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의학석사 학위논문

**S100A8 and S100A9 induction is
involved in inflammation-associated
hepatocarcinogenesis under hypoxic
conditions via the JNK pathway**

저산소증 환경에서 S100A8과 S100A9의 발현이
JNK 경로를 통해 염증 관련 간세포암의 발생에
미치는 영향

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conditions via the JNK pathway**

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Abstract

S100A8 and S100A9 induction is involved in inflammation-associated hepatocarcinogenesis under hypoxic conditions via the JNK pathway

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Background: S100A8 and S100A9 are two heterodimeric members of the cytoplasmic S100 Ca²⁺ signaling protein family. They act as proinflammatory signals and play a role in tumorigenesis as they affect inflammation, proliferation, and invasion of tumor cells in various types of cancer, including hepatocellular carcinoma (HCC). Hypoxia plays an important role in inflammation-associated tumorigenesis, but the direct role of S100A8 and S100A9 in hepatocarcinogenesis has not yet been elucidated, particularly with regard to the impact of hypoxia. The

present study investigated the role of S100A8 and S100A9 in HCC under hypoxic conditions.

Methods: The expression of S100A8 and S100A9 was quantified using real-time polymerase chain reaction. Human HCC cells (SNU-761 and SNU-3058) were grown with either S100A8/A9 treatment or control, under either normoxic or hypoxic conditions. MTS and invasion assays were performed to evaluate the role of S100A8 and S100A9 in HCC. Immunoblot analyses were performed to investigate the signaling pathway that is involved in the activity of S100A8 and S100A9.

Results: S100A8 and S100A9 mRNA expression increased under hypoxic conditions compared with normoxic conditions. S100A8 and S100A9 treatment significantly increased the invasion of HCC cells, especially under hypoxic conditions, whereas no significant changes in HCC cell proliferation were observed. S100A8 treatment significantly enhanced the expression of interleukin-8 (IL-8), an inflammatory cytokine that is involved in tumor progression, under hypoxic conditions. IL-8 siRNA transfection attenuated the S100A8-induced invasion of HCC cells. S100A8 treatment attenuated bile acid-induced HCC cell apoptosis under hypoxic conditions. Moreover, S100A8 increased the protein expression of mesenchymal markers, including vimentin, matrix metalloproteinase 9 (MMP-9), and MMP-2, which were attenuated by a c-Jun N-terminal kinase (JNK) inhibitor.

Conclusion: Hypoxia-induced S100A8/A9 expression increased the invasion of HCC cells and attenuated bile acid-induced HCC apoptosis by increasing IL-8 under hypoxic conditions. Considering the enhanced expression of epithelial-mesenchymal transition (EMT) markers, the JNK pathway may be involved in

controlling the activity of S100A8 and S100A9. These results indicate that S100A8 and S100A9 may serve as therapeutic targets for the treatment of HCC under hypoxic conditions.

Keywords: S100A8; S100A9; IL-8; JNK; hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide(1), and the burden of HCC is expected to increase in the future.(2) The lethality of HCC is mainly attributable to the advanced stage of the malignancy at the time of diagnosis and limited therapeutic options.(3) Although various treatment strategies have been proposed, tumor recurrence remains a significant limitation for most treatment modalities, and the survival benefit to patients with advanced HCC has remained unsatisfactory.(4, 5) Therefore, it is necessary to investigate the potential molecular mechanisms that are involved in the development of HCC in order to discover novel treatment strategies.

Since HCC is typically accompanied by neovascularization and hypervascularity, hypoxia is considered a strong stimulus for the genesis and progression of HCC.(6) Hypoxia enhances proliferation, angiogenesis, metastasis, chemoresistance, and radioresistance and suppresses the differentiation and apoptosis of HCC cells.(7) Thus, hypoxia has become a central issue in tumor physiology and the treatment of HCC.

S100 proteins are a group of multigenic, non-ubiquitous cytoplasmic Ca^{2+} binding proteins that are differentially expressed in a wide variety of cell and tissue types.(8) S100A8 and S100A9 are two heterodimeric members of the S100 protein family that are endogenous Toll-like receptor 4 agonists.(9) S100A8 and S100A9 are predominantly expressed in myeloid cells (e.g., neutrophils and monocytes),

and their expression in other cell types (e.g., epithelial cells) is inducible in response to stress under specific condition. S100A8 and S100A9 exhibit marked changes in expression in many types of cancer, and they are thought to contribute to the development and progression of cancer.(10) S100A8 and S100A9 have been shown to function as danger signals, in which they promote immune responses and repair mechanisms during inflammation and in tumors.(11) Different protumorigenic effects of S100A8 and S100A9 have been identified, and they are thought to promote inflammation-associated carcinogenesis by inducing inflammatory responses and creating a proinflammatory microenvironment.(12) Furthermore, S100A8 and S100A9 have been reported to activate key genes and pathways that are involved in tumor progression and metastasis.(13) The direct tumor-promoting effects of S100A8 and S100A9, such as the stimulation of cellular proliferation and migration, have been demonstrated in various tumor cell lines.(10, 14)

S100A8 and S100A9 are upregulated in HCC, and their expression correlates with poor tumor cell differentiation.(15, 16) Several studies have reported that S100A8 and S100A9 play critical roles in carcinogenesis in HCC. One study reported that S100A8 and S100A9 are novel nuclear factor κ B (NF- κ B) target genes in HCC cells during inflammation-associated hepatocarcinogenesis through the activation of reactive oxygen species-dependent signaling pathways and protection from cell death.(15) Another study reported that the S100A9-induced proliferation and invasion of HepG2 cells are partially mediated by activation of the mitogen-activated protein kinase (MAPK) signaling pathway.(17) However, the

direct role of S100A8 and S100A9 in hepatocarcinogenesis remains ambiguous and has not yet been elucidated, particularly with regard to the impact of hypoxia. Therefore, the aim of the present study was to investigate the role of S100A8 and S100A9 in HCC cells under hypoxic conditions and the ability of S100A8 and S100A9 to serve as potential markers of the progression of HCC.

Materials and Methods

Cell lines and cultures

Two human HCC cell lines were used in this study: SNU-761 (18), a poorly differentiated HCC cell line, and SNU-3058 (19), a hypovascular HCC cell line which was deposited to the Korea Cell Line Bank. Cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 100,000 U/L penicillin and 100 mg/L streptomycin. In all experiments performed in this study, cells were serum-starved overnight to avoid the effects of serum-induced signaling. According to the experimental design, the cells were subsequently incubated either under standard culture conditions (20% O₂ and 5% CO₂ at 37°C) or under hypoxic culture conditions (1% O₂, 5% CO₂ and 94% N₂ at 37°C).

Materials and reagents

S100A8 and S100A9 were obtained from Proteintech Group (Rosemont, IL, USA). The following primary antibodies were used: CK19 and Vimentin from BioGenex (Rocklin, CA, USA), Caspase 9, c-Jun N-terminal kinases (JNK), phosphorylated JNK, phosphorylated p42/44, matrix metalloproteinase-9 (MMP-9) and MMP-2 from Cell Signaling Technology (Beverly, MA, USA), Caspase 8 from BD Biosciences (San Jose, CA, USA), CAP43 from Abcam (Cambridge, MA, USA)

and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SP600125, an inhibitor of JNK, was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

Cell proliferation assay

Cell proliferation was measured using CellTiter 96 Aqueous One Solution cell proliferation assays (Promega, Madison, WI, USA). This test is based on the cellular conversion of the colorimetric reagent MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes, which are only found in metabolically active, proliferating cells. Following each treatment, 20 μ L of dye solution was added to each well in a 96-well plate and incubated for 1 hour. Subsequently, absorbance was recorded at 490 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Immunoblot analysis

Cells were lysed for 20 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40, 0.25% sodium deoxycholate; 150 mM NaCl; 1 mmol/L EDTA; 1 mM phenylmethylsulfonyl-fluoride; 1 mM Na_3VO_4 ; 1 mM NaF; and 1 μ g/mL each of aprotinin, leupeptin, and pepstatin) and then were centrifuged at 14,000 g for 10 min at 4°C. Samples were resolved by SDS-PAGE, transferred to nitrocellulose

membranes, blotted with appropriate primary antibodies, and incubated with peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA). Bound antibodies were visualized by using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film. The primary antibodies used were as above.

Invasion assay

In vitro invasion assay was performed using 24-well chambers. Inserts were transferred into wells and coated with Matrigel (BD Biosciences, Billerica, MA, USA) for 30 min at 37°C. SNU-761 and SNU-3058 cells were suspended in serum-free medium and seeded on the Matrigel-coated upper chambers (5×10^4 cells/chamber), and DMEM containing 10% FBS was added to the lower chambers. The cells were incubated for 6 h at 37°C and then incubated in the presence or absence of S100A8 (0.5 µg/mL) or S100A9 (0.5 µg/mL) for 24 h and then stained with 4 µg/mL calcein AM (BD biosciences, Billerica, MA, USA) in Hank's Balanced Salt Solution (HBSS) at 37°C for 90 min. Invasion ability was determined as relative fluorescence units measured at an excitation of 494 nm and an emission of 517 nm using a multifunctional plate reader (EnVision Multilabel Reader; PerkinElmer, Waltham, MA, USA).

Real-time PCR analysis

Total RNA was extracted by AxyPrep Multisource Total RNA Miniprep Kit according to the manufacturer's recommendations (Axygen Biosciences, Union City, CA, USA). cDNA templates were synthesized using oligo-dT random primers and Moloney Murine Leukemia Virus reverse transcriptase. The cDNA templates were used to determine gene expression levels by real-time PCR (Thermal Cycler Dice™ Real Time System; Takara Bio, Shiga, Japan) using SYBR green (Takara Bio).

Transfection of siRNA

The cells were seeded in a 6-well culture plate (2×10^5 cells/well) in 2-mL antibiotic-free medium supplemented with 10% FBS. At 60–80% confluence, the cells were transfected with siRNA using the siRNA Transfection Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions. The cells were treated with siRNA for 6 h at 37°C, and growth medium containing 20% FBS and antibiotics was subsequently added. After 18 h, the medium was aspirated and replaced with fresh medium containing 10% FBS and antibiotics. Twenty-four hours after the transfection, the cells were treated for further experiments.

Quantification of apoptosis

The degree of apoptosis was evaluated by using the nuclear binding dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) to quantify apoptotic cells with fluorescence microscopy (Leica Microsystems, Mannheim, Germany). DAPI was added to the treated cells for 30 min and the cells were examined by fluorescence microscopy. Apoptotic cells were defined as those containing chromatin condensation and nuclear fragmentation. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted $\times 100$. A minimum of 400 cells was counted for each treatment.

Statistical analysis

All cell-based experimental data are presented as mean \pm standard deviation (SD) of at least three independent experiments, unless otherwise indicated. Statistical evaluations of numeric variables in each group were conducted using the Mann-Whitney U test. All statistical analyses were performed using PASW 21.0 for Windows (IBM, Chicago, IL, USA). Differences with a P value less than 0.05 were considered statistically significant.

Results

Induction of S100A8 and S100A9 expression by hypoxia

We first investigated the expression of S100A8 and S100A9 in HCC cells using real-time PCR. S100A8 and S100A9 were overexpressed in both SNU-761 and SNU-3058 cells under hypoxic conditions compared with normoxic conditions (Fig. 1A, B).

Increased invasion of HCC cells by S100A8 and S100A9

MTS and invasion assays were performed to evaluate the role of S100A8 and S100A9 in HCC. We first performed the MTS proliferation assay after S100A8 and S100A9 treatment. No significant changes in the proliferation of HCC cells were observed under either normoxic or hypoxic conditions (Fig. 2A, B). We then evaluated whether S100A8 and S100A9 promote the invasion of HCC cells. We measured the percent change in the increase in invasion of HCC cells that were treated with 0.5 µg/mL S100A8/A9 compared with untreated cells. S100A8 and S100A9 treatment increased the invasion of SNU-761 and SNU-3058 cells under hypoxic conditions ($P < 0.05$, vs. control; Fig. 2C). These results indicate that S100A8/A9 induced the invasion of HCC cells while maintaining cell viability under hypoxic conditions compared with normoxic conditions.

IL-8 induction by S100A8 under hypoxic conditions

Since IL-8 is an inflammatory cytokine that is involved in tumor progression, we evaluated the role of IL-8 in inflammation-associated hepatocarcinogenesis. S100A8 treatment significantly enhanced the expression of IL-8 compared with controls only under hypoxic conditions ($P < 0.01$; Fig. 3A). After IL-8 treatment, the invasion of SNU-761 cells increased compared with controls ($P < 0.05$; Fig. 3B). With exogenous IL-8 treatment, no significant effect of S100A8 treatment on HCC cell invasion was observed. These results suggest that S100A8 acts through IL-8 because the presence of IL-8 was sufficient for the role of S100A8. To confirm the functional role of IL-8 in HCC cell invasion, we performed an invasion assay by knocking down IL-8 using small interfering RNA (siRNA) transfection under both normoxic and hypoxic conditions. With IL-8 siRNA transfection, no significant changes in cellular invasion were observed after S100A8 treatment compared with cells that were not treated with S100A8 (Fig. 3C).

Attenuated bile acid-induced apoptosis of HCC cells by S100A8 under hypoxic conditions

We next evaluated whether bile acid-induced apoptosis is modulated in HCC cells that are treated with S100A8. S100A8 treatment (0.5 $\mu\text{g/mL}$) significantly attenuated bile acid-induced hepatocyte apoptosis compared with controls, especially under hypoxic conditions ($P < 0.01$; Fig. 4A.). When the cells were treated with S100A8 (0.5 $\mu\text{g/mL}$), the expression of caspase 9 and caspase 8 (i.e.,

markers of apoptotic signaling) significantly decreased under hypoxic conditions compared with normoxic conditions (Fig. 4B).

S100A8-related hepatocarcinogenesis associated with the JNK pathway-dependent epithelial-mesenchymal transition (EMT)

To elucidate the possible mechanism of action of S100A8 on hepatocarcinogenesis, we explored the signaling pathways that are activated in HCC cells that were treated with 0.5 $\mu\text{g/mL}$ S100A8. S100A8 increased the protein expression of mesenchymal markers, including vimentin, MMP-9, and MMP-2, especially under hypoxic conditions (Fig. 5A). When HCC cells were treated with S100A8, phosphorylated c-Jun N-terminal kinase (JNK) expression significantly increased under hypoxic conditions (Fig. 5B). These findings indicate that the JNK pathway may be involved in the activity of S100A8.

To verify the involvement of the JNK pathway in the effects of S100A8 on hepatocarcinogenesis, we performed an invasion assay using a JNK inhibitor. The JNK inhibitor significantly reduced HCC cell invasion that was triggered by S100A8 treatment ($P < 0.05$, vs. control; Fig. 5C). With S100A8 treatment, the expression of vimentin in HCC cells was attenuated by the JNK inhibitor (Fig. 5D).

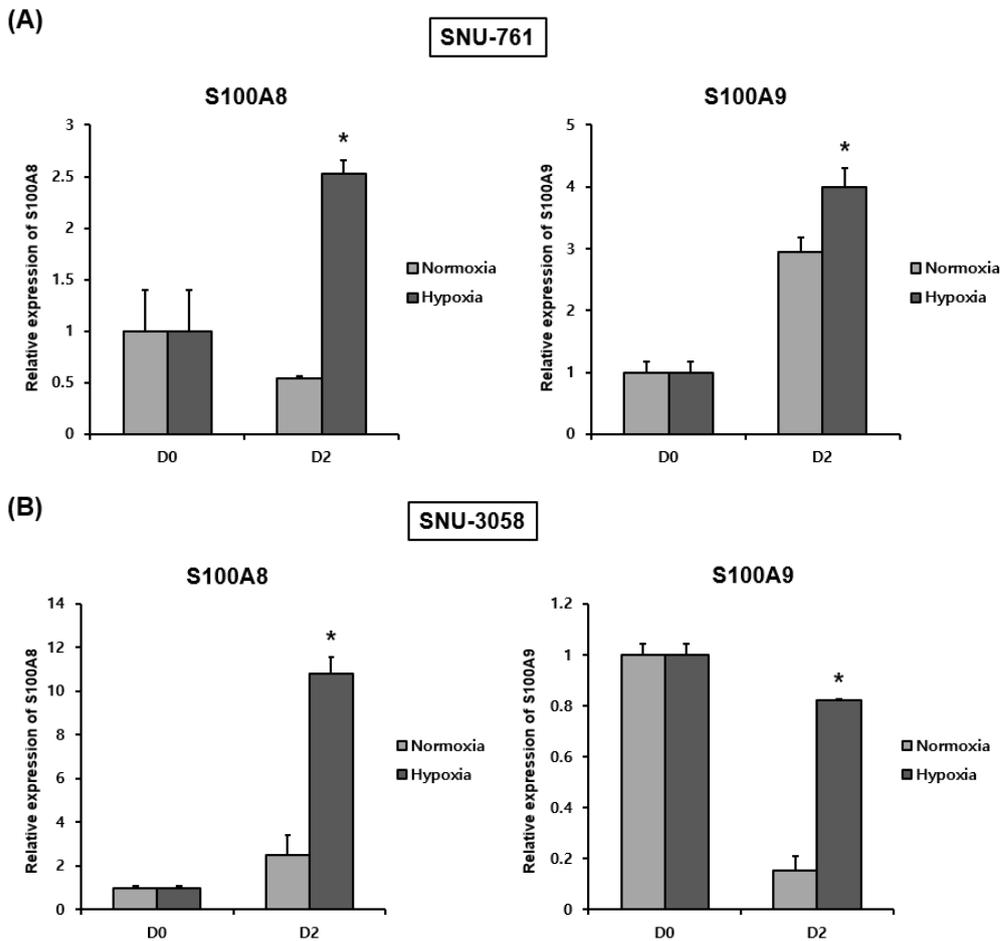


Figure 1. Induction of S100A8 and S100A9 expression by hypoxia. (A) The relative expression of S100A8 and S100A9 was assessed using real-time RCR in SNU-761 cells. After serum starvation for 24 h, SNU-761 cells were incubated either under standard culture conditions (20% O₂ and 5% CO₂ at 37°C) or under hypoxic culture conditions (1% O₂, 5% CO₂, and 94% N₂ at 37°C). After incubation for the time indicated, quantitative real-time PCR was performed for S100A8 and S100A9. (B) SNU-3058 cells were cultured under normoxic or hypoxic conditions, and the expression of S100A8/A9 was measured.

* $P \leq 0.05$ versus the normoxic conditions; columns represent means; bars represent SDs.

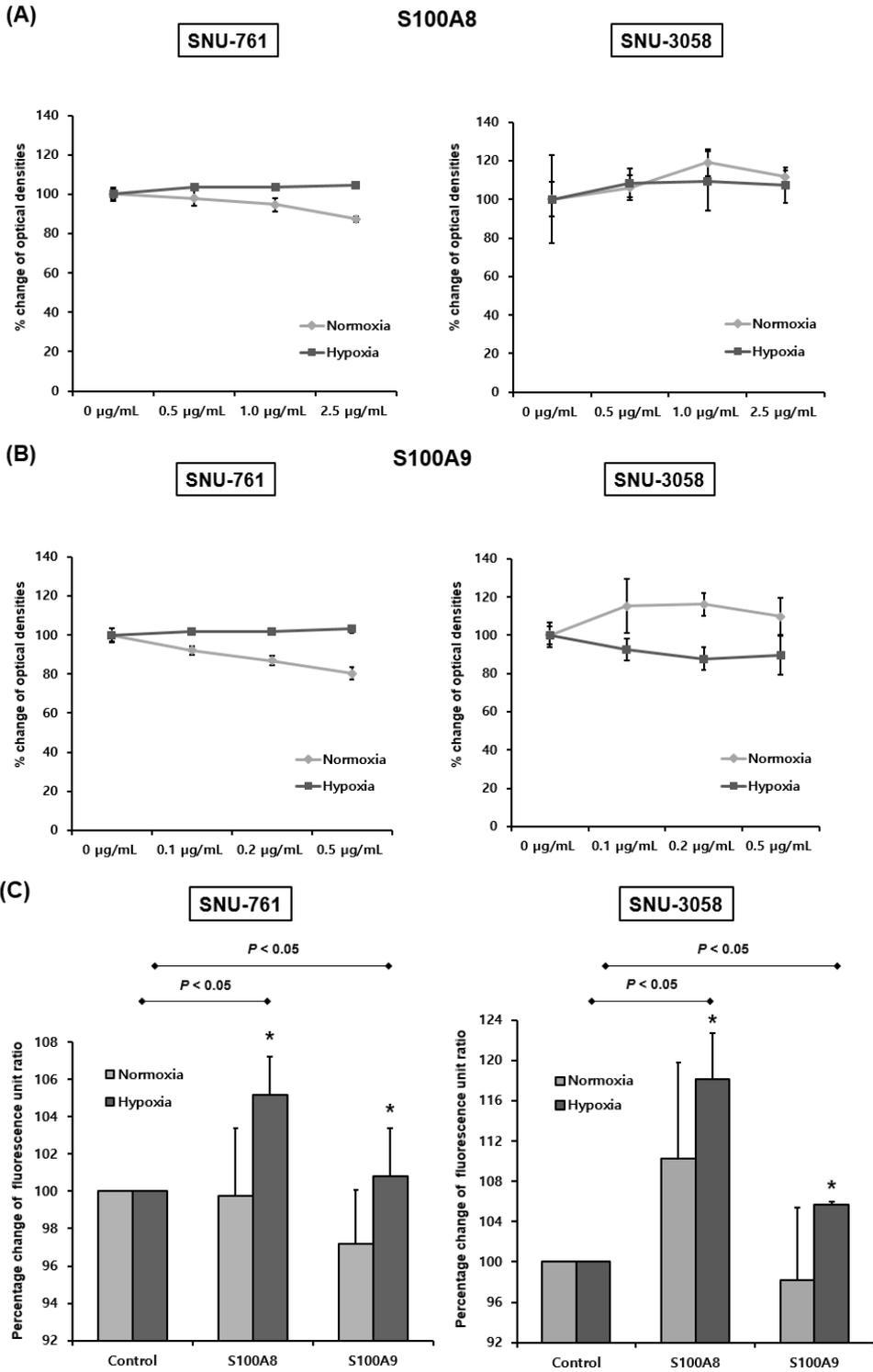


Figure 2. Increased invasion of HCC cells by S100A8 and S100A9. (A) SNU-761 and SNU-3058 cells were cultured with the indicated concentrations of S100A8 (0, 0.5, 1.0, and 2.5 $\mu\text{g/mL}$). The 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay was performed after 48 h. The data are expressed as the mean percent change in optical density compared with the control (set at 100%). (B) SNU-761 and SNU-3058 cells were cultured with the indicated concentrations of S100A9 (0, 0.1, 0.2, and 0.5 $\mu\text{g/mL}$), and the MTS assay was performed after 48 h. The data are expressed as the mean percent change in optical density compared with the control (set at 100%). (C) The invasion ability of S100A8 and S100A9 in SNU-761 and SNU-3058 cells was measured by the invasion assay. SNU-761 and SNU-3058 cells were seeded on Matrigel-coated upper chambers (5×10^4 cells/chamber), and DMEM that contained 10% FBS was added to the lower chambers of a 24-well plate. Cells were cultured under normoxic or hypoxic conditions. After incubation for 6 h, the cells were incubated in the presence or absence of S100A8 (0.5 $\mu\text{g/mL}$) or S100A9 (0.5 $\mu\text{g/mL}$) for 24 h. Invasion ability was determined as relative fluorescence units that were measured with a fluorescence plate reader using BD Calcein AM Fluorescent dye. The results are expressed as the percent change in fluorescence units compared with the control.

* $P \leq 0.05$ versus the normoxic conditions; columns represent means; bars represent SDs.

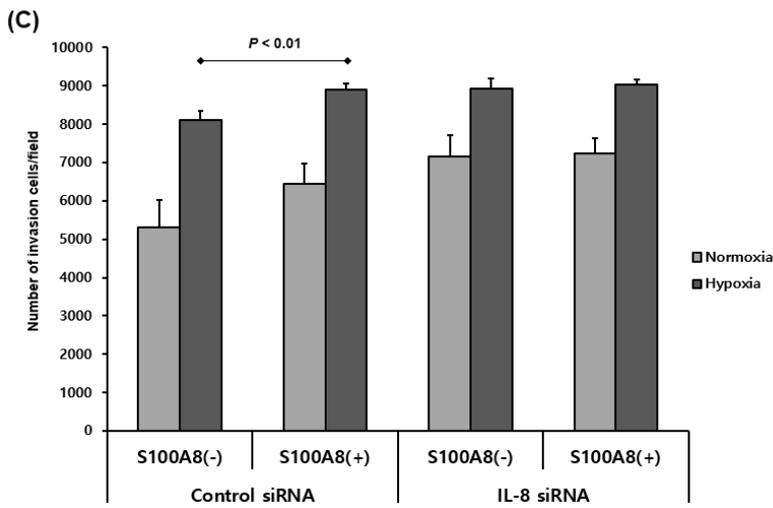
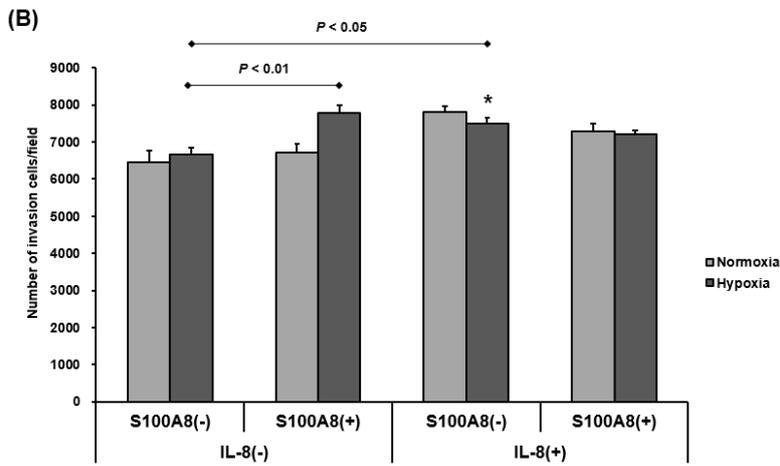
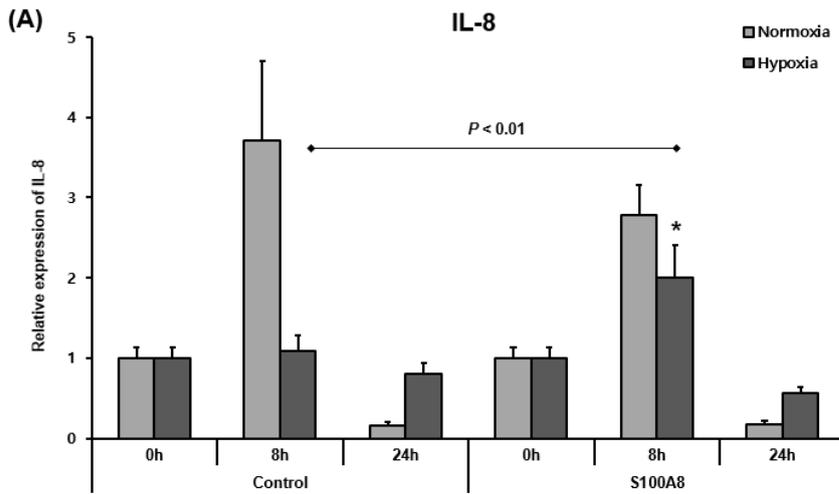


Figure 3. IL-8 induction by S100A8 under hypoxic conditions. (A) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) for 24 h under normoxic or hypoxic conditions. The cells were harvested after treatment at the time points indicated, and real-time RCR was performed for IL-8. The invasion assay was performed, and invasion ability was determined as relative fluorescence units that were measured with a fluorescence plate reader using BD Calcein AM Fluorescent dye. The results are expressed as the percent change in fluorescence units compared with the control. (B) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) and with or without IL-8 (100 ng/mL) for 24 h under normoxic or hypoxic conditions. The invasion assay was performed, and the results are expressed as the number of invaded cells per field. (C) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) following transfection with IL-8 siRNA or control siRNA under normoxic or hypoxic conditions. The invasion assay was performed, and invasion ability was determined as relative fluorescence units that were measured with a fluorescence plate reader using BD Calcein AM Fluorescent dye. The results are expressed as the number of invaded cells per field. Columns represent means; bars represent SDs.

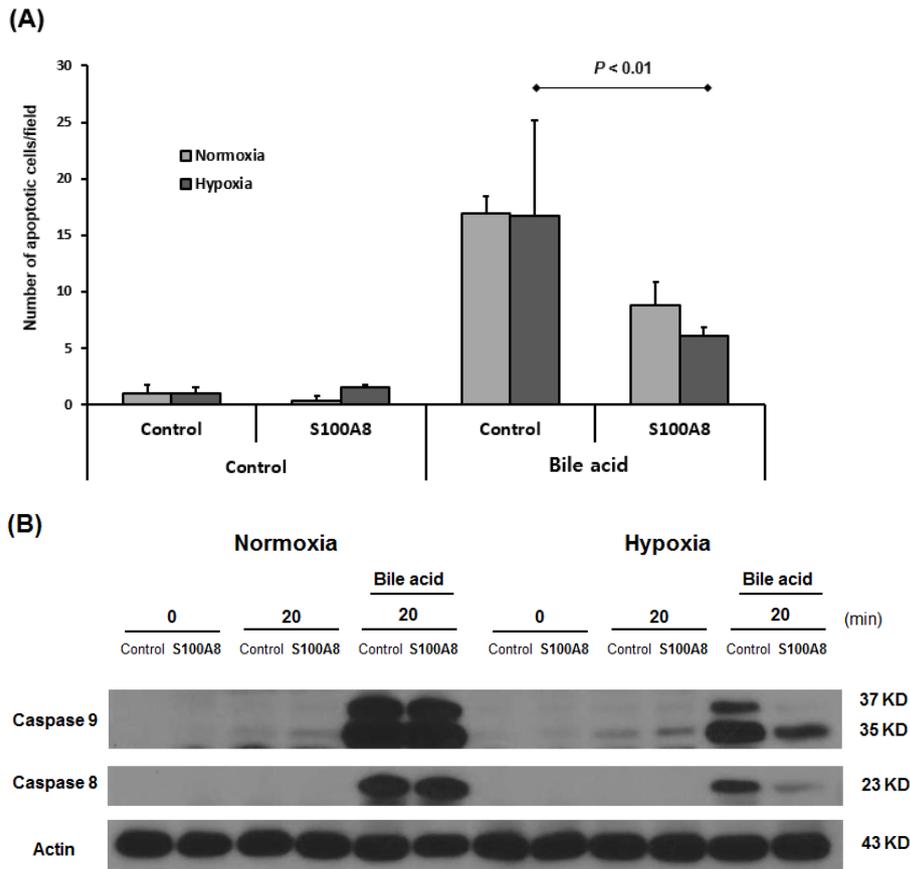


Figure 4. Attenuated bile acid-induced apoptosis of HCC cells by S100A8 under hypoxic conditions. (A) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g/mL}$) for 24 h prior to the addition of deoxycholate (DC) under normoxic or hypoxic conditions. The cells were then treated with DC (200 μM) for an additional 5 h. Apoptosis was quantified by DAPI staining and fluorescent microscopy. (B) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g/mL}$) and with or without DC (200 μM) for the indicated time periods under normoxic or hypoxic conditions. Immunoblot analyses were then performed using anti-caspase 9, anti-caspase 8 and anti-actin antibodies.

Columns represent means; bars represent SDs.

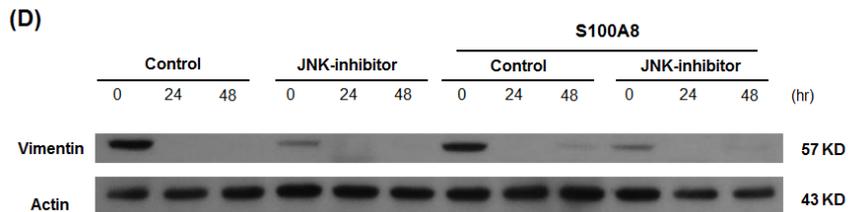
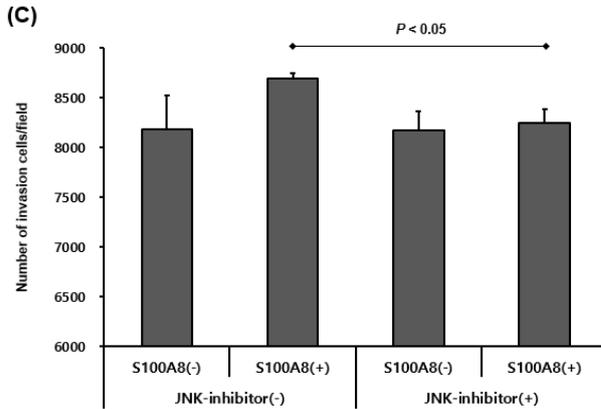
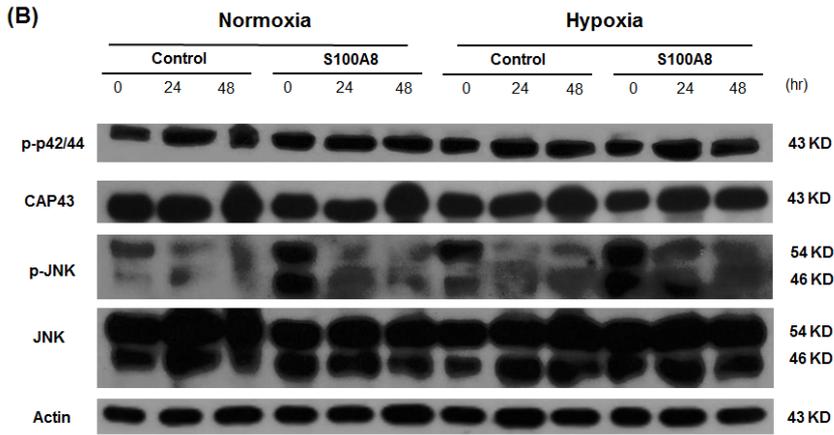
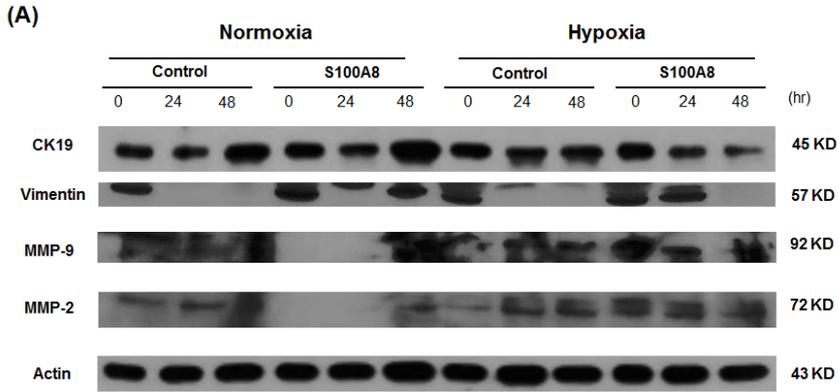


Figure 5. S100A8-related hepatocarcinogenesis associated with the JNK pathway-dependent epithelial-mesenchymal transition (EMT). (A) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) under normoxic or hypoxic conditions. Immunoblot analyses were then performed for CK19, vimentin, MMP-9, and MMP-2. An immunoblot analysis for actin was performed as a control for protein loading. (B) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) for the indicated time periods under normoxic or hypoxic conditions. The cells were lysed and immunoblot analyses were performed for phospho-p42/44, CAP43, phospho-JNK, JNK and actin. (C) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) in the presence or absence of the JNK inhibitor (0 and 10 μM) for 24 h under hypoxic conditions. The invasion assay was performed, and invasion ability was determined as relative fluorescence units that were measured with a fluorescence plate reader using BD Calcein AM Fluorescent dye. The results are expressed as the number of invaded cells per field. (D) SNU-761 cells were treated with or without S100A8 (0.5 $\mu\text{g}/\text{mL}$) and with or without the JNK inhibitor (10 μM) for the indicated time periods under hypoxic conditions. The cells were lysed, and immunoblot analyses were performed for vimentin and actin.

Columns represent means; bars represent SDs.

Abbreviation: J-I, JNK inhibitor; MMP, matrix metalloproteinase

Discussion

Persistent inflammation is known to promote and exacerbate cancer.(20) HCC is a clear example of an inflammation-related cancer, in which more than 90% of HCCs arise in the context of hepatic injury and inflammation.(21) These tumors arise at sites of chronic inflammation that can be caused by chronic hepatitis B and C viral infection, chronic alcohol consumption, and aflatoxin B1-contaminated food.(22) This chronic injury damages the normal hepatic blood system, leading to a shortage of blood circulation and thus hypoxia. Furthermore, the high proliferation of tumor cells can also induce local hypoxia inside HCC.(7) HCC is generally a hypervascular tumor, (6) but advanced infiltrative HCCs scarcely show hypervascularity, although they grow more aggressively than mass-forming hypervascular HCCs.(23) Moreover, locally recurrent HCC nodules after transarterial chemoembolization, which causes hypoxic insult, grow more rapidly than primary HCCs.(24) These findings suggest that hypoxia generates survival signals in HCC cells.

The expression and potential cytokine-like function of S100A8 and S100A9 in inflammation and cancer suggest that they may play a key role in inflammation-associated cancer.(25) Higher expression of S100A8 and S100A9 is evident in several cancers, including gastric, pancreatic, bladder, and breast cancer.(26-29) Our results showed that the expression of S100A8 and S100A9 increased in and SNU-761 and SNU-3058 HCC cells under hypoxic conditions. These results are

consistent with previous studies that reported that S100A8 and S100A9 are upregulated in other HCC cell lines, including HepG2, Hep3B, and HuH-7 cells.(15, 17) We also provided evidence that hypoxia causes S100A8 and S100A9 to be overexpressed. S100A8 and S100A9 play a role in the metastasis of colorectal, prostate, and breast cancer and HCC.(17, 30-32) Consistent with these observations, S100A8 and S100A9 treatment increased cell invasion, suggesting that S100A8 and S100A9 may be involved in the metastasis of HCC cells, especially under hypoxic conditions. Hypoxia is a common stress factor in solid tumors, leading to the onset of various adaptive responses, including the transcriptional induction of a series of genes that participate in angiogenesis, glucose metabolism, and cell proliferation, survival, and migration. Thus, the adaption to hypoxia promotes many key mechanisms of cancer progression and contributes to aggressive tumor behavior.(33, 34)

IL-8 is a proinflammatory chemokine that is associated with the promotion of neutrophil chemotaxis and degranulation. The higher expression of IL-8 and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that IL-8 may function as a key regulatory factor within the tumor microenvironment.(35) IL-8 that is produced by HCC cells is an angiogenic factor in HCC, although it is not a major one. IL-8 is also associated with the invasion and metastasis of HCC cells.(36) Recent studies reported that S100A8 induced IL-8 mRNA expression and secretion.(37) Another study indicated that S100A9 induced IL-8 expression in macrophages via NF- κ B activation.(38) In the present study, S100A8 significantly

increased the expression of IL-8, particularly under hypoxic conditions. IL-8 siRNA transfection attenuated the invasion of HCC cells by S100A8. IL-8 signaling activates the primary effectors phosphatidylinositol-3-kinase and phospholipase C and consequently promotes the activation of Akt, PKC, calcium mobilization, and/or MAPK signaling cascades.(35)

The present study found that S100A8 increased the protein expression of vimentin, MMP-9, and MMP-2. Vimentin has been frequently used as a mesenchymal marker of the epithelial-mesenchymal transition, a process that is often associated with the malignant progression and aggressiveness of cancer. The JNK pathway is required for vimentin expression.(39) Additionally, JNK is essential for MMP-9 expression, (40) and JNK has been specifically implicated in the production and activation of MMP-2.(36) JNKs are members of the MAP kinase superfamily and play an essential role in Toll-like receptor mediated inflammatory responses. JNKs are master protein kinases that regulate many physiological processes, including inflammatory responses, morphogenesis, and cell proliferation, differentiation, survival, and death.(41) The JNK signaling pathway has been implicated in many pathological conditions, such as cancer, stroke, heart disease, and inflammatory diseases.(42) It is increasingly apparent that the persistent activation of JNKs is involved in the development and progression of cancer. Moreover, the JNK pathway is critical for oncogene-induced cellular transformation.(43) Activated MAPK signaling plays a central role in the development of HCC,(17) and JNK promotes an inflammatory hepatic

environment that supports tumor development.(44) We found that the JNK pathway may be involved in the activity of S100A8 and S100A9.

JNK is a key regulator of many cellular events including apoptosis. While JNKs are well known to activate apoptotic signaling,(45) recent studies indicate that JNK can suppress apoptosis in IL-3-dependent hematopoietic cells. Thus, JNK has both pro- or antiapoptotic functions, depending on the cell type, nature of the cell death stimulus, the duration of its activation, and the activity of other signaling pathways.(46, 47) The present study found that S100A8 treatment attenuated bile acid-induced HCC cell apoptosis. Bile acids sensitize cells to TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis.(48, 49) Our results are consistent with previous studies in which the inhibition of TRAIL-induced apoptosis by IL-8 was mediated by the p38-MAPK pathway in ovarian cancer cells,(50) and hypoxia suppressed the apoptosis of HCC cells.(7)

In summary, S100A8 and S100A9 are proinflammatory danger signals that promote inflammation-associated carcinogenesis, increase the invasion of HCC cells and decrease bile acid-induced HCC cell apoptosis by increasing IL-8 under hypoxic conditions. Considering the higher expression of markers of EMT, the JNK pathway may be involved in controlling the activity of S100A8 and S100A9. Based on the present results, S100A8 and S100A9 may serve as therapeutic targets for the treatment of HCC.

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요약 (국문 초록)

배경: S100A8 과 S100A9 은 세포질 내 칼슘 결합 물질이자 신호 전달 단백질인 S100 의 구성원이다. 이들은 친염증성 신호로 작용하며 간세포암을 포함한 여러 종류의 암종에서 염증, 세포 증식, 침습 등에 영향을 줌으로써 종양의 발생에 역할을 하는 것으로 알려져 있다. 저산소증 환경은 염증과 관련된 간세포암의 발생에 중요한 역할을 하지만, 아직까지 S100A8 과 S100A9 가 저산소증 환경에서 간세포암의 발생에 작용하는 직접적인 역할은 잘 밝혀져 있지 않다. 본 연구에서는 저산소증 상태에서 S100A8 과 S100A9 가 간세포암 내에서 갖는 역할에 대해 알아보고자 한다.

방법: S100A8 과 S100A9 의 발현은 실시간 중합효소 연쇄 반응을 시행하여 확인하였다. SNU-761 과 SNU-3058 이라는 인간 간세포암 세포주에 S100A8/A9 처리 여부를 달리하여 정상 산소와 저산소증 조건에서 배양하였다. 간세포암 세포주 내에서의 S100A8 과 S100A9 의 역할을 평가하기 위해 MTS 분석과 침윤 분석을 시행하였다. S100A8 과 S100A9 와 관련된 신호 전달 경로를 확인하기 위해서 면역블롯법을 이용하였다.

결과: 정상 산소 조건과 비교했을 때 저산소증 조건에서 S100A8 과 S100A9 의 발현이 증가되어 있었다. S100A8/A9 처리를 하면

저산소화증 환경에서 세포의 증식에는 큰 차이가 없는 반면, 종양 세포의 침윤은 증가하였다. 종양의 촉진과 관련된 염증 사이토카인인 IL-8 의 발현이 저산소증 상태에서 S100A8 처리를 하였을 경우 증가하였다. SiRNA 를 이용하여 IL-8 의 활성을 억제한 이후에는 간세포 침습의 증가는 상쇄되었다. 또한, S100A8 의 처리는 담즙산에 의한 간세포 자멸사를 감소시켰다. S100A8 은 vimentin, MMP-9, MMP-2 를 비롯한 간염성 표지자의 발현을 증가시켰으며, 이는 JNK 억제제에 의해 상쇄됨을 확인하였다.

결론: 본 연구의 결과를 통해 저산소증 상태에서 간암세포 내의 S100A8 과 S100A9 의 과발현이 유도되고, S100A8/A9 이 JNK 경로를 통해 간세포암의 발생과 진행에 관여한다는 것을 확인하였다. 따라서 S100A8/A9 는 추후 간세포암의 새로운 치료 표적으로 적용될 수 있으리라 생각된다.

주요어: S100A8, S100A9, IL-8, JNK, 간세포암

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