Anti-tumor effects of epigallocatechin-3-gallate on head and neck cancer stem cells

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Directed by Professor Eun Chang Choi

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I dedicate this thesis to my family, my wife, Heywon, and my beloved collegues for their constant support and unconditional love.

I love you all dearly

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Anti-tumor effects of epigallocatechin-3-gallate on head and neck cancer stem cells

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Most solid cancers including head and neck squamous cell carcinoma (HNSCC) are believed to be initiated from and maintained by cancer stem cells (CSCs) that are responsible for treatment resistance, resulting in tumor relapse. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, can potently inhibit cancer growth and induce apoptosis in various cancers, including HNSCC. However, its effect on HNSCC CSCs is not well elucidated. In this study, we examined the anti-tumor effect of EGCG on HNSCC CSCs. We demonstrated that EGCG inhibits the self-renewal capacity of HNSCC CSCs by suppressing their sphere forming capacity, and attenuates the expression of stem cell markers, such as Oct4, Sox2, Nanog and CD44. EGCG treatment augmented cisplatin-mediated chemosensitivity by suppressing ABCC2 and ABCG2 transporter gene, which are putative molecules of treatment resistance of CSC. In addition, the combination treatment of EGCG and cisplatin inhibited tumor formation and induced apoptosis in a xenograft model. As one of mechanism of suppression of HNSCC CSC traits, EGCG decreased the transcriptional level of Notch, resulting in inhibition of Notch signaling. Collectively, our data suggest that EGCG in combination with cisplatin can be used for the management of HNSCC CSCs.

Key words : head and neck cancer, neoplastic stem cells, epigallocatechin -3-gallate, notch1, therapy

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Ⅰ. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with an annual incidence of more than 500,000 cases.[1] Despite recent advances in the understanding of HNSCC progression and the development of novel therapeutic targets, HNSCC is still a major cause of morbidity and mortality worldwide. Five-year survival rates for HNSCC have not improved in more than 3 decades.[2] Alleviation of HNSCC recurrence and mortality requires greater understanding of the biologic behavior and pathologic progression of HNSCC.

The cancer stem cell (CSC) hypothesis states that a subpopulation of intratumoral cells is uniquely capable of propagating the tumor, and relies on the hierarchical model to explain tumor heterogeneity and behavior.[3,4] Accumulating evidences have demonstrated that a variety of human malignancies, including HNSCC, contain a subpopulations of cells that exhibit stem cell-like properties, such as self-renewal and tumor-initiating capabilities.[5,6] It has been suggested that conventional chemotherapies kill differentiated or differentiating cells. CSCs proliferating more slowly appear to be relatively drug resistant and so can be spared, ultimately inducing tumor recurrence after the completion of treatment.[7] Thus, removal of CSCs becomes

more and more crucial to chemotherapy and drugs that selectively target CSCs offer a greater promise for cancer treatment.

Notch signaling is activated when a Notch ligands including Delta-1, -3, -4 and Jagged-1 and -2 interacts with a Notch transmembrane receptor.[8] This process usually initiates the γ-secretase mediated proteolytic release of the Notch intracellular domain (NICD) and activated NICD migrates to the nucleus.[9] The NICD in the nucleus binds to a transcription factor CSL (CBF1/RBPjk in vertebrates, suppressor of hairless in Drosophila, and LAG superfamily in *Caenorhabditis elegans*) and triggers the expression of Notch target genes such as hairy and enhancer of split (HES), which activates stem cell self-renewal.[10,11] In addition, the critical role of Notch signaling in CSCs was demonstrated in esophageal cancers and breast cancer.[12,13] Furthermore, Notch genes mutations have important roles in the carcinogenesis of HNSC.[14] Thus, targeting Notch signaling pathway seems to be a novel therapeutic approach of HNSCC CSC.

Green tea is one of the most popular beverages in the world, and receives considerable attention because it has many beneficial effects on human health. It contains many catechins such as epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC).[15] EGCG, a major polyphenolic constituent of green tea, potently inhibits cancer growth and induces apoptosis in various cancers by several mechanisms.[16-18] Some recent studies have demonstrated that EGCG induces apoptosis via ATM/p53-dependent NAG-1 expression in HNSCC[19] and inhibits HGF-induced tumor growth and invasion in oral and hypopharynx cancer, a subsite of the head and neck area.[20,21] However, the intracellular mechanisms by which EGCG inhibits growth and induces apoptosis in HNSCC CSCs have never been examined.

The main objectives of this study were to examine the anti-tumor effects of EGCG on HNSCC CSCs and to elucidate its underlying mechanism through the suppression of the Notch signaling pathway.

II. MATERIALS AND METHODS.

1. Sphere-forming cell culture from primary HNSCC specimens

HNSCC CSCs (K3, K4, and K5) was isolated from the primary surgical specimen of a HNSCC patient. Briefly, human HNSCC patient specimens were collected after the protocol was approved by Institute for Biomedical Research Ethics Committee, Research Institute of Medical Science, Konkuk University School of Medicine. Primary tumor samples were obtained within 1 h after surgery for HNSCC patients and minced with blades into small pieces. These pieces were enzymatically digested using collagenase (GIBCO), hyaluronidase (Sigma-Aldrich), and DNase (Sigma-Aldrich), and then incubated for 2 h at 37℃ with 5% CO_2. After pipetting with a 10-ml pipette every 15 min, cell disaggregates was washed twice with phosphate buffered saline (PBS), and then centrifuged with Percoll (Sigma-Aldrich) to exclude cell debris and red blood cells. Suspension was filtered through 40-㎛ cell strainer, and the resulting single cells were placed under stem cell suspension culture conditions, consisted of serum-free DMEM/F12 medium supplemented with N2 (GIBCO), B27 (GIBCO), human recombinant epidermal growth factor (EGF; 20 ng/ml, R&D systems), and human basic fibroblast growth factor (bFGF; 20 ng/ml, R&D systems). EGF and bFGF were supplemented every 3 days. As spheres (>20 ㎛ diameter) appeared in suspension culture conditions, they were dissociated with enzymes described above and expanded by reseeding in the aforementioned stem cell suspension culture conditions.

2. Sphere forming assay

To assess self-renewal in vitro, cells were dissociated into single cells, seeded in a 24-well plate at a density of 200 cells/well and cultured in serum-free media with EGF and bFGF supplementation every other day. Spheres with a diameter exceeding 10 μm were counted after 14 days of incubation.

3. Quantitative RT-PCR

Total cellular RNA was extracted from cells homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was prepared using a reverse transcriptase kit (Fermentes) according to the manufacturer's instructions. The synthesized cDNA was added to a mixture of 1 U of Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA) and the specific primers, and amplified using the MJ Research Minicycler™ (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were separated by electrophoresis in 1.5% agarose gels and were detected under ultraviolet light (Bio-Rad Laboratories). The sequences of human specific primers used were as follows:

Oct4

Forward primer: 5′-GCCGGCGGCAACCAGAAAAAC-3′

Reverse primer: 5′-CCGCCGGGGCCGGTATTTATA

SOX2

Forward primer: 5′-GTGGGCCTGAAGAAAACTATC-3′

Reverse primer: 5′-AGGGCTGTCCTGAATAAGCAG-3′

Nanog

Forward primer: 5′-TCCTCCAGTCAATACCCATCAG-3′

Reverse primer: 5′-CAGCAGTCATGTGCTTTTCCT-3′

Involucrin

Forward primer: 5′-GGACAGGCATCTCCAAGCAT-3′

Reverse primer: 5´-GCCCACTCTTCGAATAGCTG-3´

ABCB1

Forward primer: 5´-GCCCTACTTCCTCATGAGCT-3´

Reverse primer: 5´-GCAGGCAGTGACAAACAGCA-3´

ABCC1

Forward primer: 5´-GAACCTGCTGGCCTTTAGTC-3´

Reverse primer: 5´-GAGGTGAAGGCCTTTTGTGC-3´

ABCC2

Forward primer: 5´-GGTGTCATCTACAGGAAGGC-3´

Reverse primer: 5´-GCTGACCACAGCAGATTGAG-3´

ABCC3

Forward primer: 5´-GCCCTACTCTGGATGGAGAT-3´

Reverse primer: 5´-CCTGGCATCCGTGAAAGTTG-3´

ABCC4

Forward primer: 5´-GATGCCTTGGAAACAGCAGC-3´

Reverse primer: 5´-TGGGCTTCAGAGCACTCAAG-3´

ABCC5

Forward primer: 5´-GCAGAACACCTCTGTGGTAG-3´

Reverse primer: 5´-GAAGCTGTCCACATCTGGCT-3´

ABCC6

Forward primer: 5´-AGGTGGAGGCAAATCTTCGT-3´

Reverse primer: 5´-GACCCTGTTAATCCGTTCGT-3´

ABCG2

Forward primer: 5´-TGCCAGACCAACATCAAC-3´

Reverse primer: 5´-CTCATAGTCCTCGGATTGC-3´

Notch1

Forward primer: 5´-ACGAGAATGGAAACTTGAGTTC-3´

Reverse primer: 5´-AACTCCGATAGTCCATAGCAAG-3´

Hey1

Forward primer: 5´-CCAAAGACAGCATCTGAGCA-3´

Reverse primer: 5´-CATTGATCTGGGTCATGCAG-3´

Hes1

Forward primer: 5´-CCAAAGACAGCATCTGAGCA-3´

Reverse primer: 5´-CATTGATCTGGGTCATGCAG-3´

4. Transfection of small interfering RNA (siRNA)

Cells were seeded in 6 well plate and cultured overnight in a 5% CO2 atmosphere at 37^{o}C. Then the medium was replaced with Opti-MEM containing ABCC2, ABCG2, or control siRNA with Lipofectamine 200 reagent (Invitrogen) for 24 hours. The sense sequences of siRNA were: ABCC2: 5´-ACAAGGUAAUGGUCCUAGA-3´ and ABCG2: 5´-CACACAUGUGCAACCAUCA-3´

5. Knockdown of Notch1 in HNSCC CSCs.

For gene knockdown of Notch1, cells were infected with lentivirus with Notch1-shRNA which was cloned into pLKO-puro plasmid, according to t he manufacturer's protocols (Oligoengine). The target sequences were Notch1 sh-RNA: 5'-CCGGCGCTGCCTGGACAAGATCAATCTCGAGATT GATCTTGTCCAGGCAGCGTTTTT-3´.

6. Western blotting

Western blot analysis was performed as described.[22] Specific antibodies against Oct4, Sox2, ABCC2, ABCG2, CD44, cleaved caspase-3 Cells, notch1 and β-actin used for Western blot analysis were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA), and secondary antibodies, anti-rabbit IgG, or anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

6. FACS analysis

Sphere cells were dissociated into single cells, washed, and suspended in phosphate buffered saline (PBS). Cells were labeled with anti-CD44 and fluorescein isothiocynate (FITC)-labeled secondary antibodies and then subjected to flow cytometry using a FACSCalibur machine (BD Biosciences). IgG isotype was included as negative control.

7. Chemoresistance assay

Cells were plated in a 96-well plate at a density of 7 x 10^3 cells per well and then treated with dimethylsulfoxide (DMSO) or cisplatin at various concentrations (0 μM, 5 μM, 10 μM and 20 μM), or with EGCG (5 μM) combined with cisplatin at various concentrations (0 μM, 5 μM, 10 μM and 20 μM). Forty-eight hours later, 20 μl of 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added to each well and placed at room temperature for 3 h. The absorbance was then measured using a SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm.

8. Xenograft tumorigeneity

HNSC CSCs were treated for 48 hours with cisplatin (10 μM) alone, cisplatin (10 μM) plus EGCG (5 μM) or control DMSO in vitro. Then, 5 x 10^3 cells were subcutaneously injected into the flank of 8 week-old female BALB/c nude mice using a 22-gauge needle. Engrafted mice were visually inspected and palpated weekly to monitor tumor formation until 3 months post-transplantation. For limiting dilution xenograft assay, groups of mice were inoculated with K3-control cells and K3-shNotch1 cells at 1x10^3, 1x10^4, and 1x10^5, respectively.

Engrafted mice were inspected weekly for tumor appearance by visual observation and palpation until postinjection 8 weeks. As a orthotopic model, each mouse underwent submucosal injection cells ($1x10^3$ cell) directly into anterior tongue using a 50μl Hamilton syringe (Hamilton Co.) and mice were then examined every other day for the development of tongue tumors. All animal studies were approved by the Institutional Animal Care and Use Committee of Konkuk University.

9. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay

Nude mouse tumor tissue from HNSC CSCs treated with DMSO, cisplatin (10 μM) or cisplatin (10 μM) plus EGCG (5 μM) were used for TUNEL assays. Sections (4 μm) from formalin fixed, paraffin-embedded tumors were deparaffinized and rehydrated using xylene and ethanol, respectively. The slides were rinsed twice with PBS and treated for 15 min at 37℃ with proteinase K (15 μg/ml in 10 mM Tris/HCl, pH 7.4–8.0). Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol at room temperature for 10 min. The tissue sections were then analyzed with an *in situ* Cell Death Detection Kit-POD (Roche) in accordance with the manufacturer's instructions.

10. Isolation of $ALDH^{high}$ and $ALDH^{low}$ cell from HNSC CSCs.

Cells were obtained from freshly dissociated spheres and were analysed using an Aldefluor assay kit (Stem Cell Technologies, Durham, NC, USA). Cells were suspended in Aldefluor assay containing ALDH substrate(BAAA, 1 mol/l per 1 x 10^6 cells). A sample of cells was stained under the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) as a negative control. Flow cytometric sorting was conducted using a FACS Aria (Becton Dickinson, CA). The sorting gates were established using as negative controls the cells stained with PI only, for viability, the antibody alone.

11. Luciferase reporter gene assay

Notch transcriptional activity in HNSC CSCs treated with EGCG or DMSO was determined by analyzing the relative luciferase activities of the pGL3-CSL plasmid cloned with Notch promoter sequence (1.5 kb upstream from transcription start site) using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Transfection efficiency was normalized with the activity of Renilla luciferase, according to the instructions of the manufacturer (Promega).

12. Statistics

Statistical analyses were done using commercially available software (SPSS 12.0; SPSS, Chicago, IL, USA) was used. Experimental data were statistically assessed with two-tailed Student's t-test or one-way analysis of variance with post-hoc analysis. Univariate analysis of Correlation between Notch1 expression and clinical/pathological parameters was analyzed using Fisher's exact test and Pearson Chi-square test. Significant variables from the univariate analysis were included in the multivariate analysis using a binary logistic regression analysis. The survival analysis of human samples and mice was performed using the Kaplan-Meier survival curve and the log-rank test. A p value of <0.05 was required for statistical significance.

III. RESULTS

1. Squamosphere formation from primary HNSCC specimens.

We attempted to employ stem cell suspension culture conditions to establish sphere-forming cells (squamospheres) using single-dissociated tumor cells derived from 47 primary HNSCC specimens—25 larynx, 12 oral cavity, 5 hypopharynx, 4 oropharynx and 1 maxillary sinus. Forty cases (85%) were primary tumors and the remaining 7 cases (15%) were recurrent tumors. Only 3

samples (6%) gave rise to sphere-like clusters within 2 weeks of seeding, but the other 44 samples failed to form squamospheres. One of these 3 cases able to form squamospheres were recurrent tumor. These squamospheres showed distinct morphological appearance (Fig. 1). One squamosphere had a well-defined circular shape, whereas the others appeared as irregular cell clusters (alike bunch of grape) without evident marginal rims. As the size of squamospheres grew beyond 20 ㎛ in diameter, the squamospheres were enzymatically dissociated and then replated as single-dissociated cells in the stem cell suspension culture condition. We found that these cells were able to form squamospheres over 10 passages that we tested. We used one of three squamospheres to characterize cancer stem cell (CSC)-like properties.

2. Stem cell marker expression of HNSCC-driven squamospheres.

First, we performed real time PCR to determine whether squamospheres expressed stem cell (CK5, OCT4, SOX3, and Nestin) or differentiated cell (CK18 and Involucrin) markers in serum-free undifferentiated and serum-induced differentiated conditions. We found that the mRNA expression levels of CK5, OCT4, SOX2, and nestin were significantly higher in undifferentiated squamospheres, compared to differentiated counterparts. Conversely, mRNA levels of CK18 and Involucrin was slightly lower in undifferentiated squamospheres, compared to differentiated ones (Fig. 2a). In addition, immunofluorescence analysis revealed that undifferentiated squamospheres exhibited high levels of OCT4 and SOX3 protein expression, and barely detectable levels of involucrin expression (Fig. 2b). However, as squamospheres were allowed to growing in serum-induced differentiation culture condition for 2 weeks, OCT4 and SOX2 expressions were dramatically diminished, whereas involucrin expression was increased (Fig. 2b). Taken together, these results suggest that stem cell suspension culture condition imparts HNSCC-driven squamospheres to possess stem cell feature.

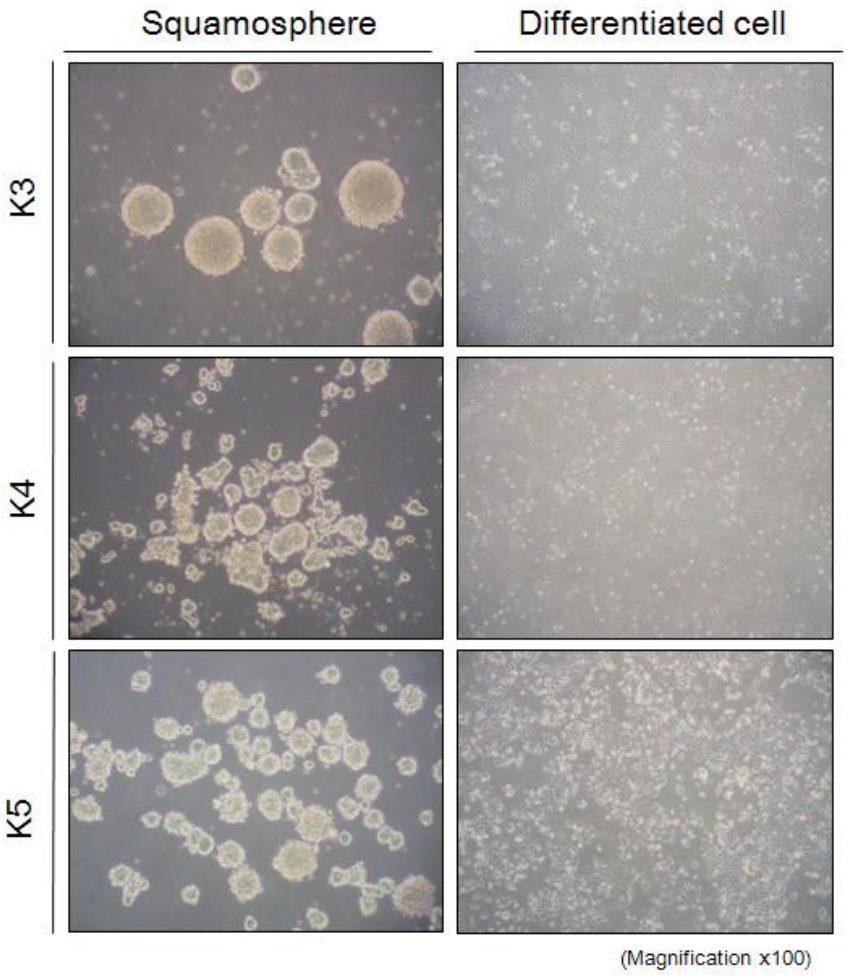


Fig.1. Primary undifferentiated squamospheres and differentiated squamosphere cells. Single-dissociated cells from primary HNSCCs generate non-adherent spherical colonies 2 weeks after seeding (right photos) in serum-free condition with defined growth factors (EGF and bFGF). Single-dissociated squamosphere cells grow in an adhered fashion, and display epithelial-like cuboidal and aggregated morphologies in serum-induced differentiating culture conditions (in the presence of FBS for 2 weeks; left photos). Magnification: 100X.

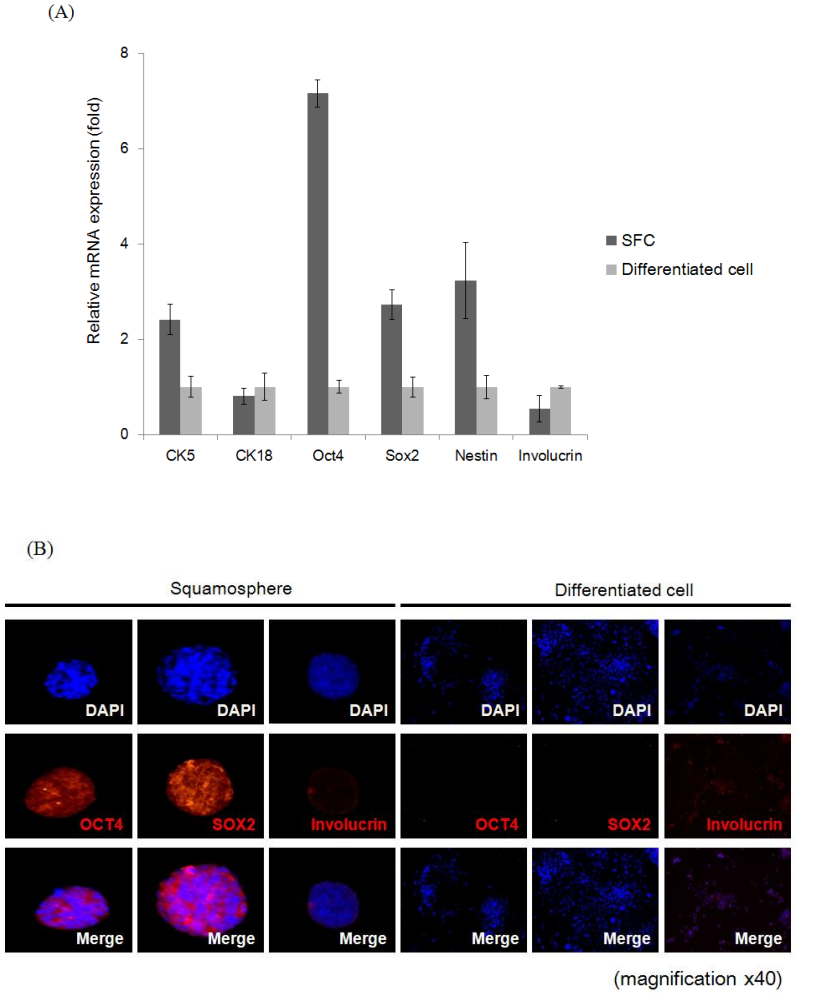


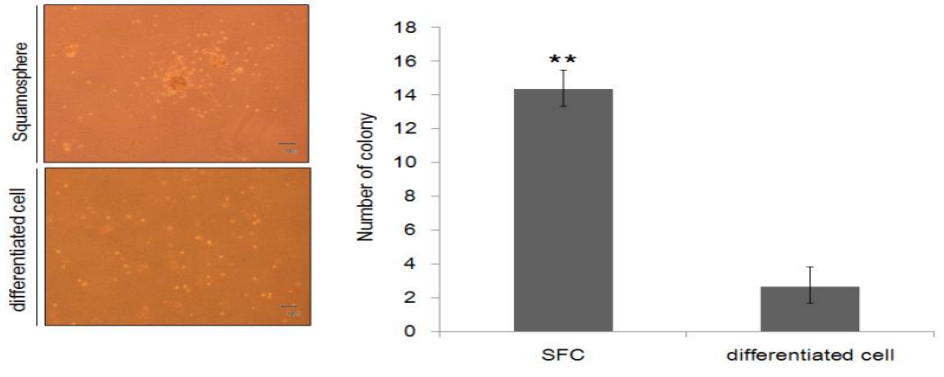
Fig.2. Stem cell marker expression in HNSCC-driven squamospheres.

(a) Real-time RT-PCR showed mRNA expression levels of stem cell markers in undifferentiated and differentiated squamosphere cells. SFC, sphere-forming cell. (b) Immunofluorescence of undifferentiated squamospheres and differentiated squamosphere cells using antibodies against stem cell markers (OCT4 and SOX2), and differentiated keratinocyte marker (Involucrin).

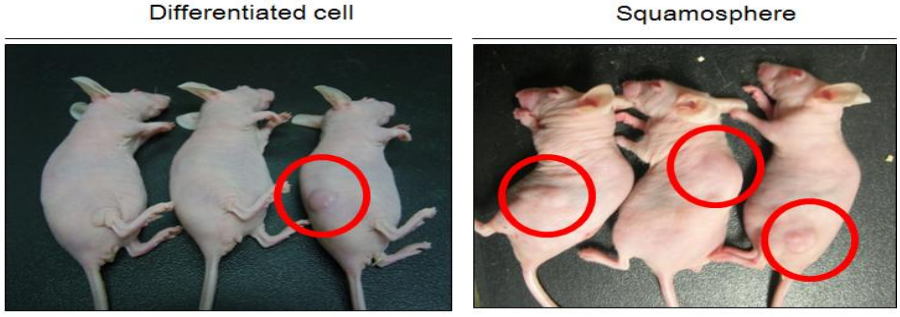
3. In vitro and in vivo tumorigenecity of HNSCC-driven squamospheres .

Next, we performed a soft agar assay to evaluate anchorage-independent growth ability, an indicator for assessing cell transformation *in vitro*. As shown in Fig. 3a, single-dissociated squamosphere cells that sustained their undifferentiated state gave rise to transformed cell colonies after 2 weeks of seeding. However, the number of colonies was dramatically diminished as serum-induced differentiated squamosphere cells allowed to grow in soft agar culture conditions. To further determine the tumorigenicity of our HNSCC-driven squamospheres *in vivo*, we subcutaneously injected different number of undifferentiated, single-dissociated squamosphere cells (10^2, 10^3, or 10^4), and serum-induced differentiated squamosphere cells (10^4, 10^5, or 10^6) in immunodeficient mice. As shown in Fig. 3b, even 10^2 undifferentiated squamosphere cells were sufficient to give rise to tumor formation 6 weeks post-injection, but only one mouse injected with 10^6 serum-induced differentiated squamosphere cells exhibited visible tumor formation. These results indicate that HNSCC-driven squamospheres possess tumor-initiating capacity, one of the most critical hallmarks of CSCs.

(A)



(B)



	Cells injected	Mice with tumors
Squamosphere	10^2	1/2
	10^3	2/2
	10^4	2/2
Differentiated cell	10^4	0/2
	10^5	0/2
	10^6	1/2

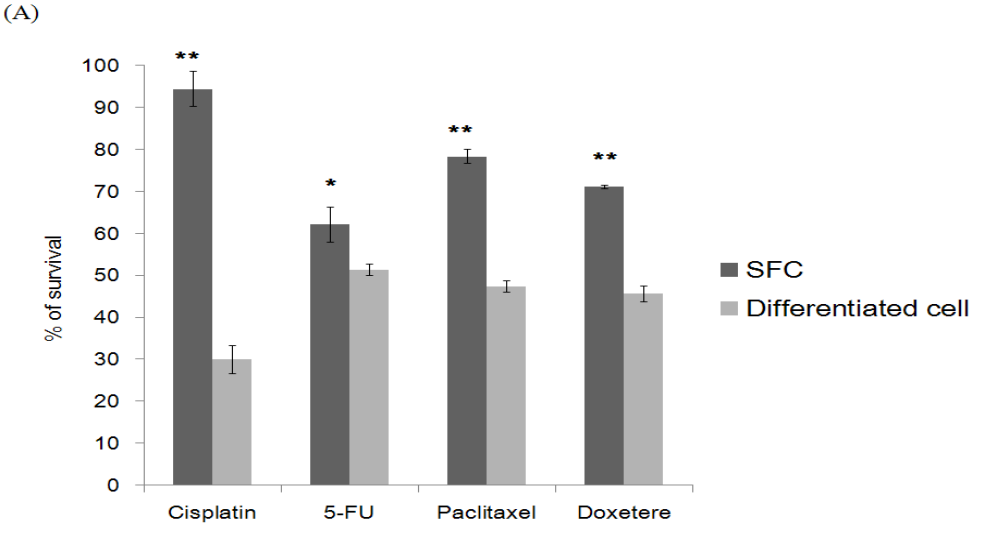
Fig.3. Tumorigenicity of HNSCC-driven squamospheres.

(a) *In vitro* soft agar assay showed anchorage-independent colony formation in single-dissociated undifferentiated and differentiated squamosphere cells. SFC, sphere-forming cell. $^{**}p < 0.01$. (b) *In vivo* tumor formation of undifferentiated and differentiated squamosphere cells. Representative photos showing BALB/c nude mice injected with single-dissociated undifferentiated squamosphere cells (upper left), and differentiated squamosphere cells (upper right). Quantitative data showing tumor formation capacity of undifferentiated (SFC) and differentiated squamosphere cells present in the bottom panel.

4. Chemoresistance and side population of HNSCC-driven squamospheres.

Since CSCs is known to be significantly resistant to various chemotherapeutic agents, we evaluated chemoresistance of our HNSCC-driven squamospheres to four chemotherapeutic agents—cisplatin, 5-Fluorouracil (FU), paclitaxel, and doxetaxel that are commonly administered for the treatment of HNSCC. We

found that undifferentiated single-dissociated squamosphere cells were significantly resistant to all chemotherapeutic agents tested, as compared to differentiated squamosphere cells (Fig.4a). A plausible explanation for chemoresistance of CSCs is the enriched side population, capable of excluding exogenous and endogenous toxic materials using ATP-binding cassette (ABC) transporters. Therefore, we investigated a side population in HNSCC-driven squamospheres by determining exclusion of Hoechst 33342 dye. Fluorescence-activated cell sorting (FACS) analysis revealed that undifferentiated single-dissociated squamosphere cells were comprised of 1.74% side population, whereas differentiated squamosphere cells were comprised of 0.11% side population (Fig. 4b). Furthermore, real-time RT-PCR showed that the expression levels of *ABCG2*, an ABC transporter up-regulated in many CSCs, were significantly higher in undifferentiated squamosphere cells, compared to differentiated squamosphere cells (Fig. 4c). Taken together, our findings indicate that HNSCC-driven squamospheres have one additional CSC property—chemoresistance.



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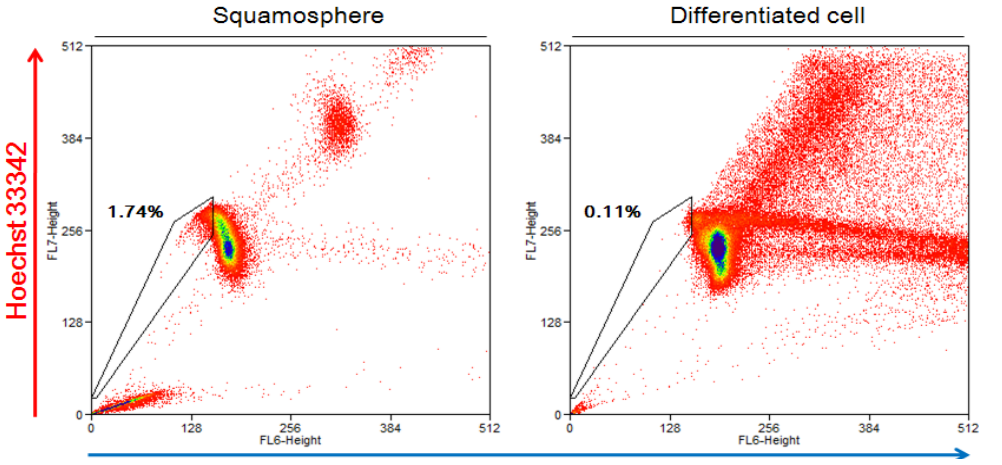


(C)

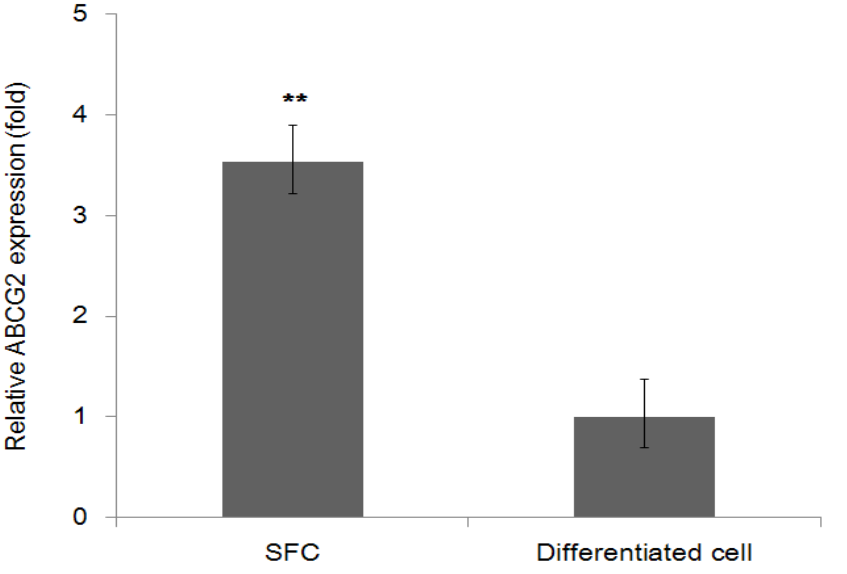


Fig.4. Chemoresistance of HNSCC-driven squamospheres

(a) MTT assay showed cell survival rates of single-dissociated undifferentiated (black columns; SFC) and differentiated (gray columns) squamosphere cells grown in serum-free culture conditions in the presence of cisplatin (10 μM),

5-FU (20 μM), paclitaxel (0.5 μM), or doxetaxel (10 nM) for 48 hrs. SFC, sphere-forming cell. *$p < 0.05$, **$p < 0.01$.(b) Flow cytometry analysis showed side population cells in single-dissociated undifferentiated and differentiated squamosphere cells stained with Hoechst 33342 dye. (c) Real-time RT-PCR showed ABCG2 expression in undifferentiated squamospheres and differentiated squamosphere cells. SFC, sphere-forming cell. **$p < 0.01$

5. EGCG attenuates HNSCC CSC traits in vitro

The self-renewal ability is one of the main characteristics of CSCs. Therefore, we examined whether EGCG could inhibit the growth of HNSCC CSCs by measuring sphere formation. Unlike most HNSCC CSCs, in which DMSO treatment generates a large-sized squamosphere, almost all HNSC CSCs treated with EGCG (5 μM) attached to culture plate, indicating a differentiated status (Fig. 5A), and EGCG treatment significantly reduced sphere formation in a dose-dependent manner (Fig. 5B, 5C, and 5D). These data suggested that EGCG can be effective in inhibiting self-renewal capacity of HNSCC CSCs. We next analyzed the expression of stem cell and differentiation markers in HNSC CSCs. Several markers including Oct4, Sox2, Nanog and CD44 are associated with CSC properties.[6, 23] Therefore, we assessed whether expression of these markers was changed by EGCG treatment. Treatment of HNSCC CSCs with 5 μM EGCG significantly reduced Oct4, Sox2 and Nonog expression and increased expression of Involucrin, a differentiated keratinocyte marker expression (Fig. 5E). Flow cytometry analysis was done to quantify CD44 expression in HNSCC CSCs because CD44+ cells possess CSC properties in HNSCCs. EGCG treatment significantly decreased CD44+ cells (Fig.5F). These results suggested that EGCG could be a potent suppressor of HNSCC CSC traits.

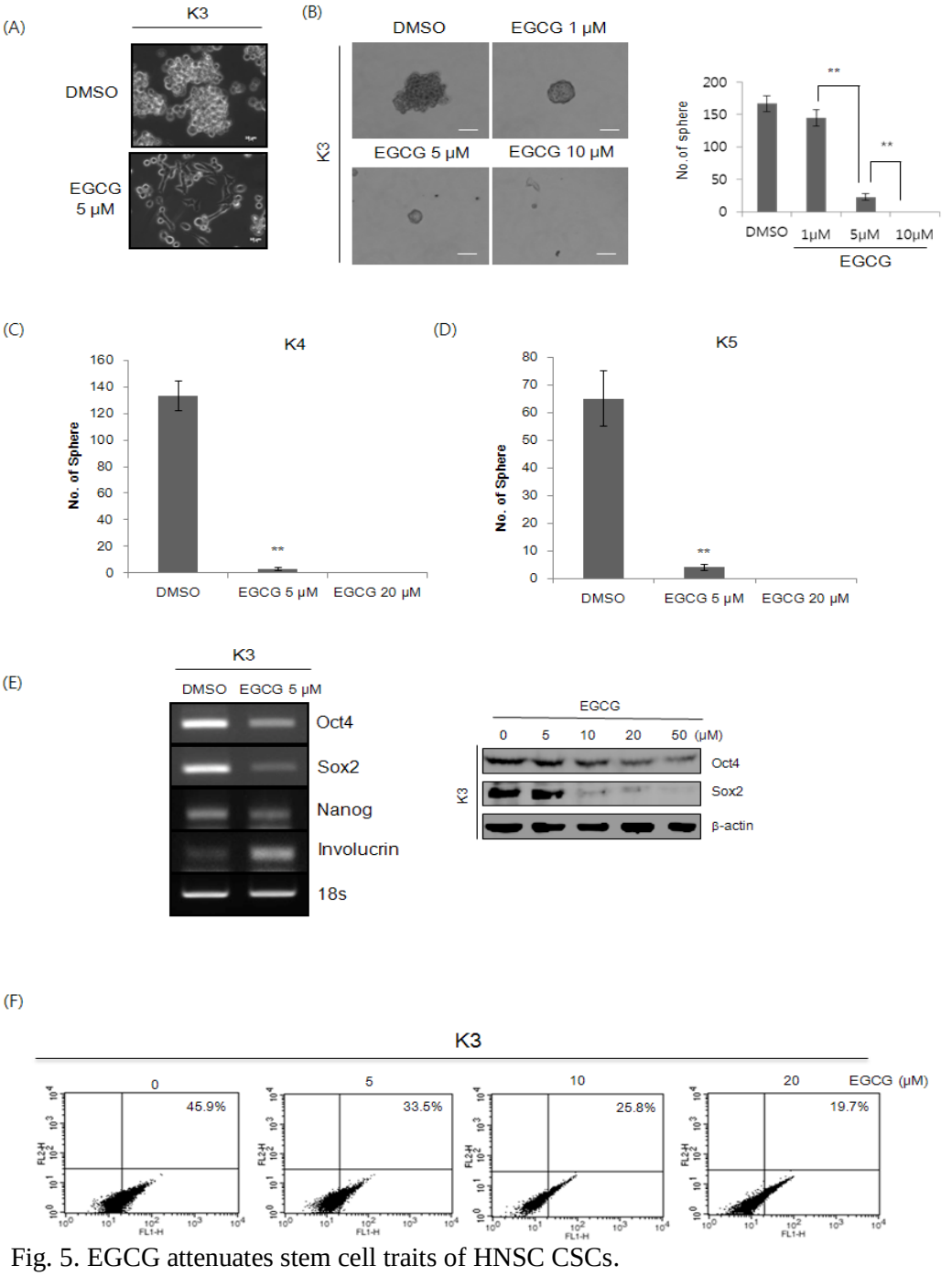


Fig. 5. EGCG attenuates stem cell traits of HNSC CSCs.

(a) Representative HNSCC CSCs morphology after DMSO or 5μM-EGCG treatment for 48 hours. (b ~ d) Sphere formation capacity in DMSO or EGCG with various concentrations-treated HNSCC CSCs (bar, 10 μm).(e) Quantitative PCR and Western blot analysis of expression of stem cell markers (Oct4, Sox2, and Nanog) and differentiation marker (Involucrin) in DMSO or EGCG treated K3 cells. (f) FACS analysis for CD44 expression in DMSO or EGCG treated K3 cells.

6. EGCG enhances chemosensitization of cisplatin for HNSCC CSCs by suppression of ABCC2 and ABCG2 transporter gene expression

CSCs are appreciably resistant to various chemotherapeutic agents including cisplatin, which is popularly used in HNSC patients. Hence, we evaluated the effect of EGCG on HNSC CSCs, especially combined with cisplatin. Specifically, we tested whether treatment of HNSC CSC by EGCG intensifies the susceptibility of cisplatin for HNSC CSC. Cisplatin plus EGCG (5 μM) increased HNSC CSC sensitivity to cisplatin compared to cisplatin alone (Fig. 6A). Next, we investigated the expression of caspase-3, which is a key indicator of apoptosis. Expression level of cleaved caspase-3 was increased after combinatory treatment with cisplatin (10 μM) and EGCG (5 μM), compared to EGCG (5 μM) or cisplatin (10 μM) alone (Fig. 6B). A plausible explanation for chemoresistance of CSCs is the increased ability of the exclusion of exogenous and endogenous toxic materials using ATP-binding cassette (ABC) transporters.[24] Therefore, we investigated the changed expression of ABC transporters in HNSC CSCs after EGCG treatment. RT-PCR and Western analysis revealed that EGCG treatment decreased ABCC2 and ABCG2 expression in HNSC CSCs (Fig. 6C and 6D). We then examined whether suppression of ABCC2 and ABCG2 resulted in chemosensitization of cisplatin for HNSC CSCs. HNSC CSCs transfected with ABCC2 and ABCG2 siRNA or control siRNA were treated with cisplatin at various concentration (Fig. 6E).

MTT assay demonstrated that transfection of siRNA against ABCC2 and ABCG2 decreased cell viability to cisplatin compared to control siRNA transfected cells (Fig. 6F). These data suggest that EGCG-induced ABCC2 and ABCG2 down-regulation may be one of the underlying mechanisms that contribute to EGCG-induced chemosensitivity to cisplatin in HNSC CSCs.

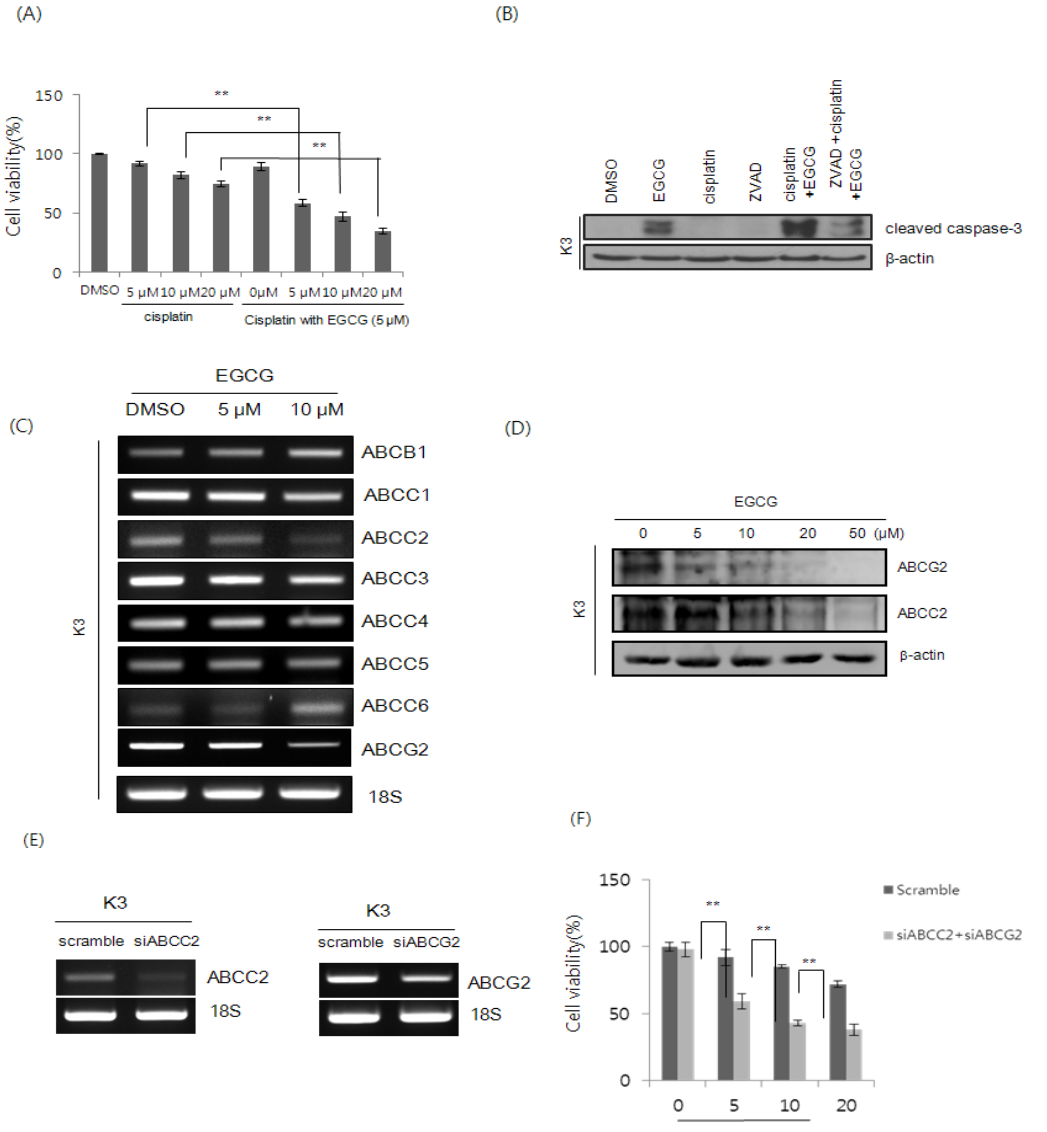


Fig. 6. EGCG enhances chemosensitization by ABCC2 and ABCG2 gene

suppression.

(a) MTT assay after cisplatin with various concentrations in DMSO or EGCG (5μM) treated K3 cells. (b) Protein levels of cleaved caspase-3 detected by Western blotting after cisplatin administration in in DMSO, zVAD(50 μM), EGCG (5μM), or combinations treated K3 cells. zVAD, a caspase inhibitor. (c) mRNA and (d) protein levels of various ABC transporter genes after EGCG (5μM) treatment in K3 cells in a dose-dependent manner. (e) Knockdown of ABCC2 and ABCG2 gene by small interfering RNA in K3 cells. (f) MTT assay after cisplatin administration with various concentrations in control siRNA-tranduced, and siABCC2 and siABCG2-transduced K3 cells.

7. EGCG combined with cisplatin inhibits tumor formation of HNSC CSCs in xenograft model

To assess whether in vitro results could be verified in vivo, the inhibitory effect of EGCG on the capacity of HNSC CSCs to propagate tumor formation in nude mice was examined. HNSC CSCs treated with 5 μM EGCG + 10 μM cisplatin generated only very small visible tumors in the nude mice, in contrast to the large tumors generated by HNSC CSCs treated with cisplatin alone (Fig. 7A). TUNEL staining revealed that apoptotic cells were significantly increased in tumors generated by treatment with EGCG + cisplatin compared to tumors generated after treatment with cisplatin alone (Fig. 7B).

8. EGCG suppresses Notch1 signaling of HNSCC CSCs

Notch signaling is an important cell signaling pathway that is vital for regulation of the balance between cell proliferation, differentiation, and apoptosis.[25] Thus, we examine whether the inhibition effects of EGCG on HNSC CSCs can be mediated by suppression of Notch pathway. We measured the change of transcriptional levels of Notch1 and its downstream target proteins Hey1 and Hes1 after EGCG treatment. EGCG treatment decreased the

mRNA levels of Notch1, Hey1 and Hes1 (Fig. 8A.). Western blot analysis showed that protein levels of Notch1 were also decreased in HNSC CSCs treated with EGCG in a dose-dependent manner (Fig. 8B). In addition, EGCG administration decreased the Notch promoter activity in a dose-dependent manner (Fig. 8C). Finally, we assessed whether blockade of Notch signaling can suppress of self-renewal capacity and chemoresistance of HNSCC CSCs. Next, treatment of DAPT, a γ–secratase inhibitor that blocks the proteolytic cleavage of the Notch receptor complex, release of intracellular fragment and modulation of Notch-specific gene expression were associated with a decrease in sphere formation of HNSC CSCs (Fig. 8D). In addition, DAPT-treated cells were susceptible to cisplatin treatment in CSCs treated with cisplatin alone (Fig. 8E). Collectively, these data suggest that EGCG can decrease HNSCC CSCs traits partly by inhibiting Notch1 pathway

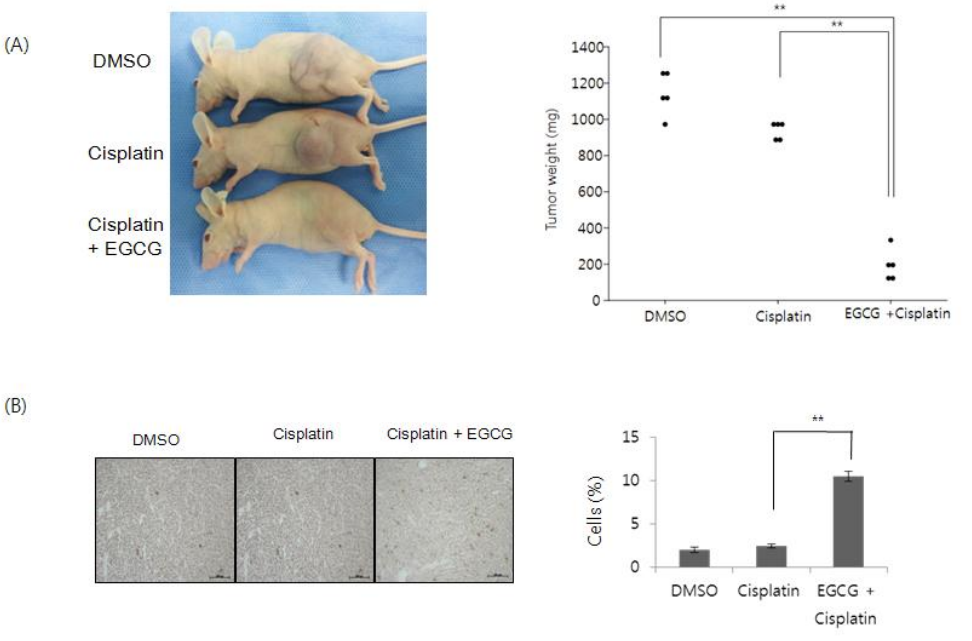


Fig.7. EGCG combined with cisplatin inhibits tumor formation of HNSC CSCs *in vivo*.

(a) Representative of tumor generated after subcutaneous injection of DMSO, cisplatin (10 μM) alone, or EGCG (5μM) plus cisplatin (10 μM) treated K3 cells in flank of nude mouse (left) and average tumor weight (right) (N=5) (b) TUNEL apoptic assay of nude mouse tumor tissue generated by DMSO-, cisplatin alone-, cisplatin plus EGCG-treated K3 cells (left) and its quantification (right).

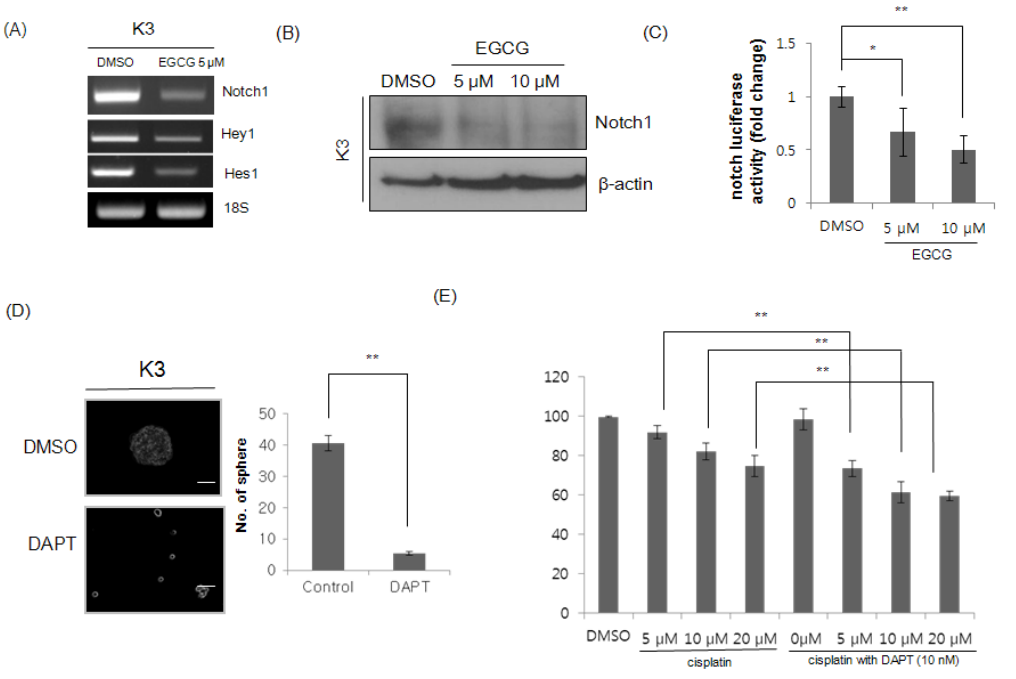


Fig. 8. EGCG suppresses Notch signaling of HNSC CSCs.

(a) mRNA levels of Notch1, Hey1, and Hes1 after EGCG treatment in K3 cells. (b) Protein levels of Notch1 after EGCG treatment in K3 cells. (c) Relative Notch transcriptional activity determined by Notch/CSL luciferase-reporter assay in DMSO or EGCG (5 μM) treated K3 cells. (d) Sphere forming assay in

DMSO or DAPT (10 nM)-treated K3 cells. (bar, 10 μm). (e) MTT assay after cisplatin with various concentrations in DMSO or DAPT (10 nM) treated K3 cells.

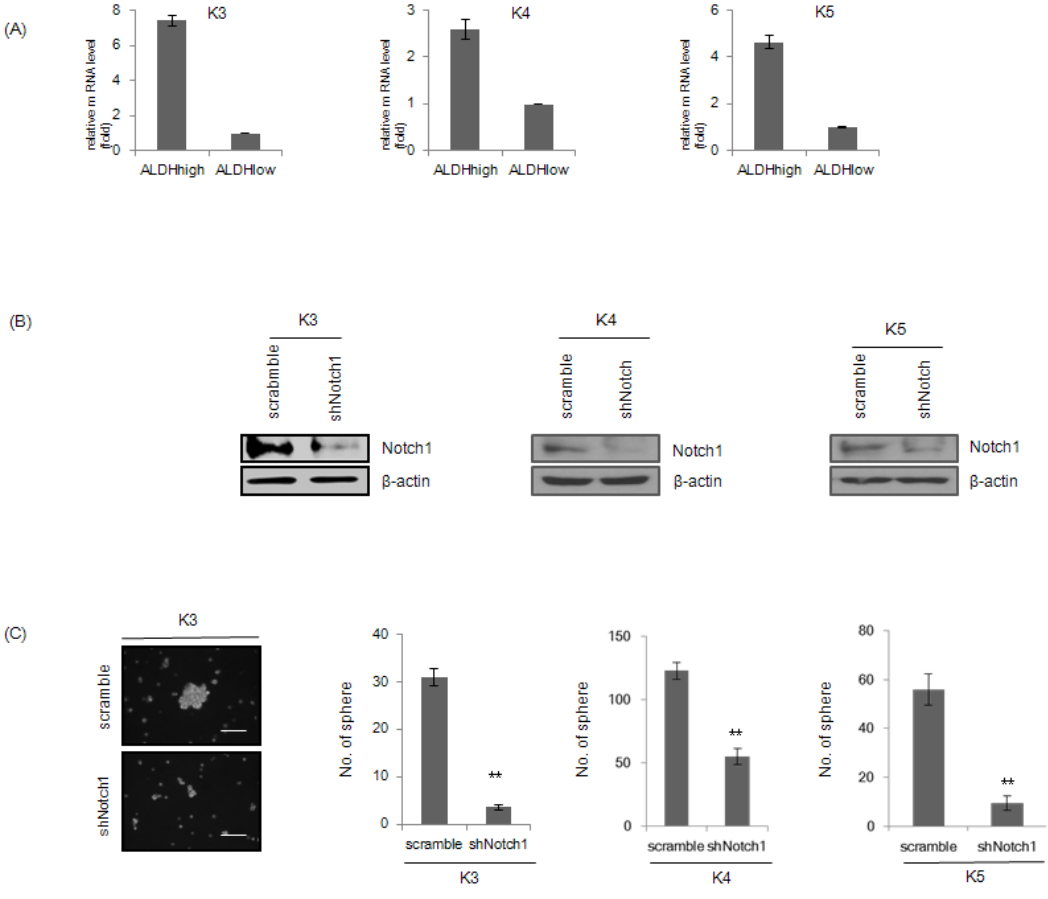
9. Knockdown of Notch1 attenuates HNSCC CSCs traits in vitro

Cancer stem cells have been identified in human HNSCC using aldehyde dehydrogenase (ALDH) activity.[26] We therefore compared Notch1 mRNA level of the $ALDH^{high}$ cells and $ALDH^{low}$ cells in HNSCC CSCs and identified the increased mRNA level of Notch1 in $ALDH^{high}$ cells in comparison to $ALDH^{low}$ cells (Fig 9A). To further determine the regulatory role of Notch1 in HNSC CSCs, we assessed the effect of Notch1 down-regulation on the cultured HNSC CSCs. For this study, HNSC CSCs were transfected with control-shRNA or Notch1-specific shRNA. Western blot analysis confirmed efficient knockdown of Notch1 expression (Fig. 9B). Contrary to Notch1 activation, knockdown of Notch1 decreased the sphere-forming ability of HNSC CSCs (Fig. 9C). Knockdown of notch1 led to decreased protein levels of CSCs markers, such as Oct and Sox2 (Fig. 9D). In FACS analysis, CD44 expression was also decreased in HNSC CSC-shNotch1 cells (Fig. 9E).

10. Knockdown of Notch1 enhances chemosensitization of cisplatin for HNSC CSCs by suppression of ABCC2 and ABCG2 transporter gene expression

Next, we conducted an MTT assay to evaluate the effect of Notch1 knockdown on the chemosensitivity of HNSC CSCs. The MTT assay revealed that knockdown of Notch1 decreased the number of viable HNSC CSCs to cisplatin compared with that to control cells (Fig. 10A). Next, we investigated the changes of ABC transporters levels in HNSC CSCs after Notch1 knockdown. As a result, Notch1 knockdown decreased the mRNA levels of ABCC2 and ABCG2 expression in HNSC CSCs (Fig. 10B). Next, to examine whether suppression of ABCC2 and ABCG2 could result in chemosensitization of cisplatin for HNSC CSCs, we transfected small interference RNA (siRNA)

into HNSC CSCs and evaluated the effect of knockdown of ABCC2 and ABCG2 on cisplatin chemosensitization. As a result, transfection of siRNA against ABCC2 and ABCG2 decreased cell viability to cisplatin compared with that to scramble-siRNA transfected cells (Fig. 10C). These data suggest that ABCC2 and ABCG2 down-regulation by Notch1 knockdown may be one of the underlying mechanisms that contribute to chemosensitivity to cisplatin in HNSC CSCs.



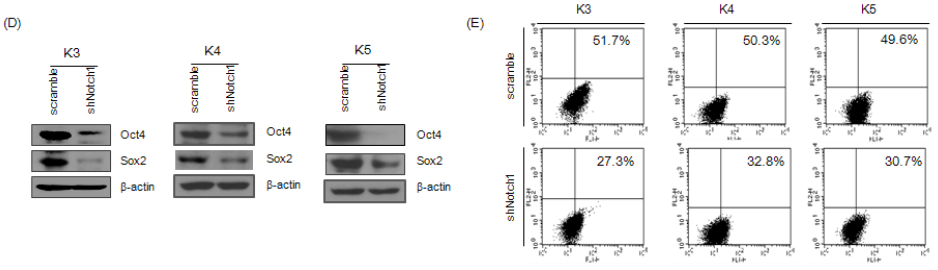


Fig. 9. Knockdown of Notch1 suppresses cancer stem cells traits of HNSC CSC.

(a) The mRNA levels of Notch 1 in ALDHhigh- and ALDHlow-HNSC CSCs. Data are means ± SD (N=3). ALDH, aldehyde dehydrogenase. (b) Verification of Notch1 knockdown in HNSC CSC-shScramble and HNSC CSC-shNotch1 cells determined by Western blot analysis. (c) Sphere forming ability of HNSC CSC-shScramble and HNSC CSC-shNotch1 cells in serum-free conditions. Bar, 10μm, Data are means ± SD (N=3). (d) The protein levels of stemness-associated genes (Oct4 and Sox2) in HNSC CSC-shScramble and HNSC CSC-shNotch1 cells determined by Western Blotting. (e) CD44 expression in HNSC CSC-shScramble and HNSC CSC-shNotch1 cells were determined by FACS analysis.

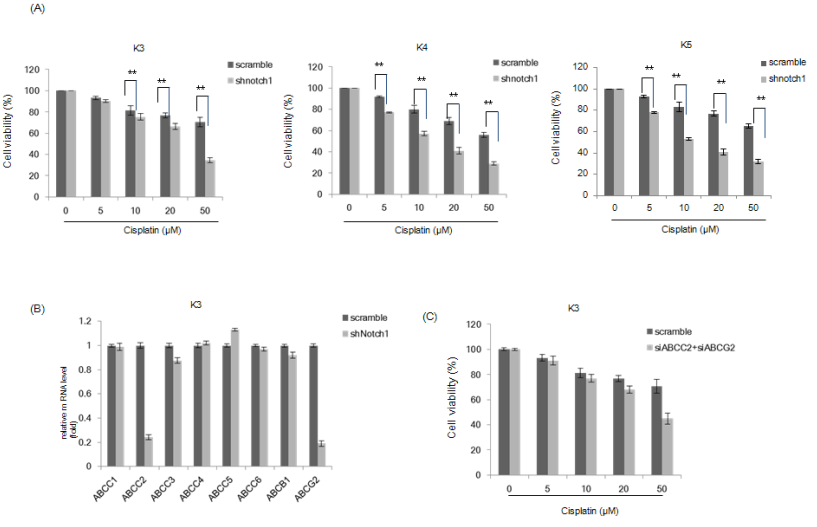


Figure 10. Targeting Notch1 enhances chemosensitization of cisplatin for HNSC CSCs

(a) MTT assay after cisplatin administration with various concentrations in HNSC CSC-shScramble and HNSC CSC-shNotch1 cells. Data are means ± SD (N=3). (b) mRNA levels of different ABC transporters in HNSC CSC-shScramble and HNSC CSC-shNotch1 cells. Data are means ± SD (N=3). (c) MTT assay after cisplatin administration with various concentrations in HNSC CSC-shScramble and HNSC CSC-siABCC2-siABCG2 cells. Data are means ± SD (N=3).

11. Knockdown of Notch1induces antitumour effects in a xenograft model of HNSC CSCs

To validate the *in vitro* findings that suppression of Notch1 decreases the stem cell traits of HNSC CSCs *in vivo*, we investigated the inhibitory effect of Notch1 knockdown on the capacity of HNSC CSCs to initiate tumor growth in a xenograft mouse model. We subcutaneously injected different numbers of HNSC CSC-shNotch1 cells (10^3, 10^4, or 10^5), and HNSC CSC-scramble shRNA cells (10^3, 10^4, or 10^5) in BALB/c mice. As shown in Figure 11A, even 10^3 HNSC CSC-scramble shRNA cells were sufficient to give rise to tumor formation 6 weeks post-injection, but only 40% (2 of 5) of mice injected with 10^5 HNSC CSC-shNotch1 cells exhibited visible tumor formation. In orthotopic model, the injection of HNSC CSC-scramble shRNA cells produced tongue tumors in all of the injected mice (n=5), but HNSC CSC-shNotch1 cells did not (Fig 11B). Next, we examined whether Notch1 knockdown had an effect on the cell proliferation rate using Ki-67 staining in sections from the xenografted tumor. We observed that Notch1 knockdown significantly decreased the expression of Ki-67 positive cells (Fig. 11C) and CD44 positive cells, a putative marker of HNSC CSC (Fig. 11D). Finally, we compared the survival rates between mice bearing HNSC CSC-shNotch1 cells or HNSC CSC-scramble

shRNA, and observed a significant increase in cumulative survival in mice bearing HNSC CSC-shNotch1 cells compared with that in HNSC CSC-scramble shRNA (Fig. 11E). Taken together, our findings suggested that Notch1 play a critical role in tumor propagation of HNSC CSCs in a xenograft model.

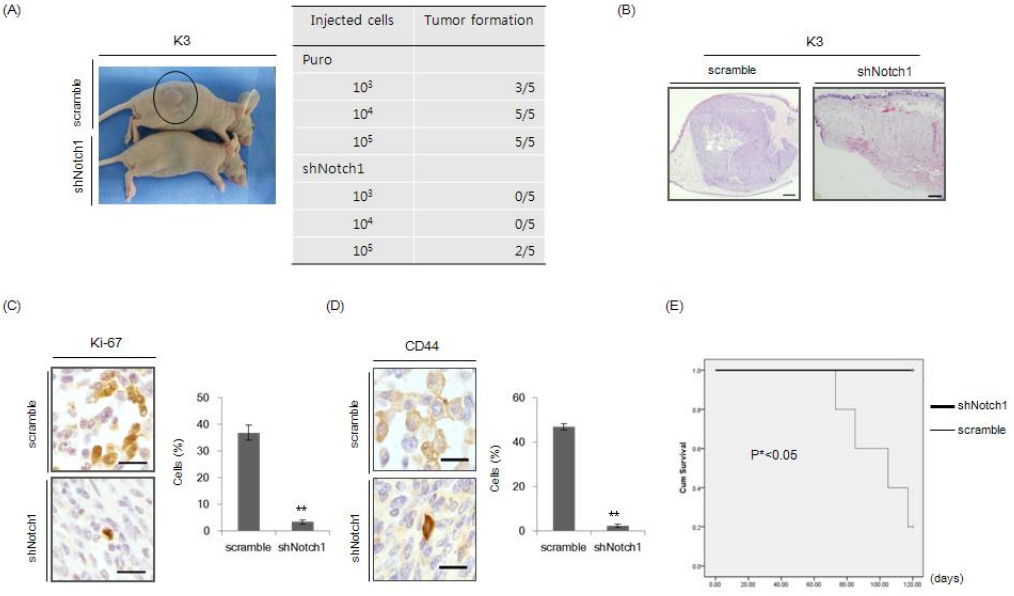


Figure 11. Targeting Notch1 suppresses HNSC CSC tumor growth and increases survival of mice bearing HNSC CSC xenografts.

(a) In vivo propagation of human head and neck cancer in nude mice by either HNSC CSC-shScramble or HNSC CSC-shNotch1cells (right panel). Representative photos showing tumor in a mouse at the cell injection site (left panel).(b) Hematoxyilin and eosin slides of tongue tumor tissue generated by injection of HNSC CSC-shScramble and HNSC CSC-shNotch1. Bar, 500μm,

Data are means ± SD (N=5). (c) Percentage of positive cells for Ki-67, a proliferation marker, in xenograft tumor tissue generated by injection of HNSC CSC-shScramble and HNSC CSC-shNotch1. Bar, 20μm, Data are means ± SD (N=2)., ***P*<0.05 (d) Percentage of positive cells for CD44, a putative HNSC CSC marker, in xenograft tumor tissue generated by injection of HNSC CSC-shScramble and HNSC CSC-shNotch1. Bar, 20μm, Data are means ± SD (N=2). ***P*<0.05. (e) Kaplan-Meier survival curves of mice implanted with HNSC CSC-shScramble and HNSC CSC-shNotch1 cells (n=5). **P*<0.05

12. Notch1 activates Wnt/ β-catenin signaling in HNSCC stem-like cells.

We previously observed that Wnt/β-catenin signaling regulates stem-like characteristics of HNSCC cells.[27] Thus, we investigated whether Wnt/ β-catenin signaling is a downstream effector of Notch1 in HNSCC stem-like cells. Knockdown of notch1 led to decreased protein levels of nuclear β-catenin expression and mRNA levels of target genes of Wnt/ β-catenin pathway, such as Axin-2, c-myc, and LEF1(Fig. 12A and B).. Also, Knockdown of notch1 decreased TOP luciferase activity and nuclear β-catenin expression in xenograft tumor tissue generated from injection of HNSCC CSCs (Fig. 12C and D). Taken together, these results show that Notch1 activates Wnt/ β-catenin signaling in HNSCC stem-like cells.

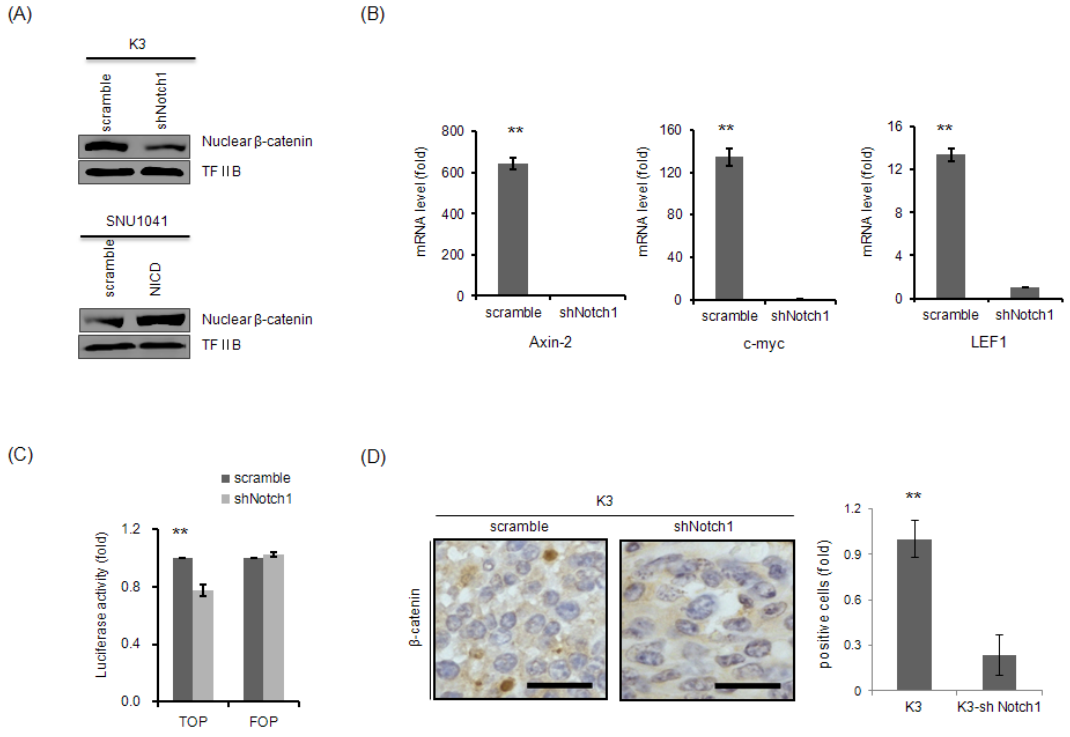
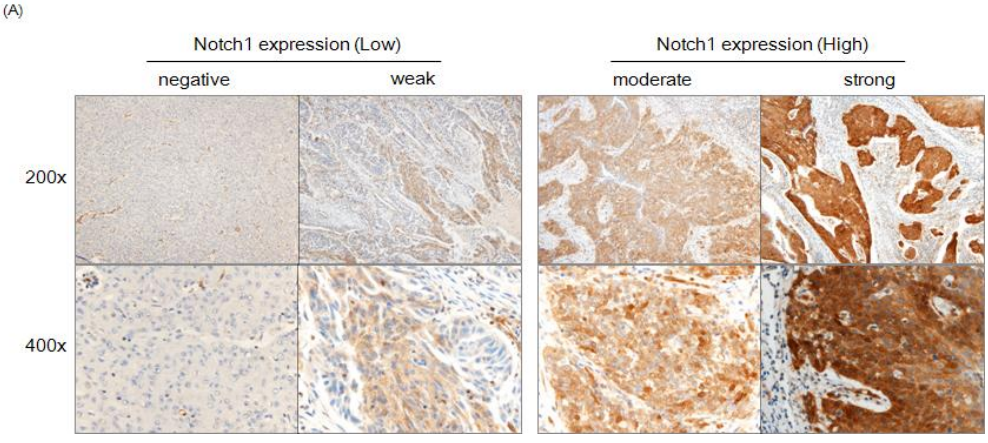


Figure 12. Notch1 activates Wnt/β-catenin signaling in HNSCC stem-like cells.

(a) The protein levels of nuclear β-catenin in HNSCC CSC-shScramble and HNSC CSC-shNotch1 cells. (b) m RNA levels of target genes of Wnt/β-catenin pathway, such as Axin-2, c-myc, and LEF1 in HNSCC CSC-shScramble and HNSC CSC-shNotch1 cells. (c) TOP/FOP luciferase activity in HNSCC CSC-shScramble and HNSC CSC-shNotch1 cells. ***P*<0.05. (d) nuclear β-catenin expression in xenograft tumor tissue generated from injection of HNSCC CSC-shScramble and HNSC CSC-shNotch1 cells. ***P*<0.05

13. Notch1 expression can predict prognosis of patients with HNSC

To explore the predictive value of the expression levels of Notch1 in the prognosis of HNSC patients, we analyzed survival data from 67 patients with HNSC based on Notch1 expression. Clinicopathological features of these patients are summarized in Table 1. Notch1 was poorly expressed in 26 samples and was highly expressed in 41 samples (Fig. 12A). Notch1 expression was significantly correlated with lymph node metastasis and tumor recurrence (p<0.05) (Table 1). Notch1 expression and Kaplan-Meier survival estimates for patients with HNSC are shown in Figure 12B, suggesting that patients with increased expression of Notch1 had a significantly worse prognosis that those with decreased expression (p<0.05). All together, these results suggest that Notch1 expression has a significant effect on the prognosis of patients with HNSCC.



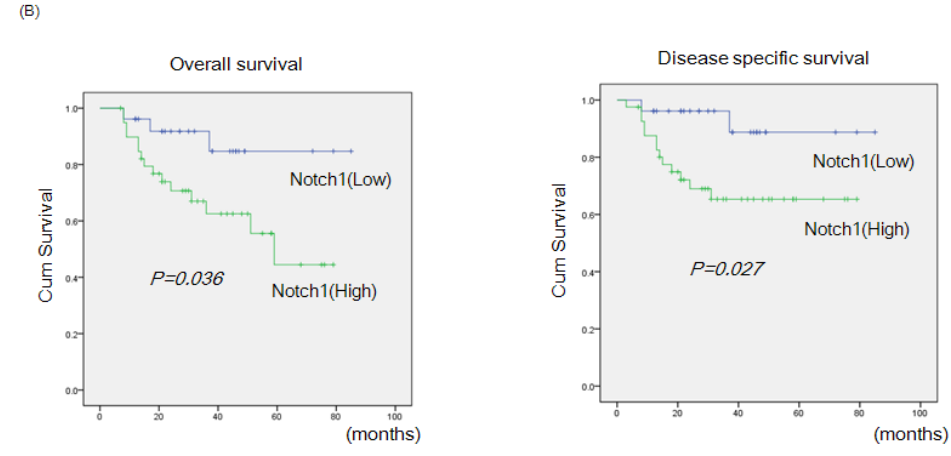


Fig. 13. Notch1 expression level is correlated with survival of patients with HNSCC.

(a) Immunohistochemical evaluation of Notch1 expression in HNSCC tissue. (b) Overall and disease specific survival using Kaplan Meier analysis based on Notch1 expression in patients with HNSCC (n=67).

Table 1. Relationship between Notch1 expression and clinicopathological parameters in patients with HNSCC.

Variables	No. of cases	Notch1 expression		*p*-Value
		Low	High	
(a) Univariate analysis				
Age (years)				0.936
>60	46	18	28	
≤60	21	8	13	
Gender				0.545
Male	54	20	34	
Female	13	6	7	
Pathological T stage				0.506
T1-T2	40	16	24	
T3-T4	27	10	17	
Pathological N stage				0.019
N0	37	19	18	
N+	30	7	23	
Recurrence				0.013
R0	51	24	27	
R+	16	2	14	

Tumor grade				0.147
WD	22	11	11	
MD or PD	45	15	30	
Tumor invasion				
Lymphatic invasion	12	3	9	0.343
Vascular invasion	2	1	1	1.000
Perineural invasion	2	1	1	1.000

(b) Multivariate analysis

Variables	*p*-Value	Odds ratio	95.0 CI Exp(B)	
			Lower	Upper
Pathologic N+	0.030	4.135	1.151	14.857
Recurrence	0.018	7.726	1.426	41.863

IV. DISCUSSION

The CSC hypothesis provides a glamorous molecular mechanism to explain chemotherapeutic resistance exhibited in many types of human malignancies. A clinical consequence of CSCs on tumor initiation, progression, metastasis, and recurrence post-treatments, however, remains largely undefined, particularly in HNSCC. In the present study**,** therefore, we employed a stem cell suspension culture condition to isolate squamospheres – the sphere-forming cells – from primary HNSCCs, as demonstrated previously in other tumors.[28,29]

We have established a number of free-floating spherical colonies in serum-free medium with defined growth factors (EGF and bFGF) from primary HNSCC specimens. However, a success rate of spheroid formation from primary tumors

was only 6% (3 of 47), which are extremely low compared to that seen in other solid tumors. For incidence, most primary brain tumors gave rise to neurospheres in stem cell suspension culture conditions within 4 weeks, which also used in the present study. This discrepancy may be due to a number of technical problems, such as a prolonged harvesting time from tumor removal, poor culture technique, or fungal infection. In fact, we experienced 4 fungal infections in stem cell suspension culture with cells derived from primary HNSCCs, even in the presence of anti-fungal agent (Fungizone). Furthermore, it is known to be difficult to establish general cancer cell lines from primary HNSCCs. For example, a previous report showed that 9 cell lines (11%) from 79 cultures using 65 HNSCC specimens were successfully established.[28] In addition, it is likely that the initial tumor mass used for stem cell suspension cultures is a crucial factor for successful establishment of CSCs, thereby one needs to use sufficient amount of tumor volume for stem cell suspension culture-based establishment of HNSCC-driven CSCs.

There are several hallmarks of CSCs: self-renewal, stem cell marker expression, aberrant differentiation, and tumor-initiating potential.[29] Our studies have demonstrated that HNSCC-driven squamospheres exhibited all the aforementioned CSC criteria: 1) HNSCC-driven squamospheres expressed a number of stem cell markers, such as CK5, OCT4, SOX2, nestin, and CD44; 2) single-dissociated squamosphere cells were able to form new squamospheres within 1 week of reseeding; 3) serum treatment led HNSCC-driven squamospheres to be non-tumorigenic differentiated cancer cells; 4) injection of as few as 100 undifferentiated squamosphere cells in nude mice gave rise to tumor formation.

In the clinical setting, one of the most important characteristics of CSCs is chemoresistance that is also considered to be a major driver factor for tumor recurrence after conventional chemotherapy in many types of human cancers.

For this, our HNSCC-driven squamospheres displayed marked resistance to four chemotherapeutic agents that are commonly used for the treatment of HNSCC patients. Furthermore, consistent to many reports that CSCs, or side population, acquire their chemoresistance by induction of membrane efflux ABC transporters,[31,32] our HNSCC-driven squamospheres exhibited enriched side population cells and increased expression of ABCG2 that is known to be a major culprit for chemoresistance in CSCs derived from many types of human malignancy.[33]

One cell surface marker that can enrich CSC population from HNSCC is CD44, a hyaluronate acid receptor.[34] As such, CD44 expression is considered an important marker for identification of CSCs in HNSCC, because CD44+ cancer cell population in primary HNSCC is comprised of less than 10% of bulk tumor, and can give rise to new tumor in *in vivo* xenotransplantation assay.[14] Similar to this previous report[34], our HNSCC-driven squamospheres also possessed enriched CD44+ cell population (53%), compared to differentiated squamosphere cells.

EGCG, the most abundant and active catechin in green tea, possesses remarkable chemopreventive and anti-cancer effects against various cancers.[18] Furthermore, EGCG can modulate the cell signaling associated with angiogenesis, apoptosis, invasion and metastasis of HNSC.[17] Although these studies have revealed multiple signaling pathways targeted by EGCG, the molecular mechanisms of EGCG anti-cancer effects in CSCs remain largely unknown.

Diverse dietary constituents such as EGCG, quercetin and retinoic acid can modify self-renewal properties of CSCs. EGCG analogs activate the AMP-activated protein kinase pathway and inhibit cell proliferation and sphere formation in breast CSCs.[35] EGCG also inhibit the sphere formation of CSC in neuroblastoma BE(2)-C cells,[36] inhibits the self-renewal capacity of pancreatic

CSC by inhibiting sonic hedgehog pathway and inhibits the expression of EMT markers.[37] In HNSC CSCs, quercetin enhances the inhibition of self-renewal, stemness markers, and migration capability and has been identified as a potent antitumor initiating agent.[3]8 Retinoic acid also suppresses the expression of the stem cell markers and inhibits the proliferation of HNSC CSCs in vitro and in vivo through the suppression of Wnt/β-catenin signaling.[22] However, concrete evidence of EGCG controlling CSC traits has been lacking.

Our study demonstrates that EGCG regulates the self-renewal capacity and chemosensitivity of HNSC CSCs. Specifically, EGCG decreased the expression of stem cell markers Oct4, Sox2, Nanog and CD44. In addition, the combination treatment of EGCG and cisplatin reduced HNSC CSC viability, and as an underlying mechanism, EGCG suppressed the expression of ABCC2 and ABCG2, putatively which have been implicated in the treatment resistance of CSC. Interestingly, EGCG administered concomitantly with cisplatin significantly inhibited tumor formation and induced apoptosis in a xenograft model.

Several therapeutic strategies have been suggested to target CSCs. Inhibiting the key signaling pathways active in CSCs is one of the most promising strategies for treatment of cancer.[39,40] Wnt and Hedgehog signaling pathways are essential to regulate the self-renewal of CSCs and are aberrantly activated in a variety of cancers.[41] Notch signaling plays a critical role in the cellular developmental pathway including proliferation, differentiation and apoptosis.[8] Notch signaling also contributes to cancer progression by activating transcription factors that promote cell survival, motility, and tumor angiogenesis.[24] Emerging lines of evidence have suggested that cancers can grow from CSCs, and the Notch pathway is believed to be deregulated, leading to uncontrolled self-renewal of CSCs that generate tumor mass. Notch may drive tumor growth through generation or expansion of CSC.[25,35] A growing body of literature has suggested the biological importance of Notch

signaling in cancer and CSCs, further suggesting that inactivation of Notch signaling by a novel approach could be useful for cancer therapy.

The Notch gene is abnormally activated in many human malignancies. Upregulated expression of Notch receptors and their ligands in cervical, lung, colon, renal and pancreatic cancers have been repoerted.[42,43] It has been recently demonstrated that Notch1 mutations are frequently found in HNSC, suggesting a potential role for this pathway in the biology of CSCs and in the etiology of head and neck cancer.[44] Zhang et al. reported high expression levels of Notch receptors and ligands in tongue cancer and suggested that Notch signaling may control cell differentiation and proliferation of tongue carcinoma cells.[45] Zeng et al. reported that HNSC may utilize the Notch signaling pathway to promote tumor angiogenesis in vivo and that the level of Jagged1 expression is associated with the development of HNSC.[46] Gu et al. reported that expression of Notch1 is associated with cisplatin resistance in HNSC.[47] In addition, Lin et al. showed high-level coexpression of Jagged-1 and Notch-1 signaling is associated with the worst prognosis in patients with HNSC.[48] Thus, Notch signaling seems to play an important role in carcinogenesis and prognosis of HNSC, However, the possible role and the consequence of the regulation of Notch signaling in HNSC CSCs have never been examined.

In this study, we investigated the role of Notch1 signaling in HNSC and HNSC CSCs. Down-regulation of Notch1 signaling led to attenuation of CSC traits in HNSC CSCs and enhancement of chemosensitization of cisplatin for HNSC CSCs by suppression of ABCC2 and ABCG2 transporter gene expression. Finally, high-level expression of Notch1 was shown to be associated with poor prognosis in patients with HNSC. Collectively, our data suggest that Notch1 signaling, as a major target for HNSC, plays an important role in HNSC and HNSC CSCs.

Current chemotherapeutic agents may target and kill the overall population of differentiated tumor cells, which constitute the bulk of the tumor. Therefore,

CSCs whose proliferation is slower and less abundantly produce drug transporters appear to be relatively drug resistant and so can be spared, ultimately inducing tumor relapse after completion of treatment. The development of new therapeutic approaches targeting CSC has become a major issue in recent cancer biology.[30] With these considerations, our results suggested that the Notch1 pathway may be an attractive target for the treatment of HNSC, because Notch1-targeting will not only kill differentiated cancer cells but could also kill CSCs.

V. CONCLUSION

In summary, we have established a number of primary HNSCC-driven squamospheres that possess the general properties of CSCs, including a self-renewal, stem cell marker expression, aberrant differentiation, tumor-initiating potential, and chemoresistance with increased side population. our results suggested that the Notch1 pathway may be an attractive target for the treatment of HNSC, because Notch1-targeting will not only kill differentiated cancer cells but could also kill CSCs. EGCG attenuates HNSC CSC traits *in vitro* and *in vivo*, and down-regulates the Notch signaling pathway. Further investigations concerning the molecular mechanism of EGCG on HNSC CSCs, based on the present results, could help to develop novel drug combinations capable of eliminating HNSC CSCs.

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ABSTRACT(IN KOREAN)

녹차추출물 epigallocatechin-3-gallate 에 의한 두경부 암 줄기세포의 항암 효과

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임 영 창

두경부 암의 침습 및 치료후 재발에 암 줄기세포의 역할이 점점 중시되고 있는데, 기존의 연구에서 항암작용이 있다고 보고된 녹차추출물 epigallocatechin-3-gallate의 두경부 암 줄기세포에 미치는 영향에 대해서는 보고된 바가 없다. 본 논문에서 epigallocatechin-3-gallate는 두경부 암 줄기세포의 자가 증식능을 감소시키고 Oct4, Sox2, Nanog과 CD44와 같은 줄기성 관련 유전자의 발현을 감소시키며, ABCC2와 ABCG2 유전자 발현의 감소를 통해 cisplatin에 대한 항암제 감수성을 증가 시킨다. In vivo에서 epigallocatechin-3-gallate는 cisplatin과의 병합 요법을 통해 누드마우스에서 두경부 암 줄기세포의 종양 형성능력을 현저히 감소시켰다. 이와 같은 epigallocatechin-3-gallate의 항암 작용 메카니즘으로는 epigallocatechin-3-gallate가 Notch1의 유전자 발현을 감소시켜 두경부 암 줄기세포의 줄기성 억제를 유도하였으며, 임상적 연관성으로 두경부 암 환자에서 Notch1 발현이 증가할수록 유의하게 생존율이 감소하였다. 결론적으로 epigallocatechin-3-gallate는 cisplatin과의 병합요법을 통해 두경부

암 줄기세포를 억제시킬 수 있는 새로운 치료전략으로 제시 될 수 있다

핵심되는 말 : 두경부암, 암 줄기세포, 녹차추출물, Notch1, 치료

PUBLICATION LIST

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