Title
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TNFα enhances cancer stem cell-like phenotype via Notch-Hes1 activation in oral squamous cell carcinoma cells

A thesis submitted in partial satisfaction of the requirement for the degree Master of Science in Oral biology by Sung Hee Lee
ABSTRACT OF THE THESIS

TNFα enhances cancer stem cell-like phenotype via Notch-Hes1 activation in oral squamous cell carcinoma cells

By

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Cancer stem-like cells (CSCs; also known as tumor initiating cells) is defined as a small subpopulation of cancer cells within a tumor and isolated from various primary tumors and cancer cell lines. CSCs are highly tumorigenic and resistant to anticancer treatments. In this study, we found that chronic exposure to tumor necrosis factor alpha (TNFα), a major proinflammatory cytokine, enhances CSC phenotype of oral squamous cell carcinoma (OSCC) cells, such as an increase in tumor sphere-forming ability, stem cell-associated genes expression, chemo-radioreistance, and tumorigenicity. Moreover, activation of Notch1 signaling was detected in the TNFα-exposed cells, and suppression of Notch1 signaling inhibited CSC phenotype. Furthermore, we demonstrated that inhibition of a Notch downstream target, Hes-1,
led to suppression of CSC phenotype in the TNFα-exposed cells. We also found that Hes1 expression is commonly upregulated in OSCC lesions compared to precancerous dysplastic lesions, suggesting the possible involvement of Hes1 in OSCC progression and CSC in vivo. In conclusion, inflammatory cytokine exposure may enhance CSC phenotype of OSCC, in part by activating the Notch-Hes1 pathway.
The Thesis of Sung Hee Lee is approved

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2012
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1. Introduction

Oral Squamous Cell Carcinoma (OSCC) is an important problem not only because of the significant mortality associated with the disease, but also because of the functional defects and disfigurement often associated with its treatment [1, 2]. Like other cancers, the development of OSCC is a multistep process with accumulation of genetic and epigenetic changes [3]. The risk factors for OSCC include tobacco smoking, alcohol exposure, chronic inflammation, and virus infection. Along with surgical approaches and conventional therapies such as radiotherapy and chemotherapy, molecular targeted therapies is another promising strategy to improve the disease control that emphasizes the understanding of molecular pathogenesis of the disease as well as further characterization of the specific molecular events involved in growth, invasion, and metastasis of OSCC [4].

Recent studies have uncovered and validated the pathophysiologic role of self-renewing cells, namely cancer stem-like cells (CSCs; also called tumor-initiating cells), in long-term sustenance of cancers [5]. Many molecular determinants of normal stem cells, such as self-renewal ability and multi-lineage differentiation capacity, are retained in CSCs [6]. CSC may arise from transformation of normal stem cells, restricted progenitors or more differentiated cells that have acquired self-renewing capacity [7]. CSCs have been isolated from various primary tumors and established cancer cell lines via cell surface markers, and they typically have the following properties: high tumorigenicity upon injection in immunodeficient mice, the ability to grow as tumor spheres in undifferentiating medium and resistance to cancer therapeutic agents [8]. Similarly, the existence of CSCs has been demonstrated in primary OSCC and cell lines [9]. CSC has a role in retaining tumor heterogeneity, and it is explained by two universally accepted CSC models. First, the stochastic model describes that tumors are homogeneous and random
stochastic influences such as a change in microenvironment or signaling pathways may cause individual cells in tumor to behave like cancer stem cell. On the other hand, hierarchy model argues that tumors are caricatures of normal tissues which are cellular hierarchies maintained by stem cells, and cellular heterogeneity is due to the cells at the apex of hierarchy that can self-renew and produce progeny that do not have cancer initiating capacity [10]. Therefore, CSCs drive the perpetuity of the disease while producing cellular heterogeneity of cancer tissues, and are becoming new targets of anti-cancer therapies [11].

The phenotypes of CSC have been reported to be maintained by several endogenous signaling pathways, such as Notch, Hedgehog, and Wnt [12-14]. Those paracrine signals by Wnt and Hedgehog pathway and signals transmitted by cell-cell contact such as Notch stresses the importance of microenvironment in determining the biological function of CSCs [15]. Activation of those pathways is frequently found in human cancers [16-18]. Especially, as Notch signaling has been implicated in a growing number of hematopoietic and solid tumors, its inappropriate activation stimulates proliferation, restricts differentiation and/or prevents apoptosis [15]. General mechanism of Notch signaling requires binding of its ligands, Jagged and Delta-like (DLL), followed by proteolytic release of the Notch intracellular domain (NICD) and its translocation to the Nucleus. NICD interacts with transcription factors and promotes transcription of downstream genes involved in various different programs [19, 39].

Among various factors, exogenous carcinogenic factors also contribute to alteration in stem cell pathways, thereby enriching CSC in cancer. For instance, nicotine enhanced CSC population in human breast cancer [20]. Furthermore, recent studies demonstrated that proinflammatory
cytokines, TGFβ and TNFα, generated CSC in breast cancer, suggesting a possible link between CSC and inflammation [21, 22].

Inflammation acts as host defense mechanism against infection or injury, but unresolved inflammatory responses results in chronic inflammation. There is increasing evidence of chronic inflammation-associated tumorigenesis [23]. Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [24]. Although the molecular and cellular mechanisms linking chronic inflammation to tumorigenesis have not been fully understood, TNFα, a major mediator of inflammation, is known to play a crucial role in the inflammation-associated cancer development. Disruption of the TNFα signaling pathway could significantly inhibit chemical induced-carcinogenesis in skin [25, 26]. Many studies suggested that TNFα promotes inflammation-associated tumorigenesis by activating the nuclear factor-κB (NF-κB) signaling [27, 28], which inhibits the death of precancerous or transformed cells during the development of inflammation-associated cancers [29-31]. In addition, TNFα has been shown to be a potential mutagen that causes DNA damage through the induction of reactive oxygen species [32].

In the present study, we investigated the effect of TNFα on CSC property in OSCC. Our study revealed that chronic exposure to TNFα enhanced CSC property, i.e., tumor sphere-forming ability, stem cell-associated genes expression, and chemo-radioreistance, in OSCC cells. Moreover, the TNFα-exposed cells with elevated CSC phenotype showed higher tumorigenic potential than the control cells. Finally, our study also demonstrated that the
enriched CSC phenotype in the TNFα-exposed OSCC cells was attributed to activation of Notch-Hes1 pathway.

2. Materials and Methods

2.1 Cell Culture and chronic exposure of cells to TNFα

The SCC-4, SCC-9, and HEP2 cancer cell lines were purchased from the American Type Culture Collection (ATCC). SCC-4 and SCC-9 were cultured in DMEM/Ham's F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gemini Bioproducts, Woodland, CA) and 0.4 μg/ml hydrocortisone (Sigma, St. Louis, MO), while HEP2 was grown in MEM (Invitrogen) supplemented with 10% FBS. The cell lines were treated with 5ng/ml of TNFα (Sigma-Aldrich, St. Louis, MO) for extended periods. All cells were incubated at 36.8 °C with 5% carbon dioxide.

2.2 Tumor sphere formation assay

The cells were grown in DMEM/F12 media with 1:50 B27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL, 10μg/mL insulin, penicillin, streptomycin, and amphotericin B in 6-well Ultralow Cluster Plates (Fisher) at a density of 1,000 cells per well. Additional 0.5ml of media was added every 5 days for 15 days. The number of tumor spheres formed were observed and counted under a microscope. Assay was done in triplicates for each tested cell type and average number of sphere was calculated.

2.3 Quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and quantity and quality of RNA was measured using NanoDrop Spectrophotometer (Thermo Fisher Scientific). cDNA was
synthesized from 5 µg of total RNA using SuperScript first-strand synthesis system (Invitrogen). We used 1 µl cDNA for qPCR amplification using SYBR Green I Master mix (Roche). The primer sequences were obtained from the Universal Probe Library (Roche), and the sequences can be available upon request. PCR amplification was performed on LightCycler 480 II (Roche Diagnostic Ltd, Rotkreuz, Switzerland). The reactions were performed by heat denaturing at 95 °C for 10 minutes, followed by 55 cycles of 95 °C for 10 seconds, 58 °C for 45 seconds and 72 °C for 10 seconds. PCR amplification of housekeeping gene GAPDH was used as internal control, and second derivative Cq value determination method was used to compare fold-differences according to the manufacturer’s instructions (Roche).

2.4 Chemo-radioresistance assays

Etoposide was purchased from Sigma-Aldrich. Chemoresistance of cells was determined by measuring cell viability using the tetrazolium salt (MTT) cell proliferation assay kit (ATCC, Manassas, VA). The cells were plated at 2 x 10³ cells per well into 96-well plate and incubated in culture medium containing the drug at 37°C for 2, 4, and 6 days. Absorbance at 570 nm was determined using a microplate reader ELx 800 (BIO-TEK Instrument).

For radioresistance assay, the cells were exposed to ionizing radiation (IR) at varying doses using the Mark I-30 Cesium-137 irradiator (JL Shepherd & Assoc., San Fernando, CA) with the delivery rate of 4.86 Gy/min. Cells were exposed to varying dose of IR from 0 to 10 Gy and seeded in 6-well plates at low density (200 cells per well) after 24 hrs post-IR. The cells were maintained in culture for 10 additional days to allow colony formation. Visible colonies consisting of at least 50 cells were stained with Giemsa stain (Sigma) for 90 minutes and counted.
2.5 Anchorage-independent growth in soft agar

To determine colony-forming efficiency in semi-solid medium, 1 x 10⁴ cells were seeded in culture medium containing 0.3% agarose over a base layer of serum-free medium containing 0.5% agarose. Three weeks after incubation, colonies were counted. The experiment was performed in triplicates with 60-mm dishes.

2.6 Western blotting

Whole cell extracts (WCEs) from the cultured cells were isolated using the lysis buffer (1% Triton X-100, 20 mM Tris –HCl pH 7.5, 150 mM NaCl, 1mM EDTA, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1 µM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mg/ml PMSF). The WCEs were then fractionated by SDS-PAGE and transferred to Immobilon protein membrane (Millipor, Billerica, MA). After membranes were blocked in 5% nonfat milk (Bio Rad) and incubated successively with the primary and the secondary antibodies, they were exposed to the chemiluminescence reagent (Amersham, Buckinghamshire, England) for signal detection. We used the following primary antibodies for this study: NICD (Val 1744; Cell signaling Technology, Danvers, MA), Hes1 (H-20; Santa Cruz Biotech.), and GAPDH (Santa Cruz Biotech.). Horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotech.

2.7 Organotypic raft cultures

OSCC cells were grown as organotypic raft cultures using techniques established previously [33] with slight modification. Briefly, 10⁶ cells were seeded on the submucosal equivalents consisting of type I collagen and normal human oral fibroblasts. The cells were grown to
confluence, submerged in the culture medium, and then exposed to the liquid-air interface by lowering the medium level. The medium contained DMEM/F12, Insulin (5 µg/ml), Hydrocortisone (0.4 µg/ml), Triiodo-thyronine (0.02 nM), Adenine (0.18 mM), Transferrine (5 µg/ml), Cholera toxin (0.1 nM), L-Glutamine (2 mM), 5% FBS, and Gentamycin (5 µg/ml). The cultures were maintained in this “rafting” fashion for 14 days and were harvested by fixing in 10% buffered formalin. Subsequently, hematoxylin-eosin (H&E) staining was performed on thick (6 μm) sagittal sections of each reconstructs to reveal the histological features. Sample processing, paraffin-embedding, sectioning, and H&E staining were performed at the UCLA’s Translational Pathology Core Laboratory (TPCL).

2.8 Determination of tumorigenicity in vivo

Four million OSCC cells were were subcutaneously injected into the flank of immunocompromised mice (strain nu/nu, Charles River Laboratories). The animal study was performed according to the protocol approved by the UCLA Animal Research Committee. The kinetics of tumor growth was determined by measuring the volume in three perpendicular axes of the nodules using micro-scaled calipers. The efficiency of tumor formation per each tested cell type was determined by the number of the mice (out of the total number of mice injected) bearing palpable tumors exceeding after 6 weeks post-injection.

2.9 Knock down of active Notch1 protein (NICD) by γ-Secretase inhibitor

In order to verify the importance of Notch activation in CSC property of SCC9/TNF, endogenous active Notch1 protein (NICD) expression was inhibited using γ-Secretase inhibitor X (Calbiochem). SCC9/TNF cells were pretreated with 10µM of γ-Secretase inhibitor X for three days prior to a Sphere Formation Assay.
2.10 Knockdown of endogenous Hes1 by siRNA

Endogenous Hes1 expression was knocked down with duplex siRNA targeting Hes1 or the control, scrambled siRNA (Santa Cruz), which was introduced using Lipofectamine RNAimax (Invitrogen). SCC9/TNF cells (2 x 10⁵) were plated in 60-mm dishes and transfected with 15 µg siRNA. Hes1 or the control was mixed with Lipofectamin RNAimax and culture medium and added to the cells. The cultures were harvested after three days post-transfection for biochemical analyses.

3. Results

3.1 Chronic TNFα exposure increases CSC property in OSCC

In order to investigate the effect of TNFα on CSC phenotype of OSCC, we treated three OSCC cell lines (SCC4, SCC9, and HEp2) with the cytokine (5-10ng/ml) for extended periods and examined its effects on undifferentiated tumor sphere formation, which is considered as CSC population and property [34]. After 2 months of exposure to TNFα, all tested cell lines showed enhanced tumor sphere-forming ability (Fig. 1A). Increased tumor sphere formation was first evident after the 2 month exposure. Importantly, it should be noted that after the 2 month exposure, we withdrew TNFα from culture medium and performed all the experiments in the absence of TNFα. In doing so, we may exclude the immediate effect of TNFα on biological behaviors of tested cell lines. Interestingly, chronic TNFα induced a robust increase in tumor sphere-forming capacity of SCC9. SCC9 displayed negligible or no tumor sphere-forming ability, whereas the TNFα-exposed SCC9 (SCC9/TNF) acquired significant sphere-forming capacity even compared to SCC4 and HEp2 (Fig. 1A and 1B). Because of these results, further experiments were focused using SCC9/TNF compared to SCC9.
Quantitative PCR pathway analysis showed enriched expression of stem cell-associated genes, including cyclin D2 (CCND2), epithelial cell adhesion molecule (EPCAM), lin-28 homolog B (Lin28B), CD44, Sox2, Myc, aldehyde dehydrogenase 1 (ALDH1) in the SCC9/TNF cells (Fig. 1C). An important characteristic of CSCs is their resistance to chemo-radiotherapy [29]. Thus, we treated SCC9 and SCC9/TNF with etoposide and performed an MTT assay. The assay revealed that SCC9/TNF cells were more resistant to the drug than control SCC9 cells (Fig. 1D left). We also performed sensitivity assays with ionizing radiation (IR) on SCC9 and SCC9/TNF. The SCC9/TNF cells displayed increased radioresistance than control cells (Fig. 1D right). These results demonstrated that chronic TNFα treatment enriched CSC population and property in SCC9.

3.2 Chronic TNFα exposure further enhances tumorigenic potential of OSCC in vitro and in vivo

Hallmarks of CSC include high tumorigenic potential. Therefore, we examined whether chronic TNFα enhances anchorage independent growth ability of SCC9 by performing a soft agar assay. The assay revealed that SCC9/TNF showed greatly increased anchorage independent growth ability compared to SCC9 (Fig. 2A). Also, SCC9/TNF formed bigger cell colonies than the control SCC9 cells (Fig. 2A). Consistent with previous report, SCC9 showed extremely low anchorage independent growth ability [36]. We also employed an in vivo-like 3D organotypic cell culture system. To recapitulate the tissue microenvironment, SCC9 and SCC9/TNF were overlaid on an extracellular matrix gel mixed with normal human oral fibroblasts, and after 14 days, a liquid-air interface was created, thereby leading to stratification of the squamous epithelium, which resembles the human squamous epithelium [37, 38]. As shown in Figure 2B,
organotypic culture of SCC9/TNF demonstrated malignant histomorphology with invasive characteristics into subepithelial layer and increased epithelial thickness, and mitotic cells compared to organotypic culture of SCC9. Furthermore, we evaluated the tumorigenicity of SCC9 and SCC9/TNF in vivo. We injected SCC9 and SCC9/TNF into nude mice and monitored tumor formation over six weeks. SCC9/TNF developed tumor in 2 of 5 mice, whereas SCC9 failed to form tumor in tested animals (Fig. 2C). Altogether, our data indicate that chronic TNFα enhances tumorigenicity of SCC9.

3.3 Chronic TNFα exposure activates Notch1 signaling pathway

Activation of Notch1 signaling pathway is critical for the maintenance of CSC and requires binding of its ligands, Jagged 1 (JAG1), JAG 2 and Delta-like (DLL), followed by proteolytic release of the Notch intracellular domain (NICD) and the activation of NICD downstream target genes [19, 39]. Since our data showed increased expression of Notch ligands, i.e., DLL1, JAG1, and JAG2 (Fig. 1C) in the TNFα-exposed cells, we explored activation status of Notch1 pathway in SCC9/TNF by examining the expression of NICD, an activated form of Notch protein. Western blot revealed that the expression of NICD was significantly elevated in SCC9/TNF compared to SCC9 (Fig. 3A). Furthermore, various NICD downstream target genes, including Hes1 (hairy and enhancer of split 1), were upregulated in SCC9/TNF compared to the control SCC9 cells (Fig. 3A and 3B). These findings clearly indicate the activation of Notch1 signaling pathway by chronic TNFα exposure in SCC9.

3.4 Effect of Notch-Hes1 pathway on CSC property in the TNFα-exposed cells

To investigate the role of Notch1 signaling pathway in the CSC property of SCC9/TNF, we examined the effect of a pharmacological Notch1 inhibitor (γ-secretase inhibitor, DAPT) on
tumor sphere-forming ability of SCC9/TNF. Because 10 µM of DAPT has no effect on SCC9/TNF cell growth (data not shown), we treated the cells with the concentration and measured NICD expression. DAPT had significantly reduced NICD expression compared to control DMSO treatment (Fig. 4A). Similarly, DAPT also downregulated the NICD downstream target Hes1 (Fig. 4A). We then assessed tumor sphere formation under DAPT treatment. The assay revealed that DAPT treatment significantly suppressed tumor sphere-forming ability of SCC9/TNF (Fig. 4B and 4C). This finding indicates that Notch1 activation is essential for tumor sphere-forming ability of SCC9/TNF.

To further understand the role of Hes1 in the CSC property, we suppressed the expression of endogenous Hes1 in the SCC9/TNF cells by transfecting them with siRNA against Hes1 (Fig. 5A). Then, we compared the sphere-forming ability of the cells transfected with control siRNA (CTLi) or Hes1 siRNA (Hes1i). The assay showed that the Hes1i-transfected SCC9/TNF cells formed significantly smaller number of tumor spheres compared to the CTLi-transfected cells (Fig. 5B and 5C). These data indicate that Hes1 is also important regulator for tumor sphere-forming capacity of SCC9/TNF, suggesting that chronic TNFα treatment increases the CSC property via a Notch-Hes1 pathway.

3.5 Expression of Hes1 in dysplastic and OSCC lesions

To further investigate the importance of Hes1 in carcinogenesis and CSC in vivo, we determined the level of Hes1 expression in laser capture microdissected dysplastic and OSCC lesions. We speculate that CSC population is enriched in OSCC lesion compared to dysplastic lesion. Quantitative PCR revealed that Hes1 is commonly upregulated in OSCC compared to precancerous dysplastic lesions (Fig. 6). Although further analysis with a larger sample size is
required, this finding suggests that Hes1 may be associated with the progression of OSCC and the enrichment of CSC population in vivo.

4. Discussion

In this study, we report that chronic exposure of OSCC cells to proinflammatory cytokine TNFα enhances CSC phenotype and tumorigenicity. Chronic TNFα exposure increased the CSC properties of OSCC, such as 1) tumorigenic potential, 2) tumor sphere-forming ability, 3) expression of stem cell-associated genes, and 4) chemo-radioresistance. Subsequently, our study found activation of Notch-Hes1 pathway in the TNFα-exposed OSCC cells. Suppression of Notch-Hes1 pathway inhibited the CSC property of the TNFα-exposed OSCC cells.

TNFα is a major mediator of inflammation. TNFα is not only widely distributed throughout the body in normal physiologic conditions, but it is involved in many pathologic processes including inflammation and carcinogenesis [40]. Since the association of chronic inflammation and cancer is well established [41, 42], the role of TNFα in carcinogenesis is well recognized. Indeed, many studies have reported the tumor-promoting effects of TNFα. First, TNFα activated oncogenic pathways [43]. Second, overexpression of TNFα increased malignant behavior of many tumor cell lines [44]. Third, TNFα and TNFα receptor 1 (TNFR1) knock-out mice are resistant to chemical induced skin carcinogenesis [35, 45]. Fourth, TNFα elevated chromosomal instability by virtue of its ability to induce ROS [46]. Consistent with those observations, chronic TNFα treatment further elevated malignant phenotypes of OSCC, i.e., anchorage independent growth ability and in vivo tumorigenic potential. We also obtained similar result that chronic TNFα exposure further elevated malignant behavior of non-tumorigenic immortalized oral keratinocytes (unpublished data). Our results clearly suggest that chronic
TNFα exposure mimicking chronic inflammatory microenvironment is an important carcinogenic factor for oral carcinogenesis.

CSCs are considered the seed of cancer for their crucial roles in malignant behavior of cancer cells, i.e., migration/invasion, resistance to anticancer therapies, anchorage independent growth ability and in vitro tumorigenic potential. However, the effect of chronic inflammation on CSC remains largely unclear. Our study show that the chronic exposure of OSCC cells to TNFα enhances CSC properties. This is important evidence supporting the role of chronic inflammation in OSCC. Our finding is consistent with a recent reports demonstrating that inflammatory cytokines, TGFβ and TNFα, increased CSC population and phenotypes in breast cancer cells [21, 22]. Furthermore, we documented that chronic TNFα treatment results in activation of Notch1 signaling pathway, a critical CSC maintaining pathway [45]. Our data demonstrated that inhibition of Notch1 pathway by DAPT suppressed tumor sphere-forming capacity of the SCC9/TNF cells with concomitant reduction of Notch1 downstream target Hes1, suggesting the importance of Notch-Hes1 pathway in the TNFα-induced CSC property in SCC9 cells. Hes1 was significantly increased in the SCC9/TNF cells, and repressed by the Notch1 inhibitor in the same cells, indicating a direct regulation of Hes1 by activated Notch1 in our cell model. In the present study, the knockdown of endogenous Hes1 in SCC9/TNF significantly inhibits tumor sphere-forming capacity, further confirming the significance of Notch-Hes1 pathway in the CSC phenotype. It is interesting to note that the effect of Hes1 knockdown on tumor sphere formation was smaller than that of Notch1 inhibition (75% vs. 36% reduction). This observation suggests the presence of Notch-Hes1 independent pathway/mechanism in the TNFα-induced CSC property in SCC9.
There is increasing evidence of the importance of Hes1 in the maintenance of progenitor cell fate. For instance, Hes1 is commonly expressed in most undifferentiated cell types in the developing mouse embryo. Moreover, Hes1 deficient mice displayed premature differentiation, progenitor cell depletion, and a consequent lethality [47]. Many studies also demonstrated the implication of Hes1 in tumorigenesis. In breast and pancreatic endocrine tumors, Hes1 was commonly repressed, whereas Hes1 is increased in osteosarcomas [48-51]. These conflicting reports suggest that Hes1 may have dual role as a tumor suppressor or an oncogene depending on the tumor types and the stages of cancer progression. However, the role of Hes1 in oral carcinogenesis has not been well documented. Our study reports for the first time that Hes1 is overexpressed in the TNFα-exposed OSCC cells with increased CSC property. Although the sample size was limited, Hes1 is greatly increased in OSCC lesions compared to precancerous lesions. Our data clearly suggest that Hes1 may be associated with cancer progression and CSC phenotype in OSCC. This also suggests the possible oncogenic role of Hes1 in oral carcinogenesis. Thus, Hes1 could be considered for a therapeutic target for CSCs in OSCC.

In conclusion, this study provides novel information of the role of chronic cytokine exposure in OSCC progression. Our results reveal a molecular mechanism that TNFα, a major proinflammatory cytokine, enhances OSCC stem cells-like phenotype, which is associated with activation of Notch-Hes1 pathway. Therefore, we speculate that the challenge of OSCC cells with chronic inflammation may further increase CSC population/phenotype and allow CSCs to subvert immune-mediated elimination, thereby permitting long term survival, which would promote malignancy of OSCC.
**Figure Legends and Figures**

**Figure 1. Chronic TNFα exposure enhanced CSC phenotype in OSCC.** Three OSCC cell lines (SCC4, SCC9, and HEP2) were exposed to 5-10ng/ml of TNFα for 2 months. (A) Tumor sphere formation assay. Five hundred cells were plated in ultralow attachment plates in serum-free tumor sphere-forming medium. Tumor spheres were counted on day 14. (B) Representative image of tumor spheres formed by SCC9 and TNFα-exposed SCC9 (SCC9/TNF). The photographs were taken at a magnification of 40X. (C) qPCR analysis of stemness-associated genes in SCC9 and SCC9/TNF. Levels of the stemness-associated genes were normalized with the level of GAPDH. (D) Chemo-radioresensitivity assay. For cell viability assay (left), 500 cells were seeded in 96-well plates and treated with 40µM of Etoposide. At each incubation period, cell viability was measured using MTT assay. For clonogenic survival assay (right), 200 cells were seeded in 6-well plates and irradiated with different doses. After 10 days, surviving colonies were stained and counted.
Figure 2. Chronic TNFα exposure increased tumorigenic potential of SCC9. (A) Anchorage independent growth assay. Five thousand cells were seeded on 0.4% soft agar and incubated for 3wks. Colonies were counted, and the photographs were taken at a magnification of 40X. (B) Ex vivo three dimensional organotypic raft cultures. Organotypic “raft” cultures were established with SCC9 and SCC9/TNF. After 14 days of air-lifting, the cultures were harvested for histological examination in sagittal orientation. (C) In vivo tumorigenic assay. SCC9 and SCC9/TNF were injected subcutaneously into five nude mice. The kinetics of tumor growth was determined by measuring the volume in three perpendicular axes of the nodules using micro-scaled calipers.
Figure 3. Notch1 signaling pathway was activated in the TNFα-exposed SCC9 cells. (A) Western blot analysis of the activated form of Notch1 (NICD) and NICD target Hes1 in SCC9 and SCC9/TNF. GAPDH was used as a loading control. (B) qPCR analysis of NICD target genes in SCC9 and SCC9/TNF. Levels of genes were normalized with the expression of GAPDH.
A

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B

mRNA level in SCC9/TNF (Fold induction against SCC9)

- SOX9
- HES1
- CCND1
- RUNX1
- HEV1
- FOS
- ID1
- MYC
- FOSL1

Bars represent the fold induction of each gene in SCC9/TNF compared to SCC9, with error bars indicating variability.
Figure 4. Suppression of Notch1 inhibited tumor sphere-forming capacity of SCC9/TNF.

(A) Synthetic γ-secretase inhibitor, DAPT (5μM) abrogated the expression of NICD and Hes1 protein in SCC9/TNF as determined by Western blot analysis. The cells treated with DMSO were included for comparison. (B) Inhibition of Notch 1 pathway reduced tumor sphere-forming ability of SCC9/TNF. The SCC9/TNF cells were treated with DAPT for 2 days and subjected to tumor sphere-forming assay. One thousand cells were plated in ultralow attachment plates in serum-free tumor sphere-forming medium. Tumor spheres were counted on 10 day. (C) Tumor spheres were photographed at a magnification of 40X.
Figure 5. Inactivation of Hes1 repressed tumor sphere-forming capacity of SCC9/TNF. (A) Endogenous Hes1 was knocked down in SCC9/TNF using siRNA against Hes1 (Hes1i). The cells transfected with control siRNA (CTLi) were included for comparison. (B) Inhibition of Hes1 reduced tumor sphere-forming ability of SCC9/TNF. One thousand cells were plated in ultralow attachment plates in serum-free tumor sphere-forming medium. Tumor spheres were counted on 10 day. (C) Tumor spheres were photographed at a magnification of 40X.
Figure 6. Differential expression of Hes1 in dysplastic and OSCC tissue. Dysplasia and OSCC tissue were dissected by Leica laser capture microdissection LMD7000 and were deparaffinized to isolate RNA. After synthesis of cDNA, the level of Hes1 mRNA was quantified using qPCR and normalized with the GAPDH expression. Higher gene expression of Hes1 was found in OSCC tissue compared to dysplasia.
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