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# Crosstalk between Raf-MEK-ERK and PI3K-Akt-GSK3β signaling networks promotes chemoresistance, invasion/migration and stemness via expression of CD44 variants (v4 and v6) in oral cancer

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ABSTRACT

*Background:* The cell-surface glycoprotein CD44 is an important oral cancer stem cell (OCSC) marker and plays significant role in oral squamous cell carcinoma (OSCC) aggressiveness, however, the regulation of CD44 is incompletely understood.

*Methods:* In the present study, 145 fresh human OSCC tissue specimens, including 18 adjacent normal, 42 noninvasive (N0), 53 invasive tumor samples ($N_{1\text{-}3}$) and 32 chemo-radiation resistant samples (RCRT), were included. The expression of CD44 standard (CD44s) and variants (CD44v4, CD44v6); the activation of pERK1/2, GSK3β, NICD (Notch) pathways; the cell viability; and the MMP-9/-2 activity were assessed using RT-PCR, immunohistochemistry, Western blotting, MTT assay and gelatin zymography. OSCC cell lines, including parental (SCC9/SCC4) and Cisplatin-resistant (CisR-SCC9/-SCC4) cells, were used. Knock down of CD44v4/CD44v6 (by siRNA) or inactivation of MAPK/PI3K pathways using specific PD98059/LY294002 was achieved for *in vitro* analysis of chemoresistance and invasion/migration.

*Results:* Elevated CD44 variants were associated with overall OSCC progression, chemoresistance and invasion. Positive correlations were observed, mainly between the expression of CD44v4 and the activation of ERK1/2 causing chemoresistance, whereas CD44v6 expression and inactivation of GSK3β caused invasiveness of OSCC. Cisplatin resistant, CisR-SCC9/SCC4 cell lines showed OCSC properties. Inhibition of MEK/ERK1/2 by SMI or knock down (KD) of CD44v4 by siRNA reversed cisplatin-resistance, whereas blocking the PI3K/Akt/GSK3β pathway by SMI or KD of CD44v6 isoforms by respective siRNA diminished invasion/metastasis potential.

*Conclusion:* Collectively, our results demonstrated that CD44v4 expression is more linked with ERK1/2 activation and promote cisplatin resistance, whereas CD44v6 expression is associated primarily with PI3K/Akt/GSK3β activation and driving tumor invasion/migration.

## Introduction

Oral squamous cell carcinoma (OSCC) is one of the most prevalent oral cancers. With a number of new cases worldwide annually, OSCC is the sixth most common human malignancy [1]. OSCCs are well known for their late stage diagnosis, rapid growth, resistance to adjuvant chemo-radiation therapy, and frequent lymph node invasion followed by regional metastases and poor prognosis. A number of biomarkers of oral premalignant epithelial lesions have been reported [2] for clinical application, but the severity of OSCC remains the same because of the initial asymptomatic nature of the neoplasm. Surgery is not always possible, and chemo-radiation therapy often leads to resistant tumors with local recurrence [3]. These OSCC cells invade nearby lymph nodes, leading to regional metastases [4,5]. Current understanding in oral cancer cells suggests there are a small subgroup of cells known as oral cancer stem cells (OCSCs); residing in the pre/malignant oral tumor. These cells contribute to tumor initiation, promote resistance to chemo-radiation treatment, support invasion/metastasis, and cause

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cancer recurrence [6]. Cluster of differentiation 44 (CD44) was first used as a surface marker to isolate cancer stem cells (CSCs) in head and neck cancer (HNSCC) [7] and has been widely studied as a stemness biomarker in OSCC [6,8].

Recent studies showed that CD44 is a promising target for cancer treatment. CD44 is known as a promising prognostic indicator in several solid tumors, including malignant glioma [9], breast cancer [10,11], lung cancer [12], prostate cancer [13], ovarian cancer [14] and HNSCC and OSCC [15–18]. CD44 is located on chromosome 11 and is a highly conserved gene in humans; it spans ∼50 kilobases and, encodes a group of proteins ranging from 85 to 200 KDa in size [19,20] due to alternative splicing. The CD44 gene contains 20 exons, and exons 1–5 and 16–20 are constant, giving CD44 standard (CD44s; 85 KDa); while, variant exons 6–15 (v1–v10) are present in different CD44v1-v10 isoforms (CD44Vs) [21,22]. The expression of CD44Vs is regulated by tissue and environment-specific factors, miRNAs, RNA binding proteins, etc. and is regulated by several oncogenic pathways [23]. Among many CD44Vs, several studies confirmed the abundance of CD44v4 and CD44v6 in OSCC [8,24]. Recent studies have demonstrated elevated levels of CD44 expression in the progression, invasion and resistance of OSCC and a correlation between the expression of exon v4 and exon v6 with poor prognosis. However, differential levels of CD44 variant expression persists in the literature, and there is still ambiguity regarding the function and role of CD44s/v4/v6 isoforms as to whether they are tumor-promoting or -suppressing factors in OSCC.

CD44 has been shown to activate a number of central signaling pathways, including the Ras-MAPK and the PI3K/Akt pathways [19,25]. CD44 forms complex networks with PI3K-Akt, MAPK (extracellular signal-regulated protein kinase 1 and 2; ERK1/2), matrix metalloproteinase, and activated protein 1 (AP1) to promote tumorigenesis [26]. Regulation of alternate splicing of CD44 by the MEK-ERK pathway has been reported [23]. The activation of MAPK (ERK1/2) is a well-established cancer promoting factor [27], and this signaling axis is linked to the progression and chemoresistance of OSCC [3]. The central role of PI3K-Akt-GSK3β has been reported in OSCC progression and invasion [28,29]. Notch and Wnt-GSK3β pathways are important signaling molecules and have been shown to be involved in CSCs in various solid tumors [30]. In addition, CD44 serves as a docking site for matrix metalloproteases (MMP), which are matrix-modifying enzymes that degrade the basement membrane and promote cell migration [4]. CD44 promotes the docking of collagen-specific MMP9, whose localization to the leading edge of migrating cells promotes collagen degradation and invasion [31].

Considering the importance of CD44 in OCSCs, its contribution to the severity of OSCC, such as chemoresistance and invasiveness, has been reported. However the detail roles of CD44, particularly the roles of CD44 variants (CD44v4 and CD44v6), in connection with the signaling pathways that can influence the biology of OCSC is not fully understood. We analyzed, fresh human OSCC tissue specimens, including adjacent normal, noninvasive primary oral tumor samples, invasive oral tumor samples and chemo-radiation-resistant samples, to determine the expression of CD44 standard (CD44s) and variants (CD44v4, CD44v6), as well as the activation of pERK1/2, GSK3β and NICD(Notch) pathways. Oral tongue SCC-derived cell lines, such as CisR-SCC9/-SCC4, that showed cancer stemness characteristics, such as drug resistance and invasiveness properties, were used as a model to investigate the effect of CD44v4 and CD44v6 expression levels (knock down by siRNA) or blocking signaling pathways (MAPK/PI3K with PD98059/LY294002) to determine functions of CD44 in OCSCs. We demonstrate that CD44v4 expression is more related with ERK1/2 activation and confers chemoresistance in OSCC, whereas CD44v6 expression is associated primarily with PI3K/Akt/GSK3β activation and drives oral tumor invasion/migration.

## Materials and methods

### Patients and tissue samples

A total of 145 tissue samples obtained from OSCC patients were used for various experiments. These included 18 adjacent normal (AN) tumor samples, 42 pretreatment tumor biopsy samples (PT/N0), and 53 invasive ($N_{1\text{-}3}$) and 32 chemo-radiation-treated with local recurrence (RCRT) samples. The $N_{1\text{-}3}$, PT and AN samples were obtained from OSCC patients before treatment, whereas RCRT samples were collected from patients with incidence of local recurrence after postoperative concurrent chemo-radiation therapy. The specimens were collected after obtaining informed consent from the patients with the approval of the Institutional Human Ethical Committee of CUJ. H&E sections were used for histological studies, and staging of these samples was conducted according to the American Joint Committee on Cancer (AJCC)/ International Union against Cancer (UICC) guidelines.

### Immunohistochemistry (IHC)

IHC was performed as discussed elsewhere [4,5] by using tissue samples (n = 145). The staining was done with EnVision FLEX Mini Kit (Code: K8023, DAKO, USA) and diaminobenzidine tetrachloride (DAB) was used to visualize the staining. For a negative control, we used BSA in place of the primary antibody, and for a positive control, samples with confirmed immunoreactivity to a particular antibody were used. Based on the staining intensity and the extent of immunoreactivity, the immunostained samples were visualized and scored (0 to 4). The staining was considered either no expression (score: 0), mild expression (score: 1–2) or overexpression (score: 3–4) of CD44 proteins.

### Western blot (WB) analysis

WB analysis was performed as previously described [3]. A total of 52 samples (12AN, 11PT/N0, 15 $N_{1\text{-}3}$, and 14RCRT) and protein extracts from SCC4/-9 were used. Tissue/cell lysates were prepared in RIPA buffer, and protein samples (40–100 μg) were resolved using 8–10% SDS-PAGE with a protein marker (Cat. No: C1992, Sigma, USA) and transferred on to PVDF membranes (by iBlot™, BioRad, USA). Immunoblot analysis was performed with the following primary antibodies from Santa Cruz Biotechnology, USA and Cell Signaling Technology, USA: CD44 (156-3C11) (CST#3570), $pS^9$GSK3β (sc-11757), GSK3β (H-76) (sc-9166), pERK1/2 (SC-16982), ERK1/2 (CST#9102), Cleaved Notch1 (D3B8) (CST#4147), Notch1 (A-8) (sc-376403), Cyclin D1 (DCS6) (sc-2044), Cyclin E (E-4) (sc-377100), MMP-9 (C-20) (sc-6840), MMP-2 (8B4) (sc-13595), Snail (C15D3) (CST#3879), E-cadherin (2Q663) (sc-71008), Bcl-2 (124) (CST#15071), Bax (P19) (sc-526), Bcl-xL (H5) (sc-8392) and β-actin (C-4) (sc-47778). The secondary antibodies (Santa Cruz Biotechnology, USA) goat anti-rabbit IgG-HRP:sc-2004, rabbit antigoat IgG-HRP:sc-2768 and donkey anti-mouse IgG-HRP:sc-2314 were applied, and the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA) was used to detect horseradish peroxidase. A developer, fixer and X-ray film (Kodak) were used to capture the signal.

### RT-PCR analysis

Total RNA from 47 samples (9 AN, 11 PT/N0, 13 $N_{1\text{-}3}$ and 14 RCRT) was isolated using the protocol described earlier [3,4] by using Trizol Reagent (Sigma Chemicals, USA) and the RNeasy Mini Kit (Cat.No-74104, Qiagen, Germany). The integrity of RNA was determined, and RNA was reverse transcribed to cDNA using the QuatiTect Reverse Transcription Kit (Qiagen Cat.No-205311), followed by RT-PCR. The primers used for RT-PCR were designed for CD44v4 and CD44v6 from NCBI reference sequences (NM_001001391.1 and NM_001202555.1) and the primers are as follows CD44v4F: 5′TGT TAA CCG TGA TGG

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