that contained Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) and Ham's nutrient mixture F12 (Gibco BRL) medium (3:1 ratio) and maintained in a cell culture incubator (5% CO_2) at 37℃. The supplement consisted of 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA), 100 U/mL of penicillin, 100 mg/mL streptomycin, $1x10^{-10}$M cholera toxin, 0.4 g/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, and $2x10^{-11}$M triiodothyronine.

4. Small interfering RNA (siRNA) transfection

Three independent small interfering RNA (siRNA) oligonucleotide pools targeting MFN2 were used to block MFN2 expression. Various numbers of cells were suspended in culture media without antibiotics and seeded in a plate. The cell density was 30-50% confluence at the time of transfection. Stealth siRNAs (Invitrogen, Carlsbad, CA, USA) targeting MFN2 were mixed with Opti-MEM media (Invitrogen, Carlsbad, CA, USA). This mixture was then mixed with an equal volum of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM media. After 20min of incubation, the final mixtures

were added to each well of the plate for a final siRNA concentration of 2 to 100 nM.

5. Cell cycle and apoptosis assay by flow cytometry

After synchronized by serum-free starvation for 24 hours and infected with adenovirus for 24h hours, OSCC cells were then harvested and stained with propidium iodide using a Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson, USA). Cell cycles were analyzed using flow cytometry with a FACScan (Becton Dickinson, USA).

Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) apoptosis detection kit (Invitrogen, USA) following the manufacturer's protocol. Briefly, cells were collected, centrifuged, and re-suspended in 500μl of 1×binding buffer in tubes. After added with Annexin V-FITC and PI, tubes were incubated darkly at room temperature for 15 minutes. Cell apoptosis assay was performed immediately on flow cytometry. Each experiment was performed at least three times.

6. Wound healing assay

Various numbers of cells were seeded with culture media in 6-well culture plates. When the cells reached a confluent state, cell monolayer were wounded by 20-200 μm pipette tip to perform wound healing assay. To remove debris, cells were washed with replaced culture meda and plates were returned to the 5% CO_2 humidified incubator. Photographs were taken at time points indicated in results. For evaluation of wound closing, three randomly selected points were marked and the horizontal distance of the migrated cells

from the initial point (time 0 hr) was measured and calculated using Image-J image analysis software (National Institutes of Health, Maryland, MD, USA).

7. Transwell invasion assay

The cell invasion ability was examined using matrigel invasion Chambers (BD Bioscience, Woburn, MA, USA). For matrigel coating, matrigel diluted in cold PBS was added on the upper chambers and incubated for 1 hour at 37 ℃ for gel formation. Then, $1X10^5$ cells in serum-free medium were placed into the coated upper chamber. Complete medium with 10% FBS were added to the lower chamber. After 24 hours, the cells remaining on the upper membrane were removed with cotton wool, whereas the cells that had invaded through the membrane were stained with 20% methanol and 0.1% crystal violet and counted.

8. Total RNA extraction and reverse transcription PCR analysis

Total RNA was purified from OSCC cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After cDNA synthesis, conventional RT-PCR analysis was performed using AccuPower PCR PreMix (Bioneer Corp., Seoul, Korea) with an annealing temperature of 58°C. β-actin was used as the housekeeping gene. Oligonucleotide primers used for the PCR were: 5'-CAACCAATTTGGAAAACAGG-3' and 5'-GTGGGAGGACAGGAGTTCTT-3' for MFN2; and 5'-

ATAGCACAGCCTGGATAGCAACGTAC-3' and 5'-CACCTTCTACAATGAGCTGCGTGTG-3' for β-actin. (Zhang, et al., 2014) Quantitative real time PCR analysis was performed was performed using 1X SYBR-Green Master Mix (Applied Biosystems), 10 pmol of each primer and 2 μl of the cDNA in an Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA) under the following conditions: initial denaturation for 10 min at 95℃, followed by 40 cycles of 95℃ for 20 sec, 50℃ for 30 sec and 72℃ for 45 sec. Oligonucleotide primers used for the PCR were: 5'- AGG CTG GCA ACA TAA CAG AG-3' and 5'- GGC ATG CAT TGA GTC TTT CT-3' for MFN2; and 5'-GAC CCC GTT ACC ACA GAA GA-3' and 5'- GCA GAA CTT TGT CCC AGA GC-3' for VEGF, and 5'- TTC CAG GAG TAC CCT GAT GA-3' and 5'- TGA GGT TTG ATC CGC ATA AT-3' for VEGFC.

9. Tube formation assay

Before tube formation analysis, HUVEC cells were transfected with 100 nM of Small interfering RNA, and then were incubated for 24 h in CO2 incubator. After 24 h, about 4 $X10^5$/well HUVEC cells were seeded in 48 well plate which was pre-coated with growth-factor-reduced Matrigel (BD Biosciences, Mississauga, ON, Canada). The result was measured at 16 h after culture. Each well was washed, fixed, stained with 1 μg/mL of Calcein AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37 ℃, and viewed through a microscope. Total tube length was measured in three fields using Image J software version 1.51 (National Institutes of Health, Bethesda, MD, USA).

10. Statistical analysis

The statistical analysis was performed using commercially available software (SPSS version 23.0; IBM Corp., Armonk, NY, USA) for statistical analysis. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Ⅲ. RESULTS

Evaluation of MFN2 mRNA expression in OSCC cell lines and tissue samples

MFN2 mRNA and protein expression were detected in all six OSCC cell lines by conventional RT-PCR analysis and western blot analysis, respectively (Figure 1). Both MFN2 mRNA and protein expression was detected in all 6 OSCC cell lines, and cell lines, YD-10B and HSC-2, which known as with tumorigenic capacities were uses in further studies. Moreover, I found that MFN2 mRNA expression was significantly increased in cell lines (YD10B & YD38) obtained from patients with LN metastasis than cell line (YD-32) obtained from patients without LN metastasis ($P<0.001$ and $P<0.05$, respectively) (Figure 2). The expression levels of MFN2 mRNA was further evaluated in the OSCC tissue samples, and found that MFN2 expression was significantly increased in OSCC tissue samples with LN metastasis than those without LN metastasis (Figure 2).

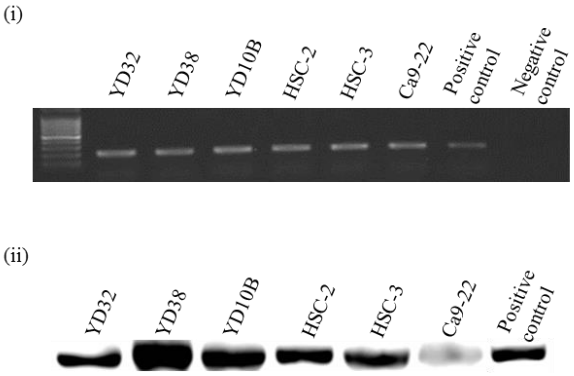


Figure 1. MFN2 mRNA (i) and protein (ii) expression in OSCC cell lines. Both MFN2 mRNA and protein expression were detected in all 6 OSCC cell lines. Hela cell was used as positive control. Distilled water replace the cDNA was considered as negative control.

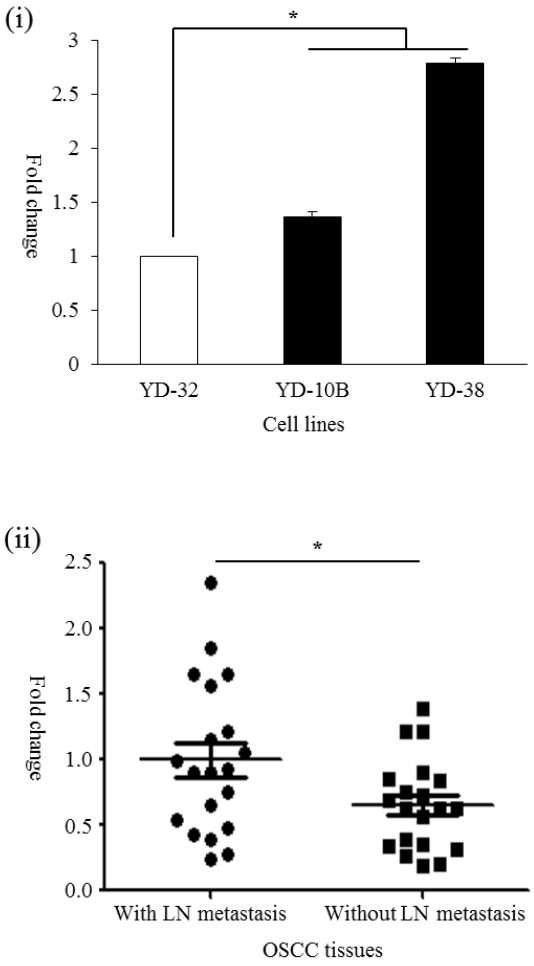


Figure 2. MFN2 mRNA expression was significantly increased in the cell lines (YD-10B & YD-38) obtained from OSCC patients with LN metastasis than cell line (YD-32) without LN metastasis (i). OSCC patients with LN metastasis showed significantly increased MFN2 mRNA expression than patients without LN metastasis (ii) (*P<.0.05)

Influence of MFN2 knockdown on proliferation of OSCC cell lines

Knockdown of MFN2 was performed in OSCC cell lines, YD-10B and HSC-2, by MFN2 siRNA treatment. I found that proliferating ability was significantly decreased in OSCC cell lines with knockdown of MFN2 than control cells (Figure 3). Number of the cells was assessed by MTT assay at 72h after MFN2 siRNA treatment. Results showed that number of the cells was significantly decreased in MFN2 SiRNA treated group than control group both in YD-10B and HSC-2 (i). Moreover, both PCNA and Ki67 expression were significantly decreased in MFN2 knockdown cells than control cells. These results indicate that knockdown of MFN2 attenuates proliferating ability of OSCC cell lines.

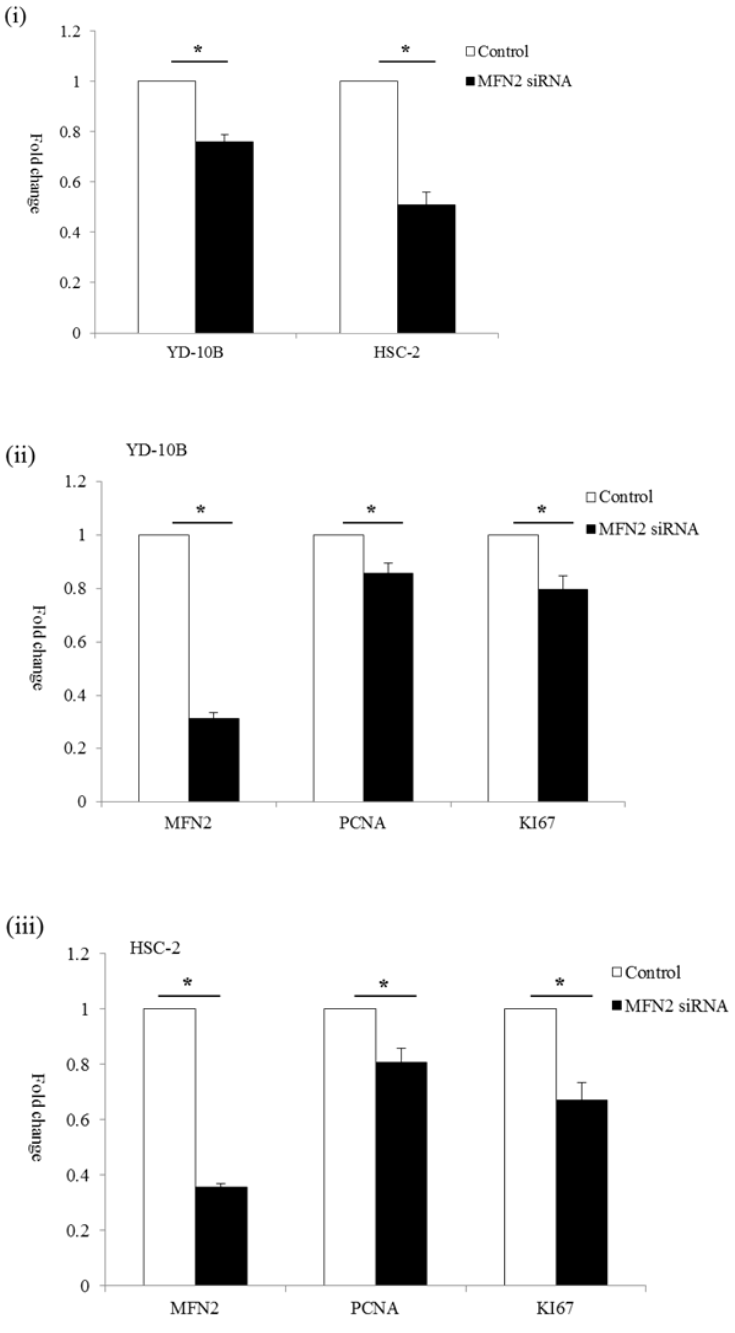
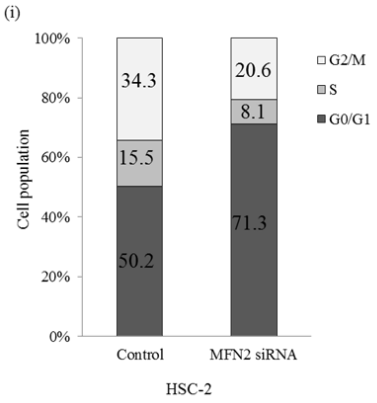


Figure 3. Knockdown of MFN2 attenuates proliferating ability of OSCC cell lines. (i) Number of the cells were assessed by MTT assay 72h after MFN2 siRNA transfection. Significant difference was found between control group and MFN2 siRNA treated group, both in YD-10B and HSC-2 cell line. Fold change of MFN2, PCNA and Ki67 mRNA expression in control and MFN2 knockdown OSCC cell lines (ii &iii). Both PCNA and Ki67 expression was significantly decreased in the cells transfected with MFN2siRNA than control group. Data represent the mean±SD of three independent samples, and the experiment was repeated three times per sample. Asterisks indicate statistical significance, *P<0.05 vs. control.

Influence of MFN2 knockdown on cell cycle arrest

To evaluate whether a decrease in cell proliferation was derived from cell cycle arrest, I performed cell cycle analysis. I found that G0/G1 phase cell population increased 12.3% and 11.5% in YD-10B and HSC-2 cell lines by knockdown MFN2 compared to the control cells, respectively (Figure 4). The results showed that MFN2 regulates cell cycle by led to G0/G1 arrest in OSCC cell lines.



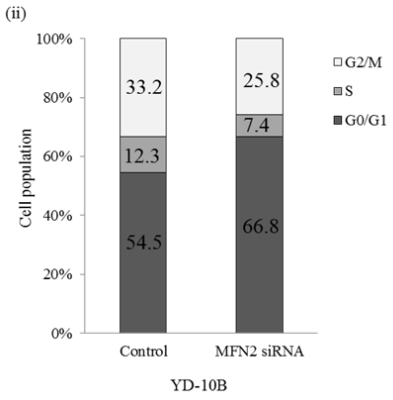


Figure 4. Effect of MFN2 knockdown on cell cycle of OSCC cell lines. After transfected with MFN2 siRNA, cell lines were labeled with PI and analyzed by flow cytometry. Both YD-10B and HSC-2 cell lines showed significant increase in G0/G1 population after MFN2 siRNA transfection. Data represent the mean ± SD of three independent samples, and the experiment was repeated three times per sample.

Influence of MFN2 knockdown in apoptosis of OSCC cell line

To further investigate the other mechanisms involved in regulating cell growth, annexin V and PI staining was performed to detect apoptotic cell death by flow cytometry analysis. Both YD-10B and HSC-2 showed 17.0% and 13.9% of apoptotic cell portion after MFN2 siRNA transfection. (Figure 5). The results showed that MFN2 knockdown mediated apoptosis can also influence cell growth in OSCC.

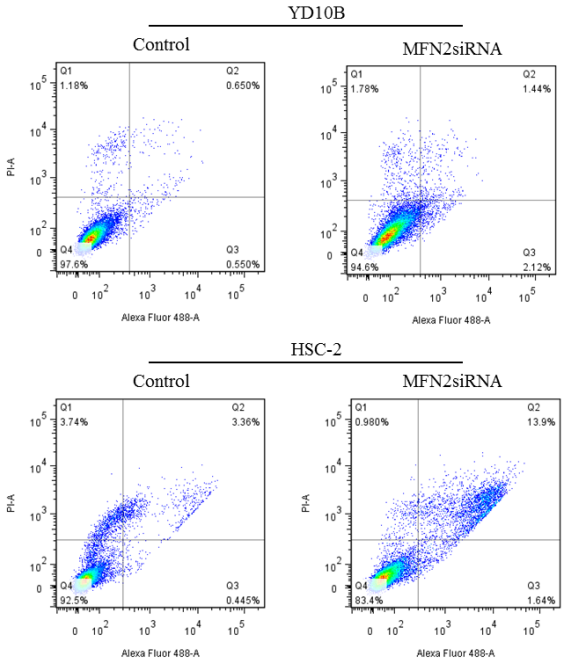


Figure 5. MFN2 knockdown promotes cell apoptosis in OSCC cell lines. (i) MFN2 siRNA transfected OSCC cell lines (YD-10B and HSC-2) and the related control cells were labeled with annexin V-FITC and PI.

Effect of MFN2 knockdown on motility of OSCC cells

To assess the influence of MFN2 knockdown in motility of OSCC cells, I performed wound healing assay using MFN2 knockdown- and control group of YD-10B and HSC-2 cells. I found that MFN2 knockdown significantly attenuates motility of OSCC cell (Figure 6).

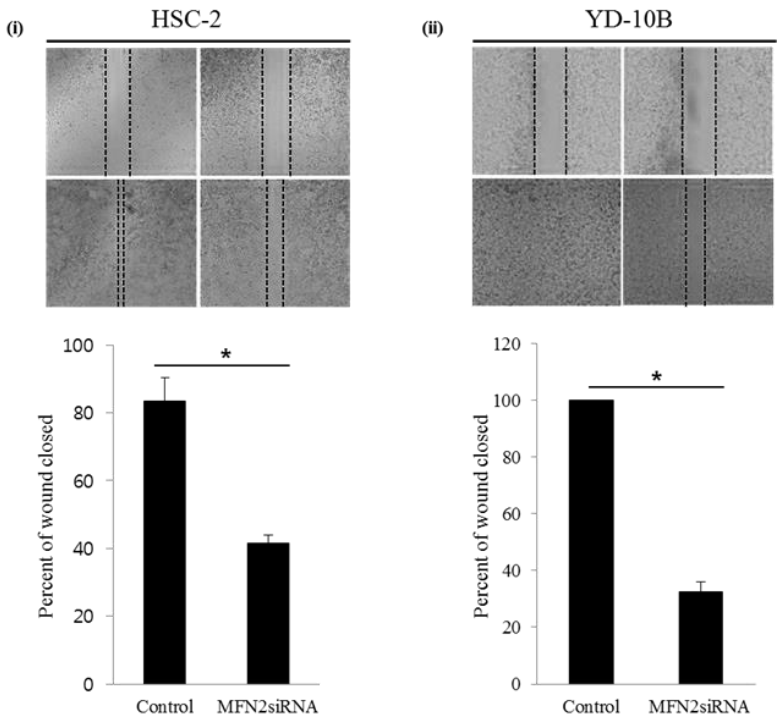


Figure 6. MFN2 knockdown significantly attenuates motility of OSCC cell lines. Wound healing assay was performed in OSCC cell lines, YD-10B and HSC-2, after the MFN2 siRNA transfection. Motility was significantly decreased after MFN2 knockdown in both YD-10B and HSC-2 cell lines. Data represent the mean±SD of three independent samples, and the experiment was repeated three times per sample (*P<0.05 vs. control).

Effect of MFN2 knockdown on invasive capacity of OSCC cells

To assess the influence of MFN2 knockdown in invasive capacity of OSCC cells, I performed cell invasion assay using MFN2 knockdown- and control group of YD-10B and HSC-2 cells. I found that MFN2 knockdown significantly attenuates invasive capacity of OSCC cell (Figure 7).

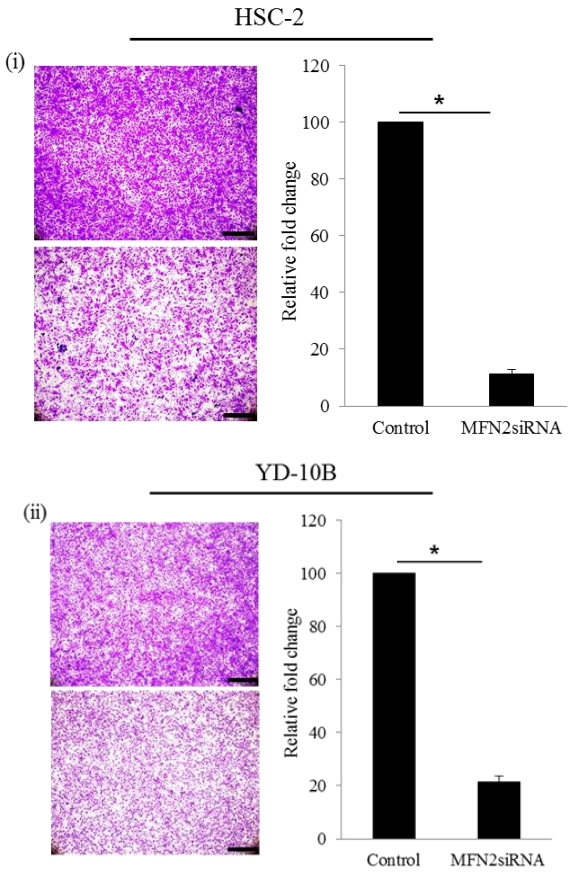


Figure 7. MFN2 knockdown significantly attenuates invasive capacity of OSCC cell. Invasion assay was conducted by matrigel coated trans-well, and invaded cells were quantified by count according to staining with crystal violet in three trans-wells after the siRNA transfection of MFN2. Invasive capacity was significantly decreased by MFN2 knockdown in both YD-10B and HSC-2 cell lines. Data represent the mean ± SD of three independent samples, and the experiment was repeated three times per sample (*P<0.05 vs. control).

In vitro, effect of MFN2 knockdown in angiogenesis of OSCC

1) Influence of MFN2 knockdown in expression of anigio-/lymphangiogenic factors

A clear correlation between tumor angiogenesis and nodal metastasis has been demonstrated in various cancers, including head and neck cancers (Petruzzelli, 2000). Some of molecular markers could strongly influence LN metastasis via the effect on angio-/lymphangiogenesis of cancers. In the public DB analysis, I found that MFN2 expression significantly related to LN metastasis in OSCC. So, I further investigated the MFN2 knockdown whether influence to angio-/lymphangiogenic activity of OSCC. I comparatively investigated the pro-angio- and lymphangiogenic factors, such as VEGF and VEGFC mRNA expression in MFN2 knockdown- and control OSCC cell lines, YD-10B and HSC-2. Interestingly, both VEGF and VEGFC mRNA expression was significantly decreased in MFN2 knockdown OSCC cell lines than control cells ($P<0.05$) (Figure 8).

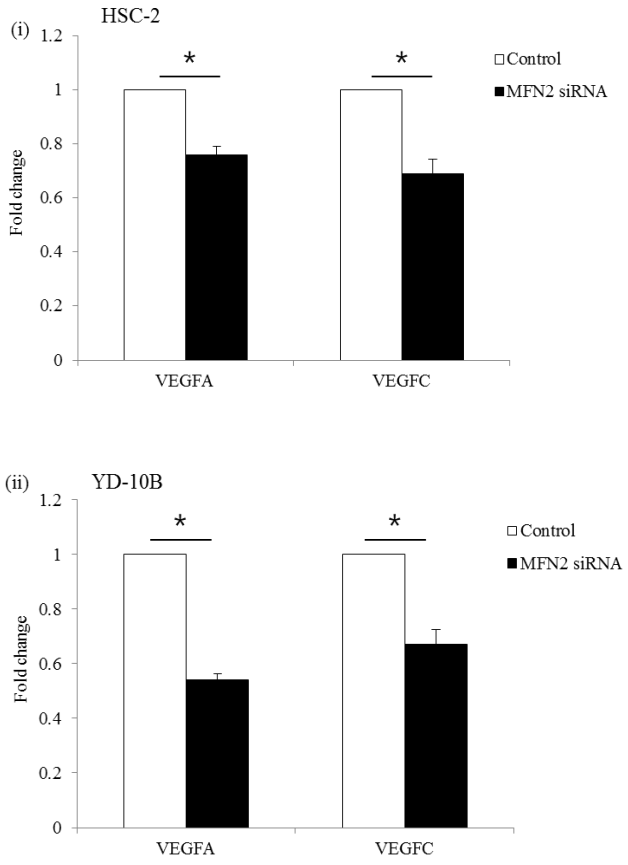


Figure 8. Influence of MFN2 knockdown in angiogenic activity of OSCC cell lines. (A) Pro-angiogenic- and lymphangiogenic factors, VEGF (i) and VEGFC (ii) mRNA expression was comparatively investigated in MFN2 depleted- and control OSCC cell lines. Compared to control group, both VEGF and VEGFC mRNA expression was significantly decreased in MFN2 depleted OSCC cell lines. Data represent the mean ± SD of three independent samples, and the experiment was repeated three times per sample (*P<0.05 vs. control).

2) MFN2 knockdown inhibit endothelial cell tube formation

Recently, some investigators showed that MFN2 are required for proper angiogenic function of HUVECs in vitro (Lugus, et al., 2011). To investigate the influence of MFN2 knockdown on tube formation of endothelial cells, I performed tube formation assay using control cells and MFN2 knockdown HUVECs. Cells were cultured on Materigel-coated six well plate and tube like structure formed after 16 hr in control group. In contrast, MFN2 depleted HUVECs resulted in disrupted, poorly connected tube networks (Figure 9).

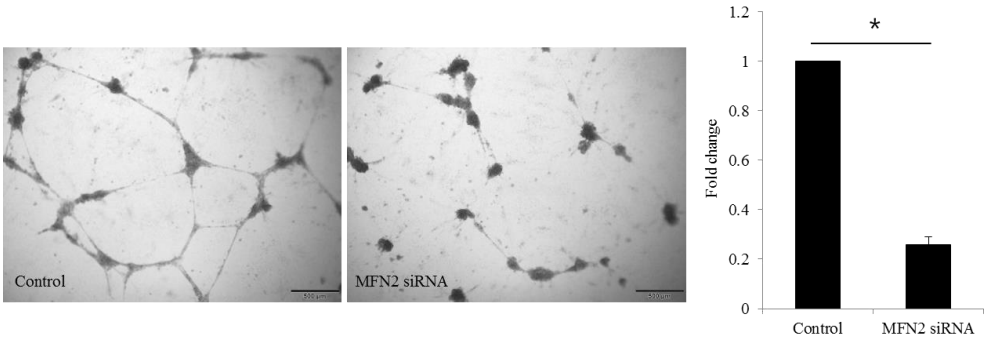


Figure 9. MFN2 knockdown inhibit tube formation of HUVECs. HUVECs were cultured on Materigel-coated six well plate. Tube like structure formed after 16 hr in control group. In contrast, MFN2 depleted HDMECs resulted in disrupted, poorly connected tube networks.

Clinicopahological significance of MFN2 expression in 103 OSCC patients

1) Association between MFN2 expression with clinicopathological parameters

MFN2 expression was detected in 10 normal oral mucosa and 103 OSCC tissue samples by immunohistochemistry. MFN2 expression was found in cytoplasm of tumor cells and vascular endothelial cells of OSCC tissues. By contrast, MFN2 expression was mainly restricted in basal layer of normal oral mucosa (Figure 10). In OSCC tissue samples, MFN2 expression significantly increased in patients with LN metastasis than patients without LN metastasis (Table 2). Moreover, patients with high MFN2 expression showed poor prognosis than patients with low MFN2 expression (Figure 11)

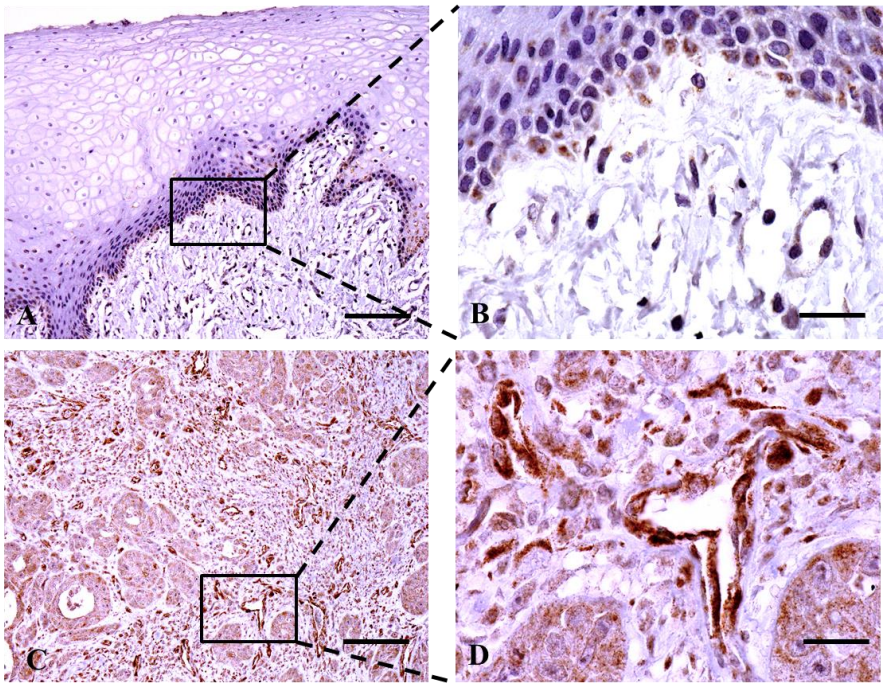


Figure 10. MFN2 expression was determined in 10 normal oral mucosa (A & B) and 103 OSCC tissue samples (C & D).

Table 2. Relationship between MFN2 expression and clinicopathological characteristics of 103 OSCC patients

Clinicopathologic variables	No. of cases (%)	MFN2 expression		P
		Low	High	
T stage				
T1-T2	43(41.7)	33(76.7)	10(23.3)	n.s.
T3-T4	60(58.3)	33(55.0)	27(45.0)	
LN status				
Negative	53(51.5)	31(58.6)	22(41.4)	0.012
Positive	50(48.5)	14(27.8)	36(72.2)	
Vascular invasion				
Absent	57(55.3)	30(52.6)	27(47.4)	n.s.
Present	46(44.7)	29(63.0)	17(37.0)	
Perineural invasion				
Absent	71(68.9)	46(64.8)	25(35.2)	n.s.
Present	32(31.1)	17(53.2)	15(46.8)	

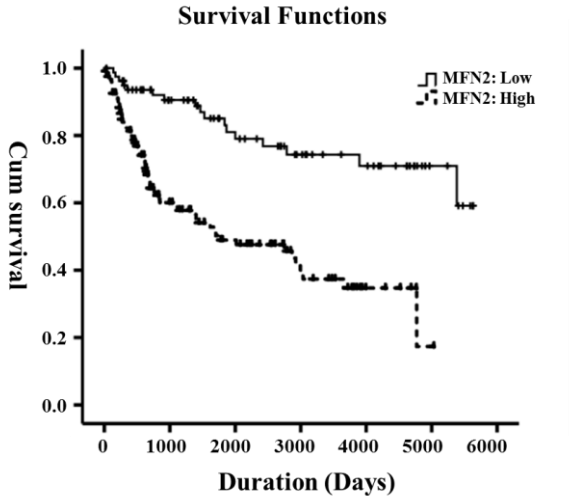


Figure 11. Overall survival period of patients with OSCC. Overall survival of 103 patients with OSCC classified into low and high MFN2 expression was analyzed by Kaplan-Meier method. Patients with high MFN2 expression showed poor prognosis than patients with low MFN2 expression ($P<0.001$).

2) Association between micro- and lymphatic vessel density with MFN2 expression in OSCC patients

Micro- and lymphatic vessel endothelial cells were highlighted by CD31 and D2-40 antibody, in this study. Micro- and lymphatic vessel density were investigated in OSCC tissue sections. Hot spots for each kind of vessel are shown in Figure 12. The association between each vessel density and clinicopathological parameters is shown in Table 3. Patients with LN metastasis showed more often of high level micro-/lymphatic vessel density than patients without LN metastasis (P=0.015 and P=0.001, respectively). Furthermore, vessel density was significantly associated with MFN2 expression in OSCC patients. I found that patients with high MFN2 expression showed increased micro-/lymphatic density than patients with low MFN2 expression (P=0.001 and P<0.001, respectively) (Figure 13).

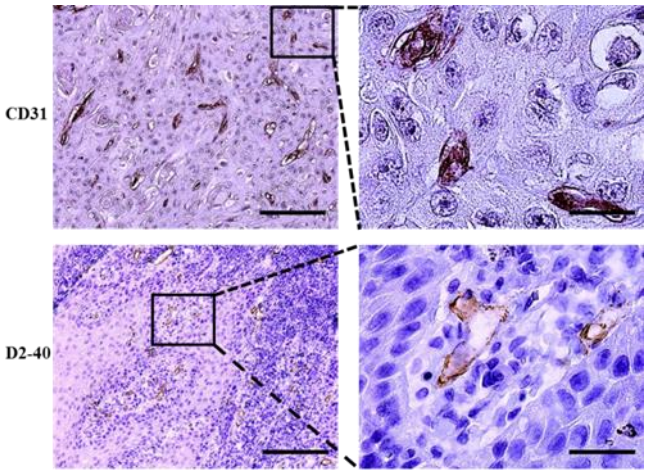


Figure 12. Example of a hot spot: (A) blood vessel endothelial cells were highlighted with CD31; (B) lymphatic vessel endothelial cells were highlighted by D2-40;

Table 3. Association between micro- and lymphatic vessel density and clinicopathological parameters

Clinicopathologic variables	No. of cases (%)	MVD		P	LVD		P
		Low	High		Low	High	
T stage							
T1-T2	43(41.7)	30(69.8)	13(30.2)	n.s.	31(72.1)	12(27.9)	n.s.
T3-T4	60(58.3)	38(63.3)	22(36.7)		38(63.3)	22(36.7)	
LN status							
Negative	53(51.5)	36(67.9)	17(32.1)	0.015	33(62.3)	20(37.7)	0.001
Positive	50(48.5)	12(24.0)	38(76.0)		13(26.0)	37(74.0)	
Vascular invasion							
Absent	57(55.3)	33(57.9)	24(42.1)	n.s.	37(64.9)	20(35.1)	n.s.
Present	46(44.7)	27(58.7)	19(41.3)		30(65.2)	16(34.8)	
Perineural invasion							
Absent	71(68.9)	43(60.5)	28(39.4)	n.s.	46(64.8)	25(35.2)	n.s.
Present	32(31.1)	20(62.5)	12(37.5)		17(53.2)	15(46.8)	

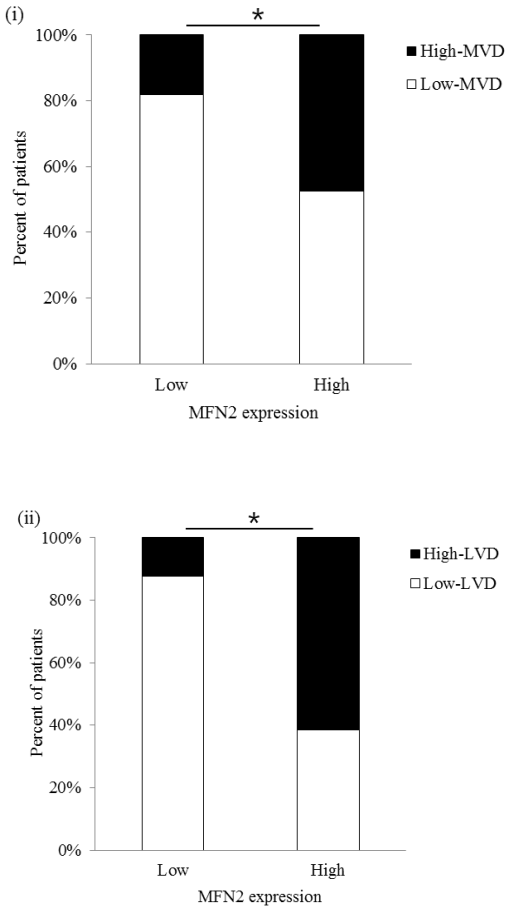


Figure 13. Association between MFN2 expression and vessel density. Significant association was found between vessel density and MFN2 expression. High micro-vessel density was found more often in patients with high MFN2 expression than patients with low MFN2 expression (P=0.023) (i). Moreover, high LVD was also found more often in patients with high MFN2 than patients with low MFN2 expression (P=0.005) (ii).

3) Clinicopathological significance of MFN2 positive vessel density in OSCC patients

MFN2 positive vessel density was determined in 103 OSCC tissue samples, and the clinicopathological significance was further investigated. High-MFN2 positive vessel density was more frequently detected in patients with LN metastasis or with vascular invasion than patients without LN metastasis or vascular invasion (P=0.032 and P=0.001, respectively)(Figure 14 i & ii). Patients with high MFN2 positive vessel density showed poor prognosis than patients with low MFN2 positive vessel density (P<0.001) (Figure 14 iii).

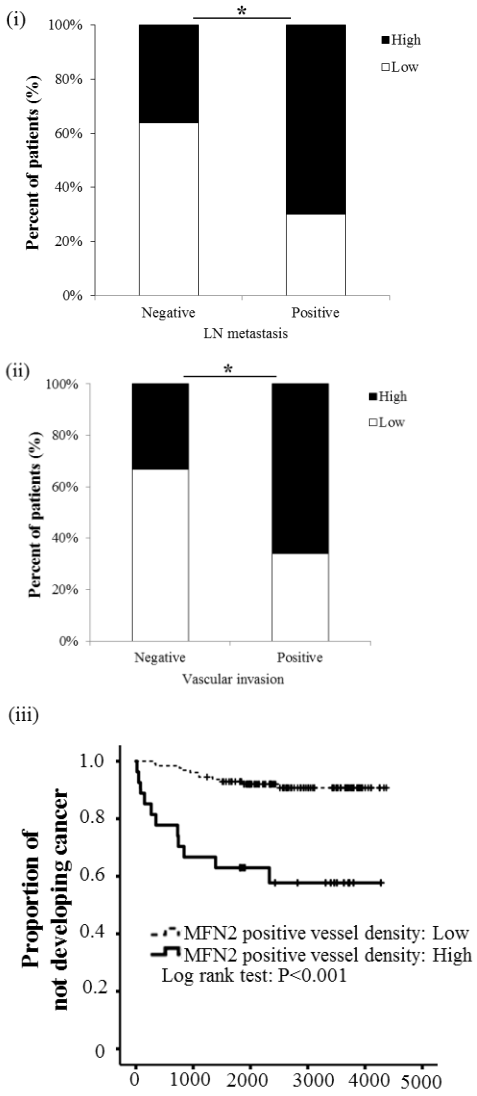


Figure 14. High MFN2 positive vessel density showed significant association with LN metastasis (P=0.032), vascular invasion (P=0.001) and poor prognosis (P<0.001).

IV. DISCUSSION

Oral squamous cell carcinoma (OSCC) is a malignant tumor that frequently invades the regional bone and lymph node and tends to metastasize to distant regions. About 500,000 new cases are occurred world wide each year and only with approximately 50% 5-year survival rate (Neville and Day, 2002). However, the underlying molecular mechanisms and the molecular target for diagnosis and treatment for OSCC were remains largely unknown.

The MFN2 gene is located on the chromosome 1p36.22 in humans, a regions containing various tumor suppressor genes (Bagchi and Mills, 2008). As a mitochondrial fusion protein, MFN2 localizes on the outer membrane of mitochondrial mediates mitochondrial function and morphology, and involved in maintenance of the mitochondrial network (de Brito and Scorrano, 2008). MFN2 contains two trans-membrane domains, a p21 (Ras) signature motif (amino acids 77-92) and a possible protein kinase A or G phosphorylation site), which plays an essential role in the signaling (de Brito and Scorrano, 2008).

The expression of the mitochondrial GTPase MFN2 in human cancers varies according to cancer type. The survey of a public human protein atlas database indicated that MFN2

protein levels are decreased in a number of human cancers, including liver cancer and bladder cancer, whereas it is elevated in lung cancer (Jin, et al., 2011; Lou, et al., 2015). However, MFN2 expression is significantly increased in OSCC tissue samples than normal oral mucosa samples. Moreover, MFN2 expression significantly associated with LN metastasis and poor prognosis. In consistent with this, previous study showed that MFN2 was downregulated in human gastric cancer tissues and that MFN2 overexpression in gastric cancer cells delayed cell proliferation and invasion (Zhang, et al., 2013). Moreover, Overexpression of the MFN2 gene in hepatocellular carcinoma resulted in tumor cell apoptosis via mitochondrial pathways mediated by calcium influx (Wang, et al., 2015). However, consistent with my results, some investigators were also showed that MFN2 knockdown can attenuates cell proliferation and invasion ability in lung cancer cell lines (Lou, et al., 2015).

To further clarify the influences of MFN2 expression on OSCC cell lines, I investigated the effect of MFN2 on biological behavior of OSCC cell lines. Both MFN2 mRNA and protein expression were detected in all 6 OSCC cell lines. I selected two cell lines that overexpress MFN2 and then performed in vitro and in vivo studies. I found that knockdown of MFN2 can inhibits proliferating ability, invasive capacity and motility of the OSCC cells in vitro. All of the findings indicated that MFN2 may have oncogenic activities in OSCC progression.

Some investigators showed that MFN2 are required for proper angiogenic function of HUVECs in vitro (Lugus, et al., 2011). Interestingly, I found that OSCC tissue samples

showed significantly increased MFN2 expressing microvessel density than normal oral mucosa. In OSCC tissue samples, tissues with high level of MFN2 showed higher micro- and lymphatic vessel density than tissues with low level of MFN2 expression. Supportively, MFN2 knockdown OSCC cells showed decreased VEGF and VEGFC expression than control cells. These data implied that MFN2 expression may be involved in angiogenesis of OSCC. A clear correlation between tumor angiogenesis and nodal metastasis has been demonstrated in various cancers, including head and neck cancers (Petruzzelli, 2001). Some of molecular markers could strongly influence LN metastasis via the effect on angio-/lymphangiogenesis of cancers. MFN2 mediated angiogenesis may further influence LN metastasis as well as prognosis of OSCC.

I concluded that MFN2 showed an oncogenic potential in OSCC via influence the biological behaviors of OSCC cells and angiogenic activity of OSCC patients.

V. CONCLUSION

I concluded that MFN2 showed an oncogenic potential in OSCC via influence the biological behaviors of OSCC cells and angiogenic activity of OSCC patients. Our findings provide further evidence for MFN2 as a novel diagnostic and prognostic biomarker in OSCC. MFN2 may also serve as a possible therapeutic target for patients with OSCC.

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ABSTRACT (IN KOREAN)

MFN2 발현이 구강편평상피암 발병기전에 미치는 영향에 대한 연구

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장 평

암종에서 구강암 사망률은 9 위를 차지하는 암종이며 그중에서 구강편평상피암은 구강암에서 가장 많이 발생하는 조직학적 유형이다.

미토콘드리아는 세포 기관에서 부단한 융합과 분열을 형성하면서 매우 중요한 역할을 하는데 여기에서 융합역할을 하는 결정적 인자 중 하나가 바로 MFN2 이다. MFN2 는 미토콘드리아의 외막에 있는 GTPase 로써 암 발생과 진행에 있어서 중요한 역할을 한다고 최근 알려졌으나 종양 억제 유전자 인자인지 아니면 종양 형성 유전자 인자인지 여부는 아직 충분히 연구되지 않았다.

본 연구에서는 구강편평상피암에서 MFN2 의 작용을 체외 혹은 체내 실험을 이용하여 연구하려 하였고 장기 추적 관찰 된 구강편평상피암 환자 조직을 이용하여 MFN2 의 발현 정도가 임상병리학적 예후에 미치는 영향을 보고자 하였다.

MFN2 는 구강편평상피암 세포주에서 빈번한 발현을 보였으며 세포의 증식, 세포사멸, 이동성 및 침습능과 통계학적으로 유의한 상관성을 나타냈다. 구강편평상피암 환자군에서 MFN2 의 발현은 림프절 전이 및 누적 생존률에서 각각 통계학적 유의성을 보였다.

결론적으로 MFN2 는 구강편평상피암에서 종양형성 유전자의 기능을 나타내며 이는 MFN2 가 구강편평상피암 환자의 진단 및 예후를 보는 생체표지자로의 의미가 있다고 할 수 있겠다.

핵심되는 말 : MFN2, 림프절 전이, 신생혈관형성, 생체표지자, 구강편평상피암