치의학박사학위논문

Prognostic significance of N-myc downstream-regulated gene 2 (NDRG2) expression and its epigenetic regulation in oral squamous cell carcinoma

구강편평세포암종에서 NDRG2 발현의 예후인자로서의 가치와 후성유전학적 발현 조절에 관한 연구

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Abstract

Prognostic significance of N-myc downstream-regulated gene 2 (NDRG2) expression and its epigenetic regulation in oral squamous cell carcinoma

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Objectives: Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity. In spite of the advances in the diagnostic and treatment modalities of OSCC, it is not sufficient to improve the prognosis of patients with OSCC. Recently N-myc downstream-regulated gene 2 (NDRG2) has been reported to be a tumor suppressor gene in various types of cancers. DNA hypermethylation was revealed to be the main mechanism of NDRG2 down-regulation, suggesting that its epigenetic regulation may be a possible new therapeutic target for the patients showing low expression of NDRG2. In OSCC, it is still unclear whether the loss of expression of NDGR2 is associated with the clinical outcome and survival, and whether

demethylating agent can regulate the functions of NDRG2 in OSCC. Therefore, I aimed to examine NDRG2 expression in OSCC tissue samples, to analyze the correlation between NDRG2 expression and clinicopathological parameters, and to evaluate its value as a prognostic marker for OSCC. In addition, I aimed to verify the tumor-suppressive roles of NDRG2 in OSCC cell lines and the effect of 5-Aza-2′-deoxycytidine (5-Aza-dC) on the cell lines with low NDRG2 expression.

Methods: Immunohistochemical study was performed using 73 OSCC tissue samples. The correlation between NDRG2 expression and clinicopathogical parameters and survival of OSCC patients were analyzed. Expression of NDRG2 in OSCC cell lines was examined using real-time PCR analysis and western blotting. To verify the roles of NDRG2 as a tumor suppressor, cell proliferation, migration, and invasion assay were performed with the treatment of anti-NDRG2 siRNA or 5-Aza-dC

Results: Low NDRG2 expression was detected in 46.6% of cases of OSCC. Low expression of NDRG2 correlated with moderately/poorly differentiated tumor (P=0.038), lymph node metastasis (P=0.007), and advanced clinical stage (P=0.017). Patients with low expression of NDRG2 showed significantly worse overall survival (OS) (P=0.001) and disease-free survival (DFS) (P=0.007). NDRG2 expression was an independent prognostic factor for both OS and DFS (P=0.022 and P=0.008, respectively). Cell proliferation, migration, and invasive abilities were significantly increased in HN22 and HSC-3 cell lines after anti-NDRG2 siRNA treatment (P<0.05). Ca9-22 and HSC-4 cells showing low NDRG2 expression were treated with 5-Aza-dC and then NDRG2 expression was recovered. Treatment of 5-Aza-dC induced significant inhibitions of the cell proliferation, migration, and invasion in both Ca9.22 and HSC-4 cells (P<0.05).

Conclusion: Results in the present study suggest that down-regulation of

NDRG2 via the epigenetic silencing plays a role in tumor progression of OSCC as a result of reducing its tumor-suppressive function. Therefore, the recovery of NDRG2 expression using demethylating agents may be a new strategy for the treatment of OSCC by suppressing the tumor cell growth, migration, and invasion. Moreover, NDRG2 can be a useful biomarker to predict clinical outcome and survival of patients with OSCC.

Keywords: NDRG2, oral squamous cell carcinoma, tumor suppressor gene, prognosis, DNA methylation, 5-Aza-dC

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Contents

I. Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of oral cavity, accounting for over 90% of all oral malignancies.[1,2] OSCC is highly aggressive cancer characterized by lower survival rate in advanced stage patients. Despite diagnostic and treatment modalities have been advanced for OSCC patients, survival of OSCC patient has been not significantly improved.[3-5] Carcinogenesis of oral cavity is a multistep process which is involved in accumulated genetic and epigenetic alterations modulated by endogenous genetic predisposition or environmental factors including the tobacco, alcohol, viral infection and chronic inflammation.[6-8] Interest in understanding mechanism of genetic and molecular alteration has gradually increased, and advanced molecular studies are helping to explore new molecular targets for early diagnosis, predicting the prognosis, and treatment for OSCC patients.[5,9,10]

The N-myc downstream-regulated gene 2 (NDRG2) is a member of NDRG family, which forms a separate homology cluster across multiple species.[11,12] With functionally diverse roles, they consist of 4 members including NDRG1, NDRG2, NDRG3, and NDRG4.[11] Human NDRG2 is located on chromosomes 14q11.2 and encodes two different protein variants of 371 aa (41kDa) and 357 aa, respectively. Its functions are involved in various processes of organ generation, differentiation, immune response, stress reaction, and hormonal response. Therefore, alteration of NDRG2 are associated with organ degeneration, immature immune system, endocrine dysfunction, and diverse neurologic dysfunctions.[13-18] Recently there have been emerging evidences that NDRG2 could function as a tumor suppressor gene in various malignancies, such as neurologic tumors, gastro-intestinal tumors, genito-urinary tumors, lung tumor, breast tumor, hematologic tumors,

and other tumors.[19,20)]

Especially, it has been well-known that NDRG2 down-regulation is significantly associated with tumor progression and metastasis in those malignancies.[20)] In OSCC, two previous reports suggested that NDRG2 could be a possible candidate tumor suppressor gene for OSCC development.[21,22)] They demonstrated that down-regulation of NDRG2 mRNA or protein was seen in the majority of OSCC cases and the loss of NDRG2 expression significantly correlated with lymph node metastasis and advanced TNM stage. To date, there has been no study evaluating clinical relevance of NDRG2 as a prognostic marker for OSCC.

In numerous molecular studies on OSCC, many researchers have taken notice of DNA methylation of various genes as the molecular mechanism which dysregulate cell growth, apoptosis, signal transducing and DNA repair in cancer cells.[23-25)] DNA methylation is mediated by DNA methyltransferase, which can act to repress gene transcription.[5,10)] Most of all, promotor hypermethylation of tumor suppressor gene such as NDRG2 can play an important roles in increasing cancer cell proliferation and invasion and/or metastasis.[26)] As this process is reversible, inhibitor of DNA methyl transferase (demethylating agent), such as 5-Aza-2′-deoxycytidine (5-Aza-dC) can reactivate the silenced tumor suppressor gene for the purpose of cancer treatment. However, there has been few research on the recovery of tumor suppressor gene NDRG2 using the demethylating agent in OSCC.

Aims of this study were to examine NDRG2 expression in OSCC tissue samples, to analyze the correlation between NDRG2 expression and clinicopathological parameters, and to evaluate its value as a prognostic marker for OSCC. In addition, I aimed to verify the tumor-suppressive roles of NDRG2 in OSCC cell lines and the effect of 5-Aza-dC on the cell lines with low NDRG2 expression.

II. Materials and Methods

Patients and tissue samples

Seventy-three of OSCC samples were collected and evaluated by immunochemistry. Tumor tissue sections were obtained from patients with OSCC and underwent tumor removal surgery in Department of Oral and Maxillofacial Surgery, Seoul National University Dental Hospital (Seoul, South Korea) between the 2000 and 2006. None of the patients received preoperative chemotherapy or radiation therapy. Total clinicopathologic data including age, gender, histological differentiation, tumor size, lymph node metastasis, clinical stage, and local recurrence, were summarized in the table 1. Clinical stages were determined according to the TNM classification by the American Joint Committee on Cancer. The study protocol was approved and performed under guideline of the Institutional Review Board of Seoul National University Dental Hospital (#CRI16002).

Immunohistochemistry

Fresh formalin-fixed, paraffin-embedded 4-μm sections were prepared for immunohistochemical staining. After deparaffinization in xylene and rehydration in graded ethanol, endogenous peroxidase activity was inactivated by incubating the sections with 3 % hydrogen peroxide for 10 min at room temperature (RT). Antigen was retrieved using microwave treatment on section in citrate buffer (pH 6.0) during 10 min and cooled at RT during 30 min. Sections were incubated in 10% normal goat serum during 30 min and then were incubated in a 1:200 dilution of with NDRG2 antibody (Abnova, Taipei, Taiwan) at 4°C overnight. The sample slides were stained using the REAL EnVision$^{TM/HRP}$ kit (DAKO, Denmark). Immunohistochemical reactions were developed with diaminobenzidine as the chromogenic

peroxidase substrate. Study slides were counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemical staining

Specific NDRG2 immunohistochemical staining was detected in the cytoplasm of tumor cells. Stained slides were scored by two pathologists, who were blinded to the clinical outcomes and clinicopathological data of patients. According to percentages of positive-staining cells, extent score was taken as 0 (< 5%), 1 (5-25%), 2 (26-50%), and 3 (>50%). Intensity was scored as 0 (colourless), 1 (weak), 2 (moderate), and 3 (strong). Cases with a multiplied score exceeding 3 were defined as high expression. All other scores were considered as low expression.[27)]

Cell lines

Ca9.22, HSC-2, HSC-3, HSC-4, and SAS cells were kindly provided by Hokkaido University (Hokkaido, Japan). HN22 cells were provided by Dankook University (Cheonan, Korea). Human Oral keratinocyte (HOK) cells were purchased from LifeLine cell technology Company (Carlsbad, CA, USA). Cancer cell lines were cultured in Dulbecco's modified essential medium (DMEM; WELGENE, Daegu, Korea) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. HOK cells were cultured in DermaLife K Medium (LifeLine cell technology).

Chemicals and antibodies

5-Aza-2′-deoxycytidine (5-Aza-dC) was supplied by Sigma–Aldrich (Sigma–Aldrich, Louis, MI, USA). Antibodies against NDRG2 and β-Actin were purchased from Cell Signaling Technology, Inc., (Charlottesville, VA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively.

RNA isolation and real-time PCR analysis

Total RNA was isolated from cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and integrity was determined by spectrophotometry. cDNAs were sythesized by reverse transcription of 1 μg total RNA using cDNA synthesis kit (Enzo Life Sciences Inc., NY, USA), and the samples were analysed using AMPIGENE qPCR Green Mix Hi-ROX with the SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, USA). The expressions of target gene was analysed according to the $2^{-\Delta\Delta Ct}$ method and normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of primers were used as follows: NCRG2 primers 5'-CACGATGTGGGACTCAACTATAA-3' and 5'-CATG AACCCGCACAAAGTTC-3'; GAPDH primers 5'-GTGGTCTCCTCTGACTT CAAC-3' and 5'-CCTGTTGCTGTA GCCAAATTC-3'.

Western blot analysis

Whole cell lysates were extracted with lysis buffer, and protein samples were quantified using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated on SDS-polyacrylamide gels by electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in tris-buffered saline and Tween 20 for 1 hr 30 min at RT and maintained overnight at 4°C with designated primary antibodies, followed by incubation with HRP-conjugated secondary antibody at RT for 1 hr 30 min. Antibody-bound proteins were detected using an ECL Western blotting luminol reagent (Santa Cruz Biotechnology).

RNA interference

For transient transfection, small interfering RNA (siRNA) specific for

NDRG2 (sc-40757) and negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Cells were seeded on 6-well plates and transiently transfected with 50 nM siNDRG2 using a Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Next, cells were incubated for 24 hr in an atmosphere of 5 % CO_2/95% humidified air at 37°C before additional analysis.

Cell proliferation assay

Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used according to the manufacturer's instruction. Briefly, 2×10^4 cells were seeded in to a 96-well plate. After the treatment of siNDRG2 or 5-Aza-dC, respectively, cell viability was evaluated with CCK-8 at daily intervals from the next 24, 48, and 72hr after seeding. Following the CCK-8 assay at 37°C for 1.5hr, then the optical density of the cells was measured using a microplate reader at an absorbance of 486nm.

Scratch wound healing assay

Both cell lines (3×10^5cells/well) were seeded in 6-well plates and cultured with DMEM supplemented with 10% FBS for 24hr. To induce the recovery of NDRG2 expression, 5-Aza-dC was added to the plates for 48hr. When reaching confluency, each well was scratched with a 200μl pipette tip. After 12hr (Ca9.22 and HSC-3), 14hr (HN22) or 16hr (HSC-4) of incubation, images of the wound areas were captured and then the distance between two cell edges was measured by Image J software (version1.51k; National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay

Cells were cultured in 24-well plates of Boyden chambers with 8-μm pore

filter inserts (Corning Life Sciences, Corning, NY, USA). The pore inserts were pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) overnight. Each cell lines (6×10^4cells/well) were suspended in 200μl DMEM supplemented without FBS and 5-Aza-dC was added to the upper chamber. 750μl DMEM with 10% FBS added to the lower chamber. After 48hr of incubation at 37°C, the cells on the top of the membrane were gently scraped using a swab. And the cells attaching to the lower surface were fixed with methanol and stained with 0.1% crystalviolet (Beyotime Institute of Biotechnology) for 30 min at RT. A total of four random high-power fields (magnification, ×100; Nikon E100; Nikon Corp, Tokyo, Japan) of each sample were selected and counted to evaluate the average number of invasive cells.

Statistical Analysis

All the statistical analyses were performed using the SPSS software version 20 (SPSS Inc., Chicago, IL, USA). To analyze the relationship between NDRG2 expression and clinicopathologic parameters of OSCC patients, data were cross-tabulated and a χ^2 test was performed. Kaplan-Meier method was used to calculate the survival curves and Cox hazard regression model was used for the univariate and multivariate analysis. Data from the cell proliferation, migration, and invasion assays were tested by t-tests or ANOVA test. A *P* value less than 0.05 was considered statistically significant.

III. Results

Correlation between NDRG 2 expression and clinicopathological parameters in OSCC

NDRG2 was expressed in the cytoplasm of epithelial cells. In normal oral mucosa, NDRG2 staining was weakly and diffusely detected through the epithelium (Fig. 1). Otherwise NDRG2 staining in the normal skin and salivary gland duct was strong positive (Fig. 2). In immunohistochemical staining for 73 samples of OSCC patients, low NDRG2 expression was detected in 34 (46.6%) of OSCC samples and high NDRG2 expression was detected in 39 (53.4%) (Fig. 3). Correlations between NDRG2 expression and clinicopathologic parameters were evaluated and comparable data were demonstrated in the table 2. Statistical analysis revealed a significant correlation between low NDRG2 expression and the histological differentiation of OSCC (P=0.038). NDRG2 expression was significantly decreased in moderately/poorly differentiated OSCC than in well-differentiated tumors. Patients with low NDRG2 expression showed the more frequent incidence of lymph node metastasis (P=0.007) and advanced clinical stage (P=0.017). However, there were no differences in age, gender, tumor size, and local recurrence rate.

Clinical significance of NDRG 2 expression for predicting survival of OSCC

The Kaplan-Meier analysis was performed to evaluate the correlation of NDRG expression level with overall survival (OS) and disease-free-survival (DFS). Patient group who had a low expression of NDRG2 showed significantly poorer OS and DFS than those who had a high expression (P=0.001 and P=0.007, respectively) (Fig. 4). Further analyses of univariate and multivariate logistic tests using cox hazard regression model were

performed to identify independent risk factors for OS and DFS (Table 3 and 4). In univariate analysis, lymph node metastasis (N1+N2) (HR 2.813, *P*=0.002), advanced clinical stage (III+IV) (HR 2.258, *P*=0.049) and low NDRG2 expression (HR 2.916, *P*=0.001) were significant risk factors for OS (Table 3). For DFS, lymph node metastasis (N1+N2) (HR 1.818, *P*=0.042) and low NDRG2 expression (HR 2.188, *P*=0.008) were significant risk factors in univariate analysis. Multivariate analysis revealed that low NDRG2 expression was an independent risk factor for both OS (HR 2.209, *P*=0.022) and DFS (HR 2.188, *P*=0.008).

Expression analysis of NDRG2 in OSCC cell lines by western blotting analysis

Expression of NDRG2 protein was evaluated in normal oral keratinocyte cell line and 6 of OSCC cell lines by western blot analysis. Western blot analysis revealed that 2 cell lines, HN22 and HSC-3, showed high expression of NDRG2 and 3 out of 6 OSCC cell lines showed very low levels of NDRG2 protein compared with human oral keratinocyte (HOK) (Fig. 5). I used HN22 and HSC-3 cells to examine the tumor suppressive function of NDRG2 in OSCC. Moreover, to examine whether down-regulated NDRG2 expression could be recovered by the demethylating agent, 5-Aza-dC, I selected two cell lines, Ca9.22 and HSC-4 cells among 3 cell lines with very low expression of NDRG2.

Effect of suppressing NDRG2 expression in OSCC cell lines using anti-NDRG2 siRNA

To verify the direct roles of NDRG2 in OSCC, cell proliferation, migration, and invasion assays were performed in HN22 and HSC-3 cell

lines with the treatment of anti-NDRG2 siRNA (siNDRG2). Western blot analysis showed significantly suppressed expression of NDRG2 protein in both cell lines after 24 hr treatment with 50 nM siNDRG2 (Fig. 6A). In both cell lines, there was no difference in the cell proliferation between the group treated with 50nM siNDRG2 and the control group after 24 and 48 hr. After 72 hr, however, there was a significant increase of cell proliferation in the HN22 and HSC-3 treated with 50 nM siNDRG2 compared with the control (P=0.005 and 0.009, respectively) (Fig 6B). In wound healing assay for evaluating migration ability, the HN22 and HSC-3 cell lines showed much more closure of wound area after transfection with 50 nM siNDRG2 compared with the control (P=0.007 and 0.008, respectively) (Fig. 7A and 7B). From the invasion assay, it was also found that significant increase in the invasion ability of the HN22 and HSC-3 cell lines (P=0.004 and 0.049, respectively) (Fig. 7C and 7D).

Effect of 5-Aza-dC on the NDRG2 expression of OSCC cells

Expression levels of NDRG2 were measured with the treatment of 5-Aza-dC as I expected that NDRG2 could be down-regulated in OSCC by DNA hypermethylation like in other malignancies. Fig. 8 shows that NDRG2 expressions in Ca9.22 and HSC-4 cells increased in a time-dependent manner with the treatment of 5-Aza-dC. There was no significant difference in the amount of recovery between the two groups treated by different concentration of 5-Aza-dC.

Effect of 5-Aza-dC on the cell proliferation of OSCC cells

The effect of 5-Aza-dC on the cancer cell proliferation was examined at the different concentrations of 1 and 2 μM for 24, 48, and 72 hr. In both cell lines, there was no difference in the cell growth between the 5-Aza-dC

treated group and control group until 24 hr. After 24 hr, there was a decreasing tendency of cell proliferation in both cell lines compared to the control (Fig. 9). But there were no significant differences of growth under two different concentrations of 5-Aza-dC except HSC-4 cells treated by 2 μM 5-Aza-dC at 72 hr (P=0.014).

Effect of 5-Aza-dC on the migration and invasion of OSCC cells

In wound healing assay for evaluating migration ability, both cell lines treated by a 5-Aza-dC showed more delayed wound healing. It means that 5-Aza-dC reduced migratory ability of both cell lines (P<0.05) (Fig. 10). From the invasion assay, it was also found that 5-Aza-dC treatment could induce a significant decrease in the invasion abilities of both cell lines at the concentration of 2 μM (P<0.05) (Fig. 11).

V. Discussion

OSCC is the most common phenotype of oral cancer and remains a lethal malignancy which can lead poor survival in over 50 % of OSCC patients.[3] Beyond prediction of cancer prognosis using clinical parameter, molecular biomarker is expected to role as a novel independent prognostic factor and therapeutic target in OSCC. In this study, patients with low expression level of NDRG2 was associated with high histological grade, advanced clinical TNM stage and lymph node metastasis. In addition, low level of NDRG2 was associated with worse chance of OS and DFS after adjusting other risk factors. These data indicate that expression level of NDRG2 is an independent prognostic marker for the patients with OSCC.

First identification of NDRG2 as the specific tumor suppressor gene was reported by Lusis et al.[28] They found out that NDRG2 on chromosome 14q were inactivated in clinically advanced meningioma. Notably,several following studies have shown that NDRG2 play a crucial role of tumor suppressor in several cancers, such as neurologic, gastro-intestinal, genitourinary, lung, and breast cancer.[20] In those malignancies, NDRG2 expression have correlated with various clinicopathologic parameters including the tumor size, histologic differentiation, lymph node metastasis or distant metastasis, and tumor stage. For example, down-regulated NDRG2 are associated with poor differentiation, lymph node metastasis and higher TNM stage in gastric cancer, hepatocellular carcinoma, colorectal adenocarcinoma and gallbladder carcinoma.[29-33] In nervous system neoplasm, glioma, and astrocytoma had shown that NDRG2 expression is inversely correlated with high grade of tumor.[34,35] Inverse correlation between the expression of NDRG2 and aggressive tumor behaviors such as high TNM

stage, metastasis, and poor proliferation also identified in renal cell carcinoma, breast cancer, and lung cancer.[36-39] In these studies, expression of NDRG2 were inversely correlated with poor clinical outcome of OSCC, suggesting that NDRG2 act as a tumor suppressor gene in common with other malignancies.

NDRG2 may be a significant factor for determining the survival of cancer patients. Consistent with the results of current study, other research also reported that decreased NDRG2 was significantly related with worse survival of patients.[30-32,35-38] Song et al reported that the survival rate of patients with low expression of NDRG2 was worse and NDRG2 expression could be an independent prognostic indicator of gallbladder carcinoma.[27] In addition, Li et al suggested that low expression of NDRG2 was an independent poor prognostic factor of lung cancer.[36] Similarly, NDRG2 was expected to be inversely correlated with the survival of patients with OSCC, but it had been not well researched. In this study, I firstly ascertained that expression level of NDRG2 is negatively associated with the survival rate in OSCC after adjusting other risk factors. Additionally, it was first revealed that NDRG2 expression can be an independent prognostic marker in OSCC.

In the present study, tumor-suppressive functions of NDRG2 were found in OSCC as well. When NDRG2 was knocked down through the treatment of anti-NDRG2 siRNA, the significant enhancement of cell proliferation and migration could be induced in HN22 and HSC-3 cell lines. These data indicate that down-regulation of NDRG2 play a possible role in the tumorigenesis and tumor progression in OSCC. According to the previous report, forced expression of NDRG2 could suppress the cell growth and migration/invasion of OSCC mainly through the PI3K/AKT signaling pathway which are essential for survival and migration.[21,22]

Alterations of gene expression could be caused from combined results

from both genetic and epigenetic events.[9,23,40] With regard to NDRG2 gene, remarkable changes of DNA sequence were not detected in mutational analysis of NDRG2 coding sequences. Therefore the epigenetic event was thought to play a major role in the development of cancer.[9,23] Among three epigenetic mechanisms including DNA methylation, histone modification and RNA-mediated silencing, DNA methylation is thought to be a main contributor of down-regulation of NDRG2.[23,24] Hypermethylation of CpG islands in the promoter region of DNA cause a transcriptional gene silencing in tumor suppressor gene, then leading to initiation and/or progression of cancer.[24,41] As expected, DNAs extracted from human OSCC tissues and precancerous tissues showed higher level of DNA methylation than those of normal oral tissues.[42] Furuta et al demonstrated that reduced expression of NDRG2 mRNA in OSCC cell lines seemed to correlate with high methylation status of the gene.[21] They also studied on the restoration of NDRG2 expression in HSC-3 and SAS cell lines using 5-Aza-dC and/or histone deacetylase inhibitor (Trichostatin). 5-Aza-dC showed more dominant effect for the restoration of NDRG2 gene. These data suggested that methylation of NDRG2 is the main cause of NDRG2 silencing in OSCC. In line with those results, 5-Aza-dC could restore the expression of NDRG2 in both Ca9.22 and HSC-4 cell lines in the present study. Further analysis of the effect of 5-Aza-dC on the cancer cell proliferation demonstrated a significant inhibition in HSC-4 cell line, but not in Ca9.22 cells. However, 5-Aza-dC could inhibit cell migration and invasion activity in both cell lines. These data of the current study suggest that the inhibitory effect of 5-Aza-dC on the OSCC cell growth and migration/invasion may be partially mediated by the recovery of NDRG2 which is one of tumor suppressor genes. Therefore, further studies should be followed in the future to confirm the direct effect of increased NDRG2 on the biological behavior of OSCC

cells.

Despite recent achievement made in the field of cancer prevention and treatment, 5-year overall survival rate for OSCC remains unsatisfied over the past decades. It may be mainly due to a lower response rate to current cancer therapy, particularly for tumor with advanced stage.[3] Since 5-Aza-Dc was first synthesized by Piskala and Sorm in 1964[43], interest in epigenetics for the development of new anticancer drug continues to rise. 5-Aza-dC (Decitabine) has been approved for clinical use in hematological malignancies.[44] Recently, there has been an increased interest in the use of decitabine as a therapy for other solid tumors as well.[45] Although there is relatively less trial to examine the anti-cancer effect of decitabine in OSCC than other malignancies, the data from the present study suggest that targeting the recovery of epigenetically silenced tumor suppressor using demethylating agent like decitabine can be a promising treatment modality for the patients with OSCC.

V. Conclusion

Correlation analysis between NDRG2 expression and clinicopathologic parameters revealed that the low expression of NDRG2 was significantly associated with moderately/poorly differentiation, positive lymph node metastasis, and high TNM stage. Patients with low expression of NDRG2 showed significantly worse OS and DFS as compared with patients with high expression of NDRG2. Multivariate analysis revealed that NDRG2 expression was an independent prognostic factor for both OS and DFS of patients with OSCC. *In vitro* study using siNDRG2 showed that down-regulation of NDRG2 increased cell proliferation and migration/ invasion, suggesting the tumor-suppressive roles of NDRG2 in OSCC. In addition, demethylating agent could induce the recovery of NDRG2 expression and simultaneously suppress the cell proliferation, migration, and invasion of OSCC cell lines.

Taken together, these data suggest that down-regulation of NDRG2 via the epigenetic silencing plays a role in tumor progression of OSCC as a result of reducing its tumor-suppressive function. Therefore, the recovery of NDRG2 expression using demethylating agents may be a new strategy for the treatment of OSCC by suppressing the tumor cell growth, migration, and invasion. Moreover, NDRG2 can be a useful biomarker to predict clinical outcome and survival of patients with OSCC.

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Table 1. Baseline characteristics and outcomes of 73 OSCC patients

Variable	
Age, mean (SD)	59.8 (±12.3)
Age range	
< 60 years	36 (49.3%)
≥ 60 years	37 (50.7%)
Gender	
Male	58 (79.5%)
Female	15 (21.5%)
Histological differentiation	
Well	59 (80.8%)
Moderately	13 (17.8%)
Poorly	1 (1.4%)
Tumor size (T)	
T1	13 (11.8%)
T2	28 (44.1%)
T3	4 (8.8%)
T4	28 (35.3%)
Lymph-node metastasis (N)	
N0	36 (49.3%)
N1+N2	37 (50.7%)
Clinical Stage (TNM)	
I	10 (13.7%)
II	8 (11.0%)
III	14 (19.2%)
IV	41 (56.2%)
NDRG2 expression	
low	34 (46.6%)
high	39 (53.4%)

Table 2. Correlation of NDRG2 expression with variable clinico pathological parameters.

	Case (N=73)	NDRG2 expression		*P* value
		lower (N=34)	high (N=39)	
Age				
<60	36	18 (50.0%)	18 (50.0%)	0.563
≥60	37	16 (43.2%)	21 (56.8%)	
Gender				
Male	58	26 (44.8%)	32 (55.2%)	0.556
Female	15	8 (53.3%)	7 (46.7%)	
Differentiation				
Well	59	24 (40.7%)	35 (59.3%)	0.038*
Moderate/poor	14	10 (71.4%)	4 (28.6%)	
Tumor size				
T1+T2	41	19 (46.3%)	22 (53.7%)	0.964
T3+T4	32	15 (46.9%)	17 (53.1%)	
Lymph-node metastasis				
N0	36	10 (31.3%)	22 (68.8%)	0.007*
N1+N2	37	24 (58.5%)	17 (41.5%)	
Clinical Stage (TNM)				
I+II	18	4 (22.2%)	14 (77.8%)	0.017*
III+IV	55	30 (54.5%)	25 (45.5%)	
Local recurrence				
Yes	20	13 (65.0%)	7 (35.0%)	0.053
No	53	21 (39.6%)	32 (60.4%)	

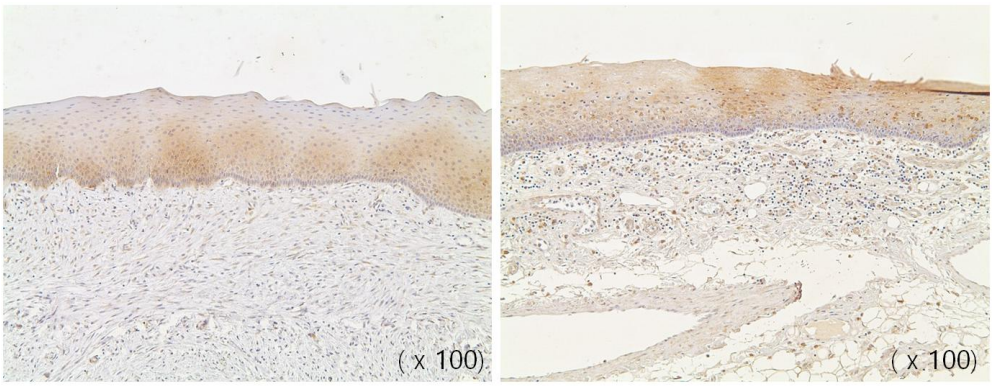
Table 3. Univariable analysis of the prognostic factors for overall survival (OS) and disease-free survival (DFS)

	OS		DFS	
	Hazard Ratio (95% CI)	*P* value	Hazard Ratio (95% CI)	*P* value
Age (≥60/<60)	0.746 (0.409-1.359)	0.338	0.994 (0.567-1.742)	0.983
Gender (male/female)	1.459 (0.647-3.290)	0.362	0.872 (0.434-1.752)	0.701
Differentiation (moderate+poor/well)	1.882 (0.962-3.681)	0.065	1.841 (0.968-3.504)	0.063
Tumor size (T) (T3+T4/T1+T2)	1.138 (0.622-2.079)	0.675	0.874 (0.494-1.546)	0.643
Lymph-node metastasis (N1+N2/N0)	2.813 (1.482-5.338)	0.002*	1.818 (1.022-3.235)	0.042*
Clinical Stage (III+IV/I+II)	2.258 (1.003-5.086)	0.049	1.196 (0.611-2.342)	0.602
NDRG2 expression (low/high)	2.916 (1.543-5.513)	0.001*	2.188 (1.225-3.907)	0.008*

Table 4 Multivariable analysis of the prognostic factors of overall survival (OS) and disease-free survival (DFS)

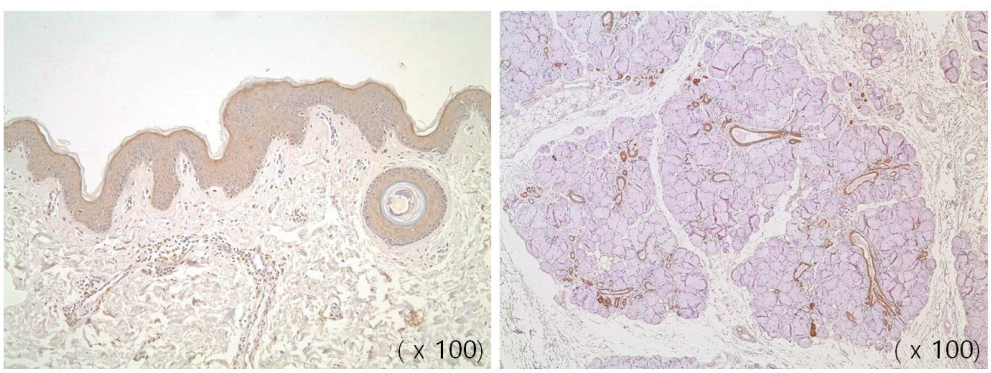
	OS		DFS	
	Hazard Ratio (95% CI)	*P* value	Hazard Ratio (95% CI)	*P* value
Lymph-node metastasis (N1+N2/N0)	2.106 (1.061-4.177)	0.033		
NDRG2 expression (low/high)	2.209 (1.121-4.355)	0.022	2.188 (1.225-3.907)	0.008

Fig. 1. NDRG2 expression in normal oral mucosa



normal oral mucosa　　normal oral mucosa adjacent OSCC

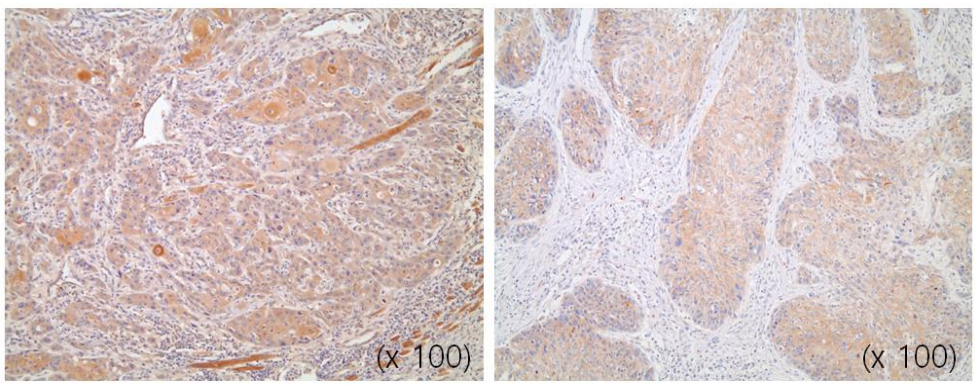
Fig. 2. NDRG2 expression in normal skin and salivary gland



normal skin　　normal salivary gland

Fig. 3. High and low NDRG2 expression in oral squamous cell carcinoma

(A) High NDRG2 expression in OSCC



(B) Low NDRG2 expression in OSCC

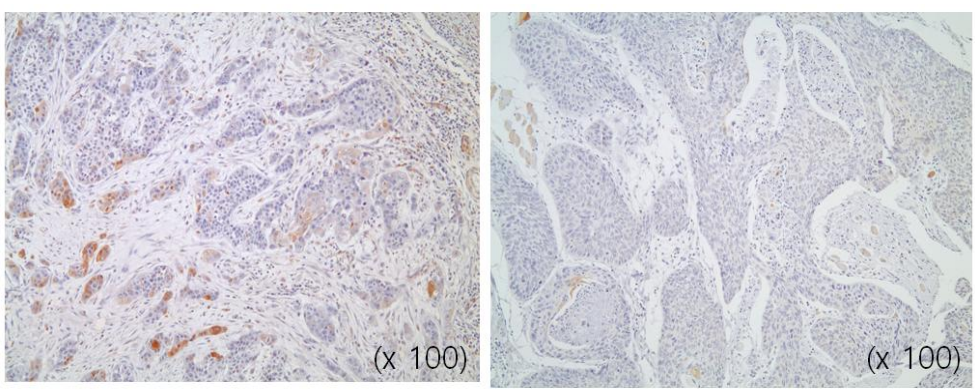


Fig. 4. The Kaplan-Meier analysis showed the correlation of NDRG expression level with overall survival and disease-free survival

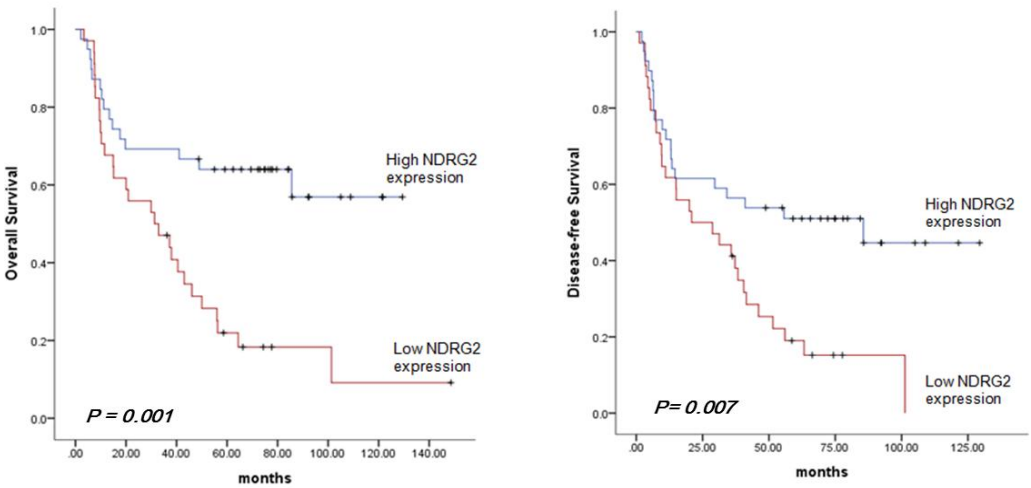


Fig. 5. Screening of six OSCC cell lines for NDRG2 protein expression.

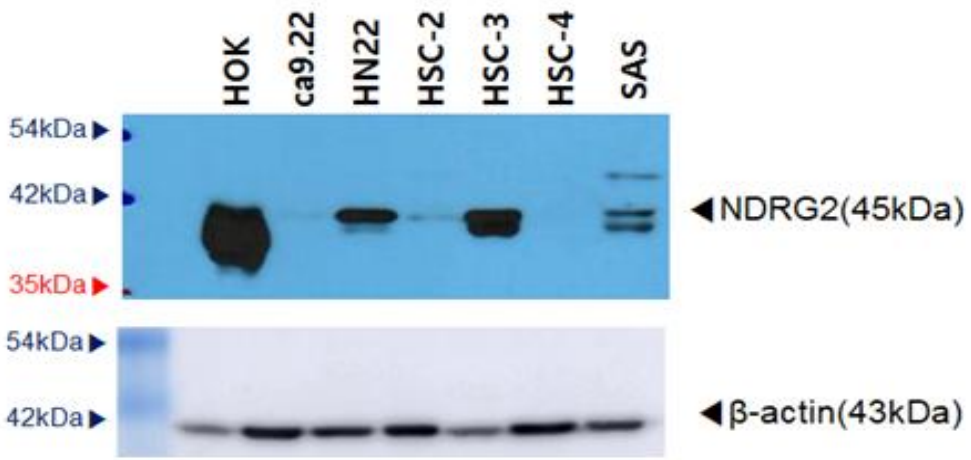
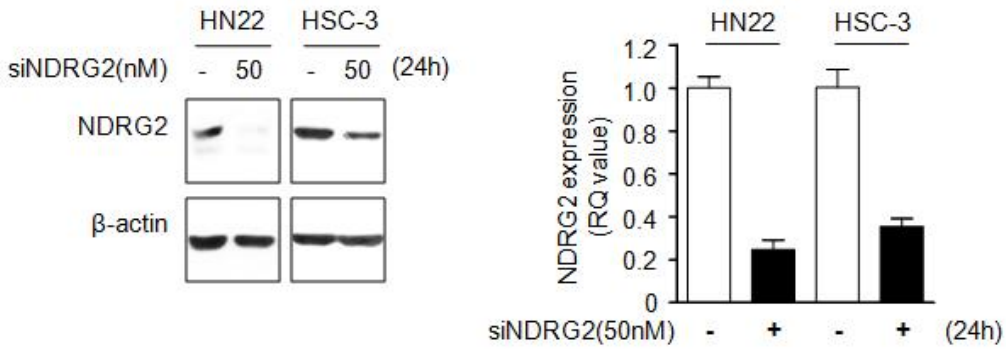


Fig. 6. Expression of NDRG2 protein and cell proliferation were evaluated in HN22 and HSC-3 after transfected with 50 nM siNDRG2

(A) NDRG2 protein expression in HN22 and HSC-3



(B) Cell proliferation in HN22 and HSC-3

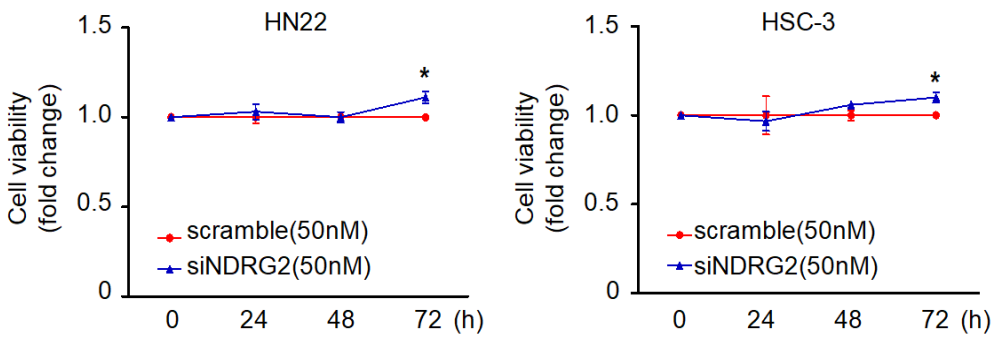
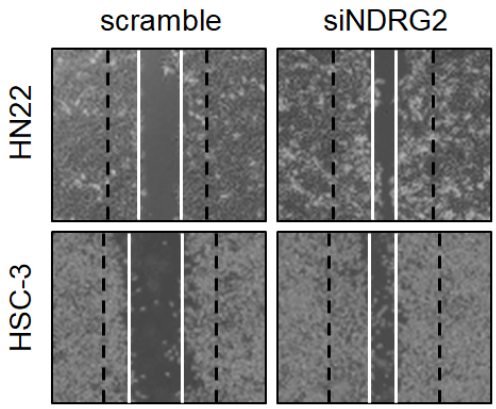
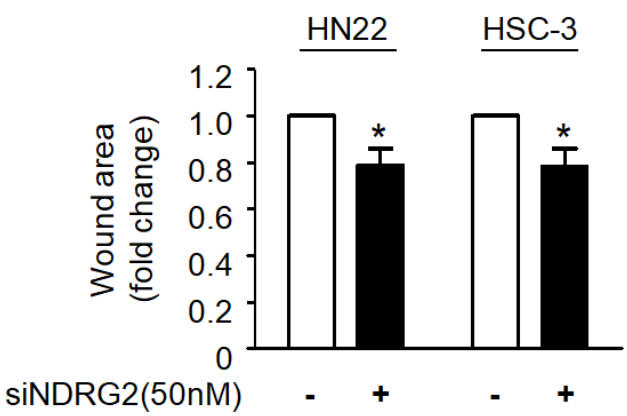


Fig. 7. The cell migration and invasion ability were evaluated in HN22 and HSC-3 after transfected with 50 nM siNDRG2

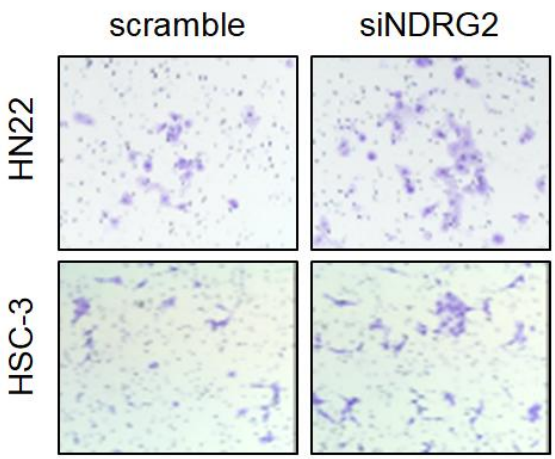
(A) HN22 and HSC-3



(B) Wound area (fold change) in HN22 and HSC-3



(C) Invasion assay in HN22 and HSC-3



(D) Invasion ability in HN22 and HSC-3

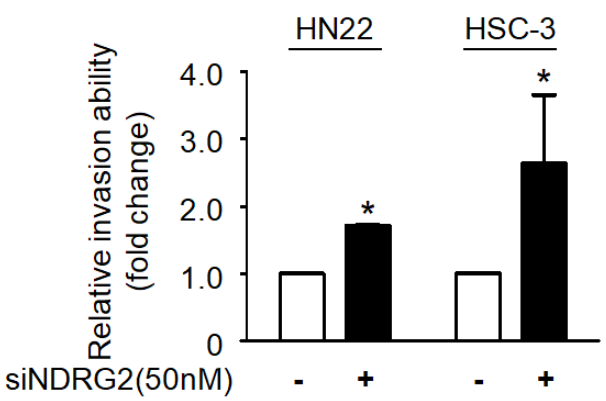
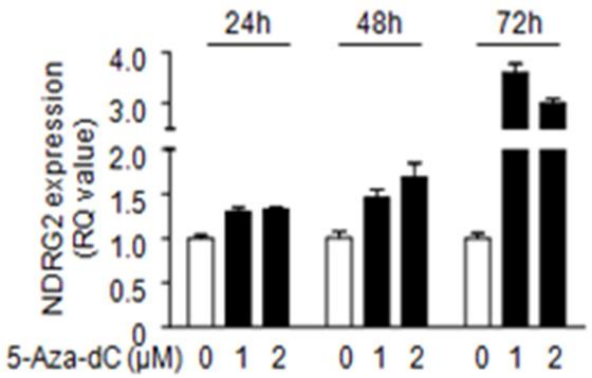


Fig. 8. Effects of 5-Aza-dC on the mRNA level of NDRG2 of Ca9.22 and HSC-4

(A) Ca9.22



(B) HSC-4

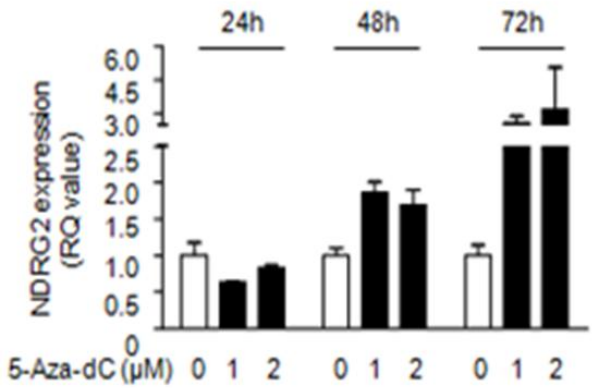
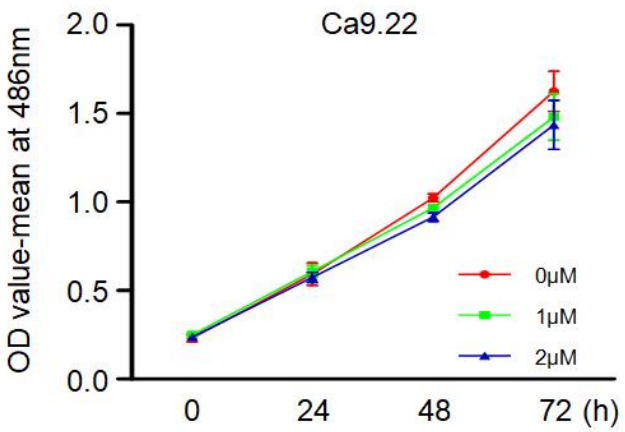


Fig. 9. Effects of 5-Aza-dC on the cell proliferation of Ca9.22 and HSC-4

(A) Ca9.22



(B) HSC-4

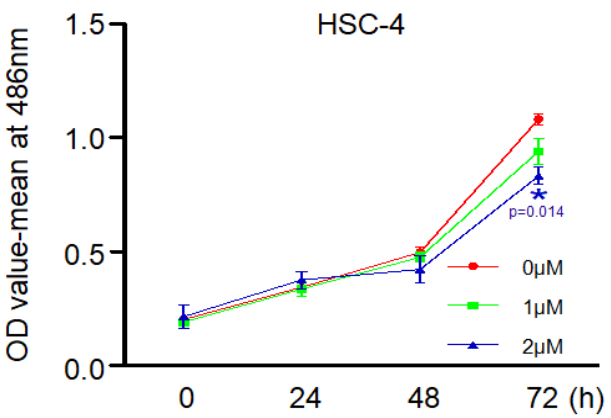
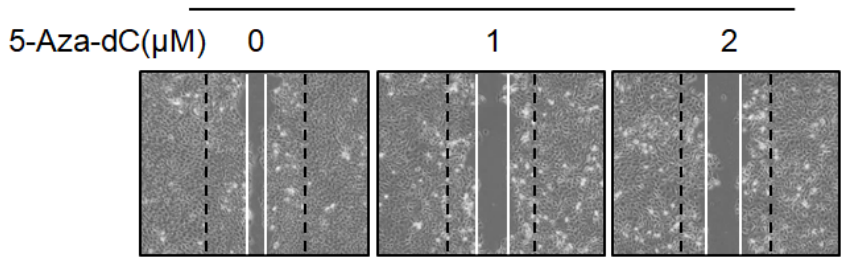
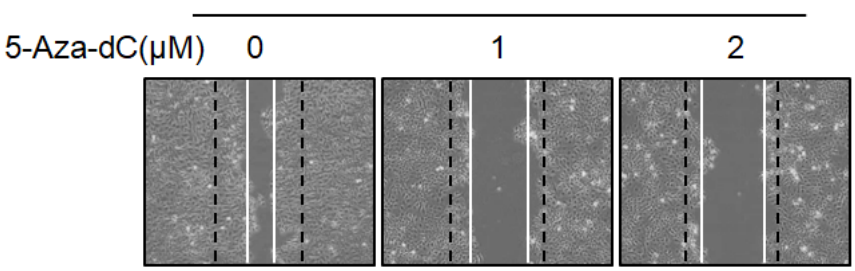


Fig. 10. Effects of 5-Aza-dC on the cell migration ability of Ca9.22 and HSC-4

(A) Ca9.22



(B) HSC-4



(C) Wound area (fold change) in Ca9.22 and HSC-4

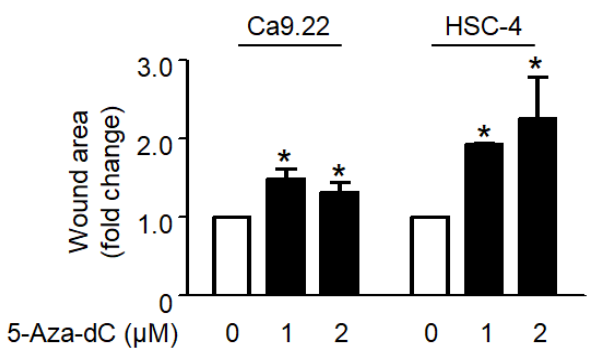
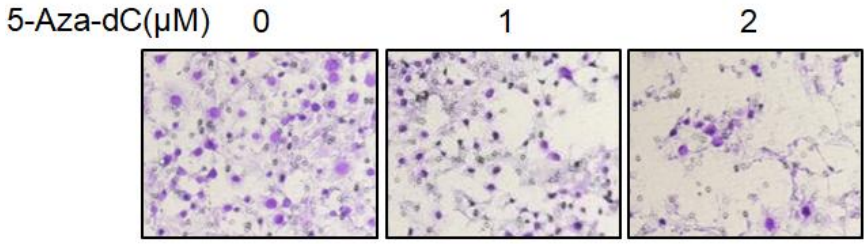
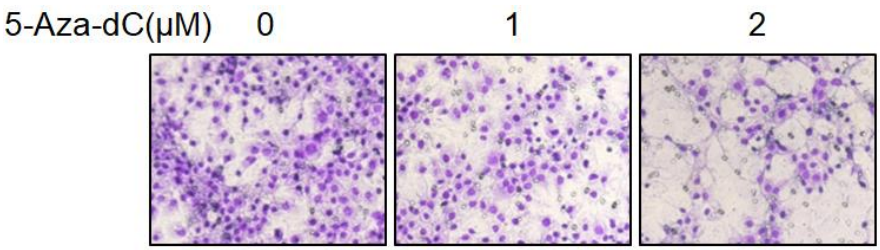


Fig. 11. Effects of 5-Aza-dC on the invasion ability of Ca9.22 and HSC-4

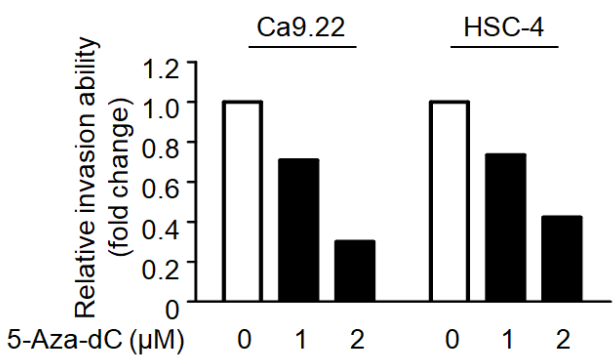
(A) Ca9.22



(B) HSC-4



(C) Invasion ability(fold change) in Ca9.22 and HSC-4



국문초록

구강편평세포암종에서 NDRG2 발현의 예후인자로서의 가치와 후성유전학적 발현 조절에 관한 연구

심 혜 원

서울대학교 대학원 치의학과 구강병리학 전공

(지도교수: 윤 혜 정)

연구개요 및 목적: 구강편평세포암종은 구강에서 가장 흔히 발생하는 악성 종양으로, 진단 및 치료 방법의 발전이 이루어져 왔으나 여전히 많은 환자에서 불량한 예후를 보여왔다. 최근 NDRG2가 다양한 암에서 종양 억제 유전자로서 작용한다는 연구가 보고되었으며, 특히 유전자의 과메틸화는 NDRG2 발현저하의 주요 기전으로 NDRG2 저발현 환자에서 후성유전학적 조절이 새로운 치료법이 될 수 있다. 그러나 구강편평세포암종에서의 NDGR2의 발현 양상에 따른 임상 경과와 생존율과의 관계, 그리고 탈메틸화 약물이 암 세포에서 NDRG2 발현 회복 및 암 억제 효과를 갖는지에 대해서는 아직 연구가 이루어지지 않았다. 따라서 본 연구에서는 구강편평세포암종 환자에서 NDRG2 발현과 임상 병리학적 지표 및 환자 생존율 간의 상관관계를 분석하고, 구강편평세포암종 환자의 예후인자로서 NDRG2의 가능성을 평가하고자 한다. 또한 구강편평세포암종

세포주에서 NDRG2의 역할 및 발현억제 기전, 그리고 유전자 탈메틸화 작용을 가진 5-Aza-dC의 구강편평세포암종 세포주에 대한 작용 효과에 대해 확인해 보고자 하였다.

연구방법: 73명의 구강편평세포암종 환자의 조직 표본을 이용하여 면역조직화학검사를 시행하였고, NDRG2 발현 정도와 임상 병리학적 지표 및 생존율간의 상관관계를 분석하였다. PCR 및 western blotting 으로 구강편평세포암종 세포주에서의 NDRG2 발현을 평가하였고, NDRG2의 종양억제유전자로서의 기능을 평가하기 위하여 anti-NDRG2 siRNA 및 5-Aza-dC 처리 후 세포 증식, 이동, 침윤 실험을 시행하였다

연구결과: 73명의 구강편평세포암종 환자 중 34명(46.6%)에서 NDRG2 저발현을 보였다. NDRG2의 저발현은 중등도/불완전 분화(P=0.038), 림프절 전이(P=0.007), 진행된 임상 병기(P=0.017)와 통계적으로 유의한 상관 관계를 보였다. NDRG2 발현이 낮은 환자는 발현이 높은 환자와 비교하여 전체 생존율(OS, P=0.001) 및 무병생존율(DFS)이 유의하게 낮았다 (P=0.007). Cox 회귀모델을 이용한 다변량 분석 결과, NDRG2 발현 여부가 독립적인 생존율 예측 인자임을 확인하였으며, NDRG2 발현이 높은 환자의 경우 낮은 환자들에 비해 약 2배 이상의 전체 생존율(P=0.022) 및 무병 생존율(P=0.008)을 보였다. NDRG2 발현을 감소시킨 HN22 및 HSC-3 세포주에서 세포 증식, 이동 및 침윤이 유의하게 증가하였다 (P<0.05). NDRG2 발현이 낮은 Ca9.22 및 HSC-4 세포주에서 5-Aza-dC 처리 후 NDRG2 발현의 현저한 회복을 확인하였고, 5-Aza-dC 처리는 두 세포주 모두에서 세포 증식과 이동 및 침윤을 현저히 억제하였다 (P<0.05).

결론: 본 연구 결과를 통해 구강편평세포암종에서 NDRG2 발현 저하는 종양 억제 기능의 저하를 유발하여 종양의 진행에 중요한 역할을 함을 유추하였다. 따라서 탈메틸화 약제를 이용한 NDRG2 발현 회복은 종양세포 증식과 이동 및 침윤을 억제하여 구강편평세포암종 환자에서 새로운 치료법이 될 수 있는 가능성을 제시하였다. 또한 NDRG2의 발현 정도

는 구강편평세포암종 환자의 임상 결과 및 예후를 예측할 수 있는 유용한 지표임을 확인하였다.

주요어: NDRG2, 구강편평세포암종, 종양억제유전자, 예후, 유전자 메틸화, 5-Aza-dC

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