Title
SOX11 Promotes Head and Neck Cancer Progression via the Regulation of SDCCAG8

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SOX11 Promotes Head and Neck Cancer Progression *via* the Regulation of SDCCAG8

A dissertation submitted in partial satisfaction of the

Requirements for the degree Doctor of Philosophy

In Oral Biology

by

Eoon Hye Ji

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ABSTRACT OF DISSERTATION

SOX11 Promotes Head and Neck Cancer Progression *via*
the Regulation of SDCCAG8

by

Eoon Hye Ji
Doctor of Philosophy in Oral Biology
University of California, Los Angeles, 2017
Professor Shen Hu, Chair

The overall goal of this project is to gain insight into the role of the enhanced expression of SOX11, a member of the SOX transcription factor family, and SDCCAG8, a tumor antigen, in oral/head and neck cancer. We hypothesize that over-expression of SOX11, an embryonic development related gene, leads to an upregulation of SDCCAG8, promoting a malignant phenotype in oral/head and neck cancer. To test this hypothesis, we have first demonstrated that knockdown of SOX11 expression inhibits the proliferation, migration and invasion of oral/head and neck cancer cells. Next, we have confirmed that SOX11 binds to the promoter of SDCCAG8 by using ChIP and luciferase assays and proven that up-regulation (or down-regulation) of SOX11 induces (or inhibits) the expression of SDCCAG8 in oral/head and neck cancer cells. To further investigate the clinical significance of SDCCAG8 over-expression in oral/head and neck cancer, we have utilized the deep sequencing data from the TCGA

database and performed a correlation analysis of SDCCAG8 gene expression with clinicopathological parameters of oral/head and neck cancer patients. The results show that high expression of SDCCAG8 is significantly associated with overall survival, tumor size and stage of the cancer patients. Taken together, our findings indicate that SDCCAG8 is a prognostic biomarker in oral/head and neck cancer and SOX11 may promote the progression of oral/head and neck cancer *via* the regulation of SDCCAG8.

The dissertation of Eoon Hye Ji is approved.

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DEDICATION

My dissertation is dedicated to my mother whom I lost to cancer and the God for always being by my side.

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1. INTRODUCTION

1.1 Proteomics and mass spectrometry (MS)

Proteomics has become one of the most commonly used tools to identify and study the characteristics, functions, and structures of proteins [1]. Proteomic analysis can characterize protein expression quantitatively and qualitatively, at specific cellular responses, as well as profile entire proteins, globally, in a sample. This allows for comparisons of protein expression levels between control groups and diseased patients which in turn provides a more informative understanding of the mechanism of the disease [2]. A commonly used proteomic tool is mass spectrometry (MS), which can identify and quantify proteins accurately, even with a relatively small quantity of the sample. A previous study discovered 3171 secreted proteins and 5570 membrane-bound proteins in major organs and tissues of the human body using proteomics [3]. When particular proteins are mutated, it may often become overly expressed, lose its normal cell expression, and/or enhance tumor growth due to its dysfunctional mechanism [3]. Studies have stated that about 260 to 290 genes were reported to be cancer driven genes among 12 to 21 tumor types [3]. In contrast to previous studies that searched for differentially expressed genes or proteins in different types of cancers, we wanted to search for proteins that might contribute to promoting the growth of oral/head and neck cancer cells. Through proteomic analysis, we have identified significantly over-expressed proteins in oral/head and neck cancer, including SOX11, which is one of the primary targets of this study.

1.2 Oral/head and neck cancer

Head and neck tumors occur in the oral cavity, oropharynx, hypopharynx, larynx, and the mouth. A most common type of head and neck squamous cell cancer (HNSCC) is oral squamous cell carcinoma (OSCC). OSCC occurs more frequently in male patients than female patients, and in patients who have been exposed to tobacco or alcohol usage [4]. In the US, there are approximately 7,900 OSCC deaths among the 39,400 newly diagnosed cases per year [5].

Oral cancer is considered the sixth most common cancer among men, and it occurs more frequently among 55 to 64 aged populations [6,7]. The main factors that cause oral cancer are smoking tobacco and drinking alcohol [8,9]. The combination of smoking tobacco and drinking alcohol result in a significant increase in the permeability of the oral mucosa, in favor of the tobacco carcinogen, nitrosonornicotine [10]. OSCC is often detected in lymph nodes due to its metastatic characteristics [11]. The way oral cancer is diagnosed nowadays is through oral examination by dentists or health care providers when they observe an abnormal area in the oral cavity [12]. Once diagnosed, oral cancer patients are treated by chemotherapy and radiotherapy. Unfortunately, studies show that patients respond poorly to these therapies at high percentages, and the OSCC reoccurs in high rates[13]. Since most of the OSCCs are diagnosed during the late phase, it is not easy to prolong their current quality of life or increase the survival rate of patients in general [13]. Most of the cancers, including OSCC, have been identified as carriers of abnormal genetics, but some consists of epigenetic changes in which the signaling pathways that involve protein or DNA functionality gets affected, and results in an abnormal phenotypic change and/or cancer development. Cancer cell proliferation and tumor growth are promoted by the presence of protein products from highly expressed oncogenes, or changes in gene expression[14]. Additionally, cancer cells may regulate negatively, to inhibit cell growth and proliferation of normal cells. In the past three decades, therapeutic targeting research has become one of the paramount aim studies to understand the mechanisms of cancer pathogenesis[14]. Many cancer drugs are directly targeting specific molecules to inhibit or activate them or to impair tumor growth and progression. Our study is focused on two target molecules, SOX11 and SDCCAG8 in OSCC/HNSCC. We looked for potential biomarkers of OSCC after selecting proteins using proteomic. For the in vitro studies, we primarily used four oral/head and neck cancer cell lines, UM1, UM2, UM5, and UM6. UM1 and UM2 cell lines were originally established from a pre-treatment patient who had single tongue carcinoma, however, the UM1

cells have a more invasive potential and higher motility than the UM2 cells [15]. Similarly, UM5 cells are much more invasive than UM6 cells [16]. Besides the in vitro studies, we have also investigated the clinical significance of SDCCAG8 in oral/head and neck cancer.

1.3 Sex-determining region Y box-containing factor 11 (SOX11)

A large body of cellular signaling pathways has been demonstrated to be involved in human carcinogenesis [17]. Understanding these signaling pathways and identifying related key regulators may provide insight into the molecular mechanisms of carcinogenesis. The results may provide new targets for therapeutic intervention. Previous studies have shown that organ morphogenesis is controlled by growth factors, signaling pathways such as the Wnt signaling pathway, and transcription factors: T-cell factor (TCF) and SOXs [18]. These signaling molecules have also been discovered to share diverse roles in various cancers, despite the fact that, oral/head and neck carcinogenesis is a multiple-step process with more tendency to be influenced by oncogenes, tumor suppressor genes, key signal transduction cascades, microRNA (miRNA); as well as genetic mutation such as gene amplification, and epigenetic modification [19,20].

The SOX genes encode a group of high-mobility protein products, which are a family of transcription factors. They have emerged as potent modulators involved in orchestrating embryonic development, cellular fate differentiation, organogenesis, stem cells maintenance, and carcinogenesis in multiple biological or pathological processes [21–24]. So far, there are 20 SOX genes that have been found in mice and humans, and they are classified into eight different subgroups, A-H [24]. Among these eight subgroups, SOXC group, which consists of SOX4, SOX11, and SOX12 [21,24,25,], plays an essential role in regulating differentiation, proliferation, and survival of cells in multiple organ lineages [21]. SOX4 plays a role in B-cell development and pancreas formation, while SOX11 is involved in the development of multiple organs, such as, the nervous system, lungs, stomach, pancreas, spleen, eyes, and skeleton

[22,24,25]. Both SOX4 and SOX11 are involved in cardiogenesis, neurogenesis, and retinal cell differentiation [21,24]. Studies have shown that depletion of SOX4 and SOX11 can be lethal or cause reduction of retinal ganglion cells in mice [21]. Moreover, SOX11 is involved in neural development [26,27], organogenesis in fetal development, regulation of embryonic development, and involved in the determination of the cell fate [28–30]. SOX11 is present at specific stages during embryo development, with highly restricted expression in adult tissue, which indicates precise regulation of transcription. As such, the depletion of SOX11 causes defects in embryo development and/or death [26]. SOX11 also participates in several other biological processes, including cell signaling[31], and has associations with various tumors [29,32–35]. It is strongly up-regulated in some malignancies and has a functional role in tumorigenesis [29,32]. SOX11 has been suggested as a tumor suppressor or promoter gene, depending on the tumor model or cell type: lymphoid and solid cancer cells, mantle cell lymphoma, gastric cancer, ovarian cancer cell, hematologic malignancies, nasopharyngeal carcinomas, prostate cancer, and breast cancer [31,33,34,35,22,25,26]. One study has shown that SOX11 over-expression is observed in an aggressive mantle cell lymphomas and promotes tumor growth [31]. On the contrary, some other studies show that, after transfecting the mantle cell lymphoma with siSOX11, they were able to observe more tumor proliferation, *in vitro,* and faster tumor growth rate, which correlated with the death rate, *in vivo* [36]. Also, over-expressed SOX11 causes suppressing migration and invasion abilities in gastric cancers [26].

Nevertheless, SOX11 has gained extensive attention as a diagnostic marker in a series of cancers [31,33,34,37]. However, to date, the potential roles of SOX11 in oral/head and neck cancer have not been investigated thoroughly. With the aim to explore differences in SOX11 regulated intracellular protein expression in oral/head and neck cancer cells, our preliminary comparative proteomic analysis revealed that SDCCAG8, a tumor antigen, might be a down-stream target gene of SOX11. Therefore, in this study, we aim to investigate if SOX11 regulates

SDCCAG8 in oral/head and neck cancer cells, promoting proliferation, invasion and migration of those cells.

1.4 Serologically defined colon cancer antigen 8 (SDCCAG8)

SDCCAG8 is a protein coding gene and has been identified as a tumor antigen with various tumor associations[38,39–41]. It is also known as CCCAP (Centrosomal Colon Cancer Autoantigen Protein), NY-CO-8 (human colon cancer antigen), NPHP10 (Nephronophthisis-related Ciliopathies 10), SLSN7 (Senior-Loken syndrome 7), and BBS16 (Bardet-Biedl Syndrome 16) [42]. This gene is located on chromosome 1, forward strand (1q43) [42] and has four isoforms [43]. These isoforms (except isoform e) are located at the end of both centrioles and co-localizes to the centrosome and centrioles [43,44]. SDCCAG8 has 18 exons [43,45] and encodes a multiple coiled-coiled domain protein [46,41]. Mutation of SDCCAG8 causes diseases such as, nephronophthisis, Bardet-Biedl syndrome, and retinal-renal ciliopathy [43,45,46]. SDCCAG8 mutation is also often observed in patients with mental retardation, cognitive impairment, and seizures [46]. Stransky et al. profiled whole-exome sequences of tumors from 74 head and neck squamous cell carcinoma (HNSCC) patients, and they found several mutations of SDCCAG8 in HNSCC. The nucleic acid mutation was common on chromosome 1, changing from Guanine to Thymine, as such, the changing of several codon sequences would occur. Changing from GCC to TCC would have a resulting outcome that modifies the protein, amino acid location 607 from alanine to serine [47], and disrupt its function. This study provided evidence that there is a mutation on SDCCAG8 in HNSCC. Recent studies have demonstrated that over-expression of SDCCAG8 was detected in human lung cells, LC5, with over-expressed MASPIN playing a role in the invasion of cancer cells [39]. Over-expression of SDCCAG8 was also observed in gastric cancer cells of patients with poor survival rates [48]; and in diffuse-type gastric cancer cells, which is defined as non-cohesive cells that are poorly differentiated, they often metastasize into the peritoneum or lymph nodes [49]. Another study has found that SDCCAG8 could be a marker to

identify and distinguish cervical cancer patients, who can benefit from getting only radiotherapy treatment, from those who would need both radiotherapy and chemotherapy [40]. The cervical cancer patients who were treated with the only radiotherapy showed much more improvement than the patients treated with radiotherapy and chemotherapy [40]. Further studies demonstrated that SDCCAG8 regulates cell cycle, mitotic G2-G2/M phases, and recruitment of centrosome proteins, and other complexes [41,44,46]. Although there have been validations of SDCCAG8 and its pleiotropic effects in cellular regulations, the underlying mechanisms of SDCCAG8 in cancers are still not well-known.

The novel observation of Serologically Defined Colon Cancer Antigen 8 as a downstream target for SOX11 is an exciting area that warrants further investigation. The expression level correlations of SDCCAG8 and SOX11 have been demonstrated in highly metastatic oral cancer tumor cell lines; with the discovery that the detailed molecular link between SOX11 and SDCCAG8 depends largely on a relationship in which SOX11 is regulating the expression of SDCCAG8. This allows us to define the function of SOX11 in tumorigenesis, however, the functional link between SOX11 and SDCCAG8, and the underlying mechanism of SDCCAG8 being involved in tumorigenesis remains to be elucidated. Furthermore, we have used molecular approaches to dissect the underlying mechanism of SDCCAG8-mediated invasion and we suspect that these results will identify a biochemical function for SOX11. We believe the results will have a significant impact on our understanding of the functional link between two distinct classes of cellular proteins that are involved in the control of cell growth, SOX11 and SDCCAG8.

There are various types of cancers which have many different cellular signaling pathways. Elucidating cellular signaling pathway of each cancer is still an ongoing process, and although we are able to diagnose different types of cancer at late stages, the discovery of biomarkers for early diagnosis or accurate prognosis are yet to be achieved. Many genes are

mutated in cancer cells and their abnormal function encourages proliferation, invasion and metastasis of cancer cells. Understanding functions and characteristics of the proteins that show differential expression levels between cancer cells and normal tissues will guide us to discover potential biomarkers and targeted treatments of cancer patients. The findings may have important implications for the mechanisms of pleiotropic phenotype through multiple tissue-specific diseases, including tumorigenesis. Identification of cell-specific factors that participates in tumorigenesis involving the SOX11-SDCCAG8 axis, will yield novel molecules for drug discovery in cancer treatment. In this project, based on our preliminary results, we have hypothesized that SOX11 regulates the expression of SDCCAG8 and consequently, these two proteins could be constituents of a regulatory pathway in oral/head and neck tumorigenesis. The following two aims will demonstrate our hypothesis and determine the interactive functional role of SOX11 and SDCCAG8 in oral/head and neck cancer.

Aim #1. To determine if SOX11 regulates the expression of SDCCAG8 in oral/head and neck cancer cells using *in vitro* approaches

Aim #2. To investigate the functional role and clinical significance of SDCCAG8 in oral/head and neck cancer

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

The OSCC/HNSCC cell lines, UM1, UM2, UM5, UM6, and UM17B, were cultured in cell culture media, Dulbecco's modified eagle medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA), supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The cells were incubated in a CO_2 incubator at 37°C with 5.0% CO_2, and the medium was changed every two days until cells reached 90-95% confluence. Cells were washed three times with Dulbecco's Phosphate-Buffered Saline (DPBS) (Invitrogen Life Technologies, Carlsbad, CA) and harvested.

2.2 siRNA knockdown

Transfection with siRNA was performed on the UM1, UM2, UM5, UM6, or UM17B cells using transfection reagent for 48-72 hours in 6-well plates according to the manufacturer's instruction. Double-stranded siRNAs of SOX11 (SC-38422, Santa Cruz Biotech, Santa Cruz, CA, USA), SDCCAG8 (SC-78905, Santa Cruz Biotech, Santa Cruz, CA, USA), or non-target control scrambled siRNAs (Santa Cruz Biotech, Santa Cruz, CA, USA) were prepared separately with a transfection reagent, Lipofectamine RNAiMAX (13778150, Invitrogen Life Technologies, Carlsbad, CA, USA). Once cells reached 70% confluency, cells were transfected with siRNAs. We mixed 20 µl of 10 µM siRNA, 10ul of Lipofectamine RNAiMax, and 470 µl of DMEM (antibiotics-free and serum-free) and incubated them for 5 minutes at room temperature. During the 5 minutes incubation, the old culture medium was removed from each well and 3mL of fresh DMEM to each well was added. Then, the siRNA-RNAiMax complex was added to each well and incubated overnight in a CO_2 incubator at 37°C with 5.0% CO_2. After a 24-hour treatment, the cells were maintained in fresh normal growth media for 24 to 48 hours for further

experiments. The siRNA targeting SOX11 sequences were as follows: 5'-GGAGAGAAUUCUACAUUUAdTd-3' AND '5'-UAAAUGAUGAAUUCUCUCCdTdT-3'.

2.3 Quantitative proteomic data analysis

To profile the protein expression changed in oral/head and neck cancer cell lines (UM1 and UM17B), which were transfected with siRNA, we used quantitative proteomics based on isobaric tags for relative and absolute quantitation (iTRAQ). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) followed the protocol as described previously [50]. Cells were collected at 72 hours post-transfection for proteomic data analysis.

2.4 Chromatin Immunoprecipitation (ChIP)

We performed ChIP assay to determine whether SOX11 binds to the promoter region of SDCCAG8. A total of $2x10^6$ cells from UM1 and UM5 cancer cells were fixed with 1% formaldehyde. Cells were lysed using 450uL of SDS lysis buffer in the kit (EMD Millipore Taunton, MA, USA) for 10 minutes and sonicated for 7 x 10 seconds in a Branson Ultrasonics Sonifier S-450 (Emerson Electric Co.,St. Louis, MO, USA). We utilized the Chromatin Immunoprecipitation (ChIP) Assay Kit (17-295, EMD Millipore Taunton, MA, USA) for sample preparation. Initially, 20ul of SDCCAG8 antibody (SC-137753, Santa Cruz Biotech, Santa Cruz, CA, USA) was added to the 1.5mL supernatant fraction sample and incubated overnight at 4°C with rotation. Afterwards, 60ul of Salmon Sperm DNA were added with Protein A Agarose Slurry (16-157C, EMD Millipore Taunton, MA, USA) for one hour at 4°C with rotation to collect the antibody/histone complex. We centrifuged down the agarose by gentle centrifugation (3000rpm at 4°C) for 1 minute and carefully removed the supernatant that contained the unbound, non-specific DNA. The protein A Agarose/antibody/histone complex was washed for 3 minutes on a rotating platform with 1mL of each of the buffers in the kit at 4°C. To amplify DNA that is bound to the immunoprecipitated histone, we ran a Polymerase Chain Reaction (PCR) assay in 65°C for 4 hours. We performed the assay with quadruplicate qPCR data. A 160 bp DNA fragment

was amplified from anti-SOX11 (H-290: SC-20096, Santa Cruz Biotech, Santa Cruz, CA, USA). The primers for the SDCCAG8 gene promoter region are forward primer, 5'-TCTGCAGCTTACACCAATCGT-3' and reverse primer, 5'-GCTTTGAAGGCAAGCCTGAT-3'. Data was normalized against beta-actin using the primers 5'-AGCGAGCATCCCCCAAAGTT-3' and 5'- GGGCACGAAGGCTCATCATT-3'. Normal rabbit IgG was used as a negative control. All experiments were performed in triplicates. Data was analyzed using the $2^{-\Delta\Delta C}$ T Method [51].

2.5 Western blotting

Western blotting was used to detect SOX11 and SDCCAG8 expressions in UM1, UM2, UM5 and UM6 that were treated with or without siRNA. Western blotting experiments were performed as described previously [52]. Equal amounts of each protein samples were separated on homemade 10% SDS-PAGE gel with 30% Acrylamide and transferred to nitrocellulose membrane (Bio-Rad). The membranes were then blocked with 5% non-fat milk (Santa Cruz Biotech, Santa Cruz, CA, USA) in Tris-buffered saline and Tween 20 (TBST) for overnight at 4°C. After the blocking step, the membranes were incubated with anti-SOX11 (H-290, SC-20096, Santa Cruz Biotech, Santa Cruz, CA, USA) or anti-SDCCAG8 (GTX115484, GeneTex, Irvine, CA, USA) primary antibodies in 2% non-fat milk overnight at 4°C. The membranes were washed with TBST 3 times for 7 minutes each and were incubated with secondary antibodies (GE Healthcare, Piscataway, NJ, USA) in 2% non-fat milk for 1 hour at room temperature. The ECL Plus Detection Kit (GE Healthcare, Piscataway, NJ, USA) was used to develop the films and detect the signal intensity of the proteins. For quantification, proteins were normalized to Actin and GAPDH. All experiments were performed in triplicates

2.6 Quantitative real-time PCR (qPCR)

To validate western blot analysis data, we performed qPCR analysis and measured the mRNA expression levels of SOX11 and SDCCAG8 in oral/head and neck cancer cells. RNA isolation was done using the Quick-RNA MiniPrep kit (R1054, Zymo Research, Irvine, CA)

according to the manufacturer's protocols. Briefly, 400ul of RNA lysis buffer was added to the cells grown on 6-well plate and collected. Samples were spun down with 15,000g using a centrifuge for 1 minute to remove gDNA. Ethanol (95%) with the same volume of the sample to the sample in RNA lysis buffer as 1:1 ratio was added into the sample. The mixture was transferred to the Zymo Spin IIICG column in the collection tube and was spun down for 30 seconds with 10,000g. Afterwards, 400ul RNA wash buffer was added to the column and the mixture was spun down for 30 seconds with 10,000g. Then, 80ul of DNase I reaction mixture was added into the column and the mixture was incubated for 15 minutes at the room temperature. After incubation, samples were spun down for 30 seconds in 10,000g and were added with 400ul RNA prep buffer. Several washing steps were applied and samples were added with 30ul of RNase-free water and spun down with the highest speed. RNA were eluted from the sample and cDNA were synthesized by Reverse Transcription SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, USA) using 1.5 ug of total RNA. Samples were incubated at 65˚C for 5 minutes and then were placed on the ice for 1 minute. cDNA synthesis mixture (10X RT buffer, 25nM MgCl2, and 0.1MDTT) was added into the sample, and the mixture was incubated at 25˚C for 10 minutes, 50˚C for 50 minutes and 85˚C for 5 minutes to complete the synthesis. Finally, 10ul cDNA was used for qPCR with SYBR Green I Master mix (Roch, Indianapolis, IN, USA) added. The condition of qPCR cycle was 1 cycle for 10 minutes at 95°C and 55 cycles for 10 seconds at 95°C, 45 second at 55°C and 10 seconds at 72°C. Specific primer sequences were used to amplify targets for SOX11: 5'-CCAGGACAGAACCACCTGAT-3' and 5'-CCCCACAAACCACTCAGACT-5', and for SDCCAG8: 5'- CCATCGAAAGACTGGTTAAAGAA-3' and 5'-CTTTTTCAAGTCGCTCCGCC-3'. Data were normalized against beta-actin using primers 5'-GCGCGGCTACAGCTTCA-3' and 5'-CTTAATGTCACGCACGATTTCC-3' or against GAPDH using primers 5'-TCTCTGCTCCTCCTGTTC-3' and 5'-GTTGACTCCGACCTTCAC-3'.

2.7 Plasmid construction and transformation

A mammalian overexpression plasmid containing FLAG-tagged SOX11 (SOX11F) and a mutant lacking the transactivation domain (SOX11FΔTAD) were kindly gifted by Prof. Kathryn M. Albers [53] and Prof. Angie Rizzino [36]. They were subsequently cloned into bacterial culture for the overexpression experiments. Sox-11F was constructed using the primers FLAGSox-11 (5-CGTGCTGGTACCGCCACCATGGACTACAAGGACGACGATGATATGGTGCAGCAGGCCGA GAGC-3) and Sox-11FTAD was constructed using the primer pair sox11TAD (5-CTCTACTACAGCTTCAAGTGAGCGGCCGCAAACATCACCAAGCAGCAG-3) as described in previous study [36].

2.8 Luciferase reporter assay

To further investigate if SOX11 acts as a transcription factor for SDCCAG8, we performed luciferase reporter assays. We utilized a plasmid for Flag-tagged SOX11 and a modified version, ΔTAD, with a stop codon inserted before the transactivation domain. A NotI site was also added to the modified version to aid in distinguishing it from the original, using restriction enzyme test-cutting. The construct SOX11F was cut off by EcoRI (5847 base pair) and XhoI (2253 base pair). SOX11FΔTAD was cut off by 1ul of EcoRI, XhoI, and NotI based on the map (Figure 12). We prepared DNA as minipreps from bacterial stocks after we received the plasmids and used an ampicillin resistant plate to grow colonies. After transformation and purification of DNA samples, 16ul of the samples were run on homemade 1% agarose gel to confirm the plasmid identification. 3ul of DNA loading buffer (6x) (C113-1, Lamda Biotech, St. Louis, MO, USA) and 10ul of 1Kb DNA Ladder (M108-1, Lamda Biotech, St. Louis, MO, USA) were used to run the gel at 80 V.

To test whether SOX11 binds to SDCCAG8 promoter region, oral/head and neck cancer cells were seeded in 24-well plates in DMEM medium and cultured as the same method described earlier. When the cells reached 50% confluency, they were transfected with 200ng of

SDCCAG8 luciferase reporter, 100ng of SOX11F or SOX11FΔTAD plasmid, and 200ng of empty promoter reporter vector using 2.0 µl of lipofectamine 2000 reagent (11668019 , Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Samples were prepared in 4 different conditions (**Figure 14**) in UM1 and UM5 cells. After washing cells with DPBS, a mixture of DNA-Lipofectamine-Plasmid with serum-free DMEM medium was added to cells and incubated at room temperature for 20 minutes. The treated cells were incubated in a CO2 incubator for 4 hours and the medium was changed after 4 hours of treating. The cells were incubated in a medium which contained FBS and antibiotics and incubated for 31 hours more to have completed 36 hours of post-transfection. After 36 hours of post-transfection, cells were lysed with 100ul of lysed buffer, which is mixed with reconstituted assay substrate from the LightSwitch Luciferase Assay Kit (LS010, Switchgear, Carlsbad, CA) to determine promoter activity. Cells were transferred to 96 wells and incubated 30 minutes at room temperature while preventing light exposure. After incubation, each well was read for 2 seconds in a plot luminometer (BioTek Instruments, Inc., Winooski, VT) with Gen5 software on Neo. We used the read height auto-adjust function which resulted in optimal signal sensitivity at 8.25mm [54]. All experiments were repeated in triplicate.

2.9 Overexpression of SOX11F and SOX11FΔTAD mutant

To investigate the phenotypes of oral/head and neck cancer cells after upregulating SOX11 and SDCCAG8 expression levels, UM1, UM2, UM5, and UM6 cells were treated with 100ng of SOX11F or SOX11FΔTAD plasmid with 2.0 µl of lipofectamine 2000 reagent (11668019, Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously in this paper. After cells reached about 90% confluency, cells were harvested after 48 hours with 400ul of RNA lysis buffer for qPCR analysis or after 72 hours with Rehydration Buffer for western blot analysis by following the protocol as described previously.

2.10 Wound healing assay

To examine migration ability after inhibiting SOX11 or SDCCAG8 in head and neck cancer cells, the wound-healing assay was performed. Oral/Head and neck cancer cells were cultured in 6 wells and treated with siRNAs of SOX11 (SC-38422, Santa Cruz Biotech, Santa Cruz, CA, USA), SDCCAG8 (SC-78905, Santa Cruz Biotech, Santa Cruz, CA, USA), or non-target control scrambled siRNAs (Santa Cruz Biotech, Santa Cruz, CA, USA) and a transfection reagent, Lipofectamine RNAiMAX (13778150, Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously. After 24 hours post-transfection, 400,000 cells were transferred into the ibidi Culture-Insert (ibidiGmbH, 82152 Planegg / Martinsried, Germany) in the 24-well. Cells were incubated overnight at 37°C with 5.0% CO_2 and the ibidi Culture-Insert was removed by sterile tweezers carefully. Cells were washed with DPBS and incubated with 2ml of DMEM (antibiotics-free and serum-free). Photos were taken under a light-microscope at 0 hour and 24 hours after taking out the ibidi-Insert.

2.11 Cell proliferation with SOX11 knockdown

To test if SOX11 effects proliferation ability of oral/head and neck cancer cells, we conducted proliferation assay in UM1 and UM5 head and neck cancer cells. Cells were cultured in 6 wells and treated with siRNAs of SOX11 (SC-38422, Santa Cruz Biotech, Santa Cruz, CA, USA) or non-target control scrambled siRNAs (Santa Cruz Biotech, Santa Cruz, CA, USA) and a transfection reagent, Lipofectamine RNAiMAX (13778150, Invitrogen Life Technologies, Carlsbad, CA, USA). After 24 hours post-transfection, 25,000 cells were transferred into a 24 well plate. Every 24 hours, cells were trypsinized with 250ul of 0.25% Trypsin (1x)(15050, Thermo Scientific, Waltham, MA USA) for 5 minutes at 37°C with 5.0% CO_2 and quenched with 300 ul of DMEM complete medium. Cells were collected in cell counting tubes and measured in numbers for four days by using a cell counter (Vi-CELL XR, Beckman Coulture, Indianapolis, IN, USA). All experiments were performed in triplicates.

2.12 Cell proliferation with SDCCAG8 knockdown

To investigate if SDCCAG8 effects proliferation ability of oral/head and neck cancer cells, we conducted proliferation assay in UM1 and UM5 oral/head and neck cancer cells. Cells were cultured in 6 wells and treated with SDCCAG8 (SC-78905, Santa Cruz Biotech, Santa Cruz, CA, USA), or non-target control scrambled siRNAs (Santa Cruz Biotech, Santa Cruz, CA, USA) and a transfection reagent, Lipofectamine RNAiMAX (13778150, Invitrogen Life Technologies, Carlsbad, CA, USA). After 24 hours post-transfection, cells were transferred into 96 wells with the same 2000 starting cell numbers. Every 24 hours, cells were quantified with Methl-3H-Thymidine (MTT) assay. Every day, cells were incubated with 100ul MTT solution for 4 hours at 37°C with 5.0% CO_2. After incubating the cells, 100ul Dimethyl sulfoxide(DMSO) was added and were measured for their absorbance at 550nm through a plate reader (BioTek Instruments, Inc., Winooski, VT) with Gen5 software on Neo. Cell proliferation was measure for four days with triplicates.

2.13 Transwell invasion assay

To understand how SDCCAG8 effects the invasion ability of oral/head and neck cancer cells, we conducted invasion assay in UM1 and UM5 cancer cells. Cells were cultured in 6 wells and treated with SDCCAG8 (SC-78905, Santa Cruz Biotech, Santa Cruz, CA, USA), or non-target control scrambled siRNAs (Santa Cruz Biotech, Santa Cruz, CA, USA) and a transfection reagent, Lipofectamine RNAiMAX (13778150, Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously. Cell invasion assay was conducted using the BD Matrigel Invasion Chamber and culture insert (354480, BD Biosciences, Bedford, MA, USA). After 72 hours post-transfection, 50,000 cells were transferred into the chamber and incubated with 0.5ml of DMEM (antibiotics-free and serum-free). Cells were washed with DPBS and the membrane was fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Pictures were taken 24 hours after staining. Assays were performed in triplicates.

2.14 Patient clinical data analysis

To determine the clinical significance of SDCCAG8 in HNSCC, we examined SDCCAG8 protein expression in HNSCC patients using the data from the Cancer Genomic Atlas (TCGA) database. We also performed mining of TCGA RNA Seq V2 data and generated mRNA expression of SDCCAG8 in HNSCC patients and healthy control. We also explored the data from the OncoLnc.org to generate Kaplan-Meier plots for the survival rate of various cancer patients who had low or high SDCCAG8 expression levels, based on TCGA database. We also investigated SDCCAG8 mRNA expression in HNSCC patients using the cBioPortal website**.**

2.15 Western blot and qPCR analysis of SDCCAG8 in pancreatic/lung cancer cells

We further investigated if SDCCAG8 is over-expressed in pancreatic and lung cancer cells compared to corresponding normal cells. As described earlier, we cultured the cells in the same way as HNSCC cells and performed Western blot and qPCR analysis of SDCCAG8 among BXPC-3, MiaPaCa2, AST, A549, HPDE and BEAS2B cells.

2.16 Statistical analysis

Experiments were typically performed in triplicates in this study. All data wrere presented as mean ±SEM. Student *t* test was used to determine the statistical significance, and the GraphPad Prism (Graph-Pad Software, CA) was used to generate data graphs and the patient survival graphs. We also used Kaplan-Meier survival curves and used log-rank tests to analyze the overall survival rate as previously described [66]. $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Role of SOX11 on the expression of SDCCAG8 in oral/head and neck cancer cells

3.1.1 Transfecting SOX11 with siRNA in oral/head and neck cancer cells caused suppression of SDCCAG8 gene expression levels

We used quantitative proteomics to profile protein expression changes in oral/head and neck cancer cell lines (UM1 and UM17B), which were transfected with small interfering RNA (siRNA). Table 1 showed many gene expression levels in UM1 and UM17B cells after silencing SOX11 ($P \leq 0.05$). Quantitatively, SDCCAG8 gene expression levels were both reduced when UM1 and UM17B cells were transfected with siSOX11.

3.1.2 SOX11 binds to the promoter region of SDCAG8 in UM1 and UM5

To investigate if SOX11 binds to the promoter region of SDCCAG8 as a transcription factor, we perform ChIP assay with anti-SOX11 in UM1 and UM5 cells **(Figure 2 and 3)**. In UM1 cancer cells, DNA fragment enriched with anti-SOX11 was amplified and produced 3.4-fold change in UM1 cells when compared to IgG control. In UM5 cells, 3.9-fold change in enrichment was observed. The ChIP assay showed an enrichment of the SDCCAG8 promoter fragment with anti-SOX11 when compared to IgG control, suggesting that SOX11 may bind to promoter of SDCCAG8 in both UM1 and UM5 cells.

3.1.3 Both protein and mRNA expression of SOX11 and SDCCAG8 were significantly higher in UM1 and UM5 cells compared to UM2 and UM6 cells

With comparative Western blot analysis of SOX11 and SDCCAG8 expressions, we identified that there was a significant similar pattern of protein expression level between SOX11

and SDCCAG8 in oral/head and neck cancer cells. In UM1 and UM5 cells, which are highly invasive and of metastatic origin, both SOX11 (47 kDa) and SDCCAG8 (82 kDa) proteins were over-expressed significantly **(Figure 4A and 5B)**. Interestingly, in UM2 and UM6 cells, which are low invasive metastatic cells, both SOX11 and SDCCAG8 barely showed any protein expression **(Figure 4A and 5A)**. To validate Western blot analysis data, we performed qPCR analysis and measured the mRNA expression levels of SOX11 and SDCCAG8 in oral/head and neck cancer cells **(Figure 4B and 5B)**. Similar to the Western blot analysis, UM1 and UM5 cells had significantly higher SOX11 and SDCCAG8 gene expression than UM2 and UM6 cells.

3.1.4 Both protein and mRNA expression of SDCCAG8 were decreased after transfecting cells with siSOX11

We further investigated the relationship between SOX11 and SDCCAG8 to confirm the direct inhibition of SDCCAG8 by down-regulating the expression of SOX11. UM1 and UM5 cells were transfected with siSOX11. qPCR analysis was performed on UM1 and UM5 cells transfected with siSOX11 to quantify SOX11 and SDCCAG8 mRNA expression levels (**Figure 6A and 7A),** and on UM1 and UM5 cells transfected with siSDCCAG8, to confirm SOX11 and SDCCAG8 mRNA expression levels **(Figure 6B and 7B)**. As we expected, in UM1 and UM5 cells, SDCCAG8 mRNA expression was suppressed after transfection with siSOX11 **(Figure 6C and, 7C)**. To confirm qPCR analysis, Western blot analysis was performed in triplicates, and both SDCCAG8 and SOX11 protein expression were inhibited after transfection with siSOX11 (**Figure 6D and 7D)**. However, when UM1 and UM5 cells were transfected with siSDCCAG8, only SDCCAG8 expression was inhibited but SOX11 protein expression was not significantly changed **(Figure 6E and 7E)**.

3.1.5 SOX11 was overrepresented on promoter region of SDCCAG8

To verify if SOX11 binds to the promotor region of SDCCAG8 in oral/head and neck cancer cell lines, luciferase assay was performed on UM1 and UM5 cells (**Figure 8**). Figure 9A and 9B showed colonies containing FLAG-tagged SOX11 (SOX11F) and a mutant lacking the transactivation domain (SOX11FΔTAD) on top of Ampicillin-resistance plates. DNA were isolated successfully and grew inside large centrifuge tubes with growth medium **(Figure 9C**). From transformation, we were able to reproduce 1250.3 ng/uL of SOX11F and 880ng/uL of SOX11FΔTAD **(Table 2).** Based on cmv5 flag sox11 map **(Figure 10),** we identified the size of enzyme restriction fragments on 1% agarose gel and confirmed our transformation and DNA purification were successfully done **(Figure11**). As shown in **Figure 11**, lanes 2, 5, 6, 9 were from SOX11F showing two fragment bands. On the other hand, lanes 3, 4, 7, 8 were obtained from SOX11FΔTAD showing three fragment bands. The plasmids in the lanes 2, 5, 6, 9 were digested by restriction enzymes EcoRI and Xhol whereas those in the lanes 3, 4, 7, 8 were digested by restriction enzymes EcoRI, Xhol, and Notl. To investigate if SOX11 could drive SDCCAG8 expression, luciferase assay was performed in UM1 and UM5 cells with plasmid contained either SOX11F or SOX11FΔTAD (**Figure 12)**. **Figure 13A and 13B** shows the result of luciferase assay of UM1 and UM5 cells transfected with SOX11F or SOX-11FΔTAD. As we expected, we were able to see an overrepresentation and underrepresentation of SOX11 on the promoter region of SDCCAG8 after transfection of the reporter gene construct into UM1 or UM5 cells. When SDCCAG8 reporter constructs were co-transfected with the SOX11F, luciferase activity was significantly overexpressed compared to cotransfection with SOX11FΔTAD in both UM1 and UM5 cells (**Figure 13A and 13B**). In other words, the luciferase activity of the SDCCAG8 reporter construct, with SOX11FΔTAD, was suppressed in both UM1 and UM5 cells. The luciferase expression in the SDCCAG8 reporter construct, by itself, showed a little bit higher expression level than luciferase activity in the empty vector. Overall, both UM1 and UM5 cells showed a similar pattern of luciferase activities under the four different conditions

3.1.6 SOX11 overexpression induced SDCCAG8 expression in oral/head and neck cancer cells

To investigate potential regulatory mechanism of SDCCAG8 by SOX11, we overexpressed SOX11using plasmid contained SOX11F in UM2 and UM6 cells and tested the protein level of SDCCAG8 by Western blotting. We also transfected SOX11 using plasmid contained SOX11FΔTAD in UM1 and UM5 cells. As we expected, our results showed that transfected cells with plasmid expressed SOX11F showed significant upregulated SOX11 expression levels (lanes 3 and 7) as well as SDCCAG8 expression levels (lanes 3 and 7) compared to wildtype cancer cells (lanes 4 and 8) (**Figure 14**). However, cells that were transfected with plasmid contained SOX11FΔTAD, showed suppressed SOX11 expression of protein (lanes 1 and 5) and SDCCAG8 protein expression levels (lanes 1 and 5) compare to wildtype cancer cells (lanes 2 and 6) (**Figure 14**).

3.2 Functional role of SDCCAG8 in head and neck cancer cells

3.2.1 Differential invasion ability among the four cancer cell lines in this study

To characterize the migration and invasion ability of the oral/head and neck cancer cell lines (UM1, UM2, UM5, and UM6) used in this study, we performed both wound healing assay and transwell invasion assay. As shown in **Figure 15**, UM1 and UM5 showed significantly higher migration and invasion abilities than UM2 and UM6 cells.

3.2.2 Knockdown of SOX11 inhibits the migration of oral/head and neck cancer cells

To investigate the impact of SOX11 on the motility of oral/head and neck cancer cells, we conducted a wound healing assay of UM1 and UM5 cancer cells transfected with siSOX11 or scrambled control siRNA. We observed that both UM1 and UM5 cells, when transfected with siSOX11, had slower rates of migration than the cells treated with siCTRL (**Figure 16A and 17A**).

3.2.3 Knockdown of SOX11 inhibits the proliferation of oral/head and neck cancer cells

In order to investigate whether SOX11 influences the proliferation of oral/head and neck cancer cells, we performed proliferation assay of UM1 and UM5 cancer cells after transfection with siSOX11. As we expected, the numbers of cells silenced with siSOX11 and their growth rates were significantly lower than the cells treated with siCTRL **(Figure 16B and 17B)**.

3.2.4 Knockdown of SOX11 inhibits the invasion of oral/head and neck cancer cells

To investigate the role of SOX11 in the invasion potential of UM1 and UM5 cells, we performed transwell invasion assay after transfecting UM1 and UM5 cells with siSOX11. After siSOX11 transfection, the number of cells invaded through the transwell insert was found to decrease about 60% when compared to the cells treated with siCTRL **(Figure 16C and 17C)**. We also counted the number of cells under a light-microscope and observed that about 90 cells less in siSOX11 treated UM1 and 120 cells less in UM5 cells ($P<0.05$) **(Figure 16D and 17D)**.

3.2.5 Knockdown of SDCCAG8 inhibits the migration of oral/head and neck cancer cells

Wound healing assay experiments were performed to investigate if SDCCAG8 has a functional role in the migration of oral/head and neck cancer cells. As expected, we observed UM1 and UM5 cells, when treated with siSDCCAG8, migrated significantly slower than the cells treated with siCTRL to close the wound (**Figure 18A and 19A**).

3.2.6 Knockdown of SDCCAG8 inhibits the proliferation of oral/head and neck cancer cells

Proliferation assays were performed to investigate if knockdown of SDCCAG8 affects the proliferation of oral/head and neck cancer cells. As shown in **Figure 18B and 19B**, after transfecting UM1 and UM5 cells with siSDCCAG8, both types of cancer cells were growing slower than the cells transfected with siCTRL.

3.2.7 Knockdown of SDCCAG8 inhibits the invasion of oral/head and neck cancer cells

To exam the effect of SDCCAG8 on the invasion capability of UM1 and UM5 cells, we knockdown the cells with siSCCAG8 and investigate their invasive characteristics. Comparing UM1 cells treated with siCTRL, approximately 70% decrease in invasion was observed after the cells were transfected with siSDCCAG8 (**Figure 18C**). Similar to UM1 cells, UM5 treated cells showed about 50% decrease in invasion when compared to siCTRL-treated UM5 cells (**Figure 19C). Figure 18D and 19D** showed quantitative data of the transwell invasion assays.

3.2.8 Overall survival rate in HNSCC patients with high SDCCAG8 expression

To determine the clinical significance of SDCCAG8 in HNSCC, we analyzed the RNA-Seq data in regards to SDCCAG8 from the Cancer Genomic Atlas database (TCGA). **Figure 20** shows that the expression level of SDCCAG8 mRNA was significantly upregulated ($P<0.001$) in HNSCC patients compared to normal controls. Among 519 HNSCC patients, the HNSCC patients who have higher expression levels of SDCCAG8 mRNA live shorter lives ($P=0.0027$) than the HNSCC patients who have lower expression mRNA level of SDCCG8 (**Figure 21**). A total of 21 different cancers was shown from the OncLnc website, based on the TCGA database. Among 21 cancers, 6 cancers correlated with high expression levels of SDCCAG8. Besides HNSCC, kidney renal clear cell *carcinoma (KIRC)* and cervical squamous cell *carcinoma* and endocervical adenocarcinoma (CESC) patients showed p-values of 0.0099 and 0.0583, respectively (**Figure 22A and 22B**) for the survival analysis. However, the following cancers did not show significant p-values: Colon Adenocarcinoma (COAD), with p-value of 0.224, *Lower*

grade glioma (LGG), with p-value of 0.775, Lung Squamous Cell Carcinoma (LUSC), with p-value of 0.633, and Rectum Adenocarcinoma (READ), with p-value of 0.366 (**Figure 22C, 22D, 22E, and 22F).** To further investigate the clinical significance of SDCCAG8 over-expression in oral/head and neck cancer, we have utilized the deep sequencing data from the TCGA database and performed correlation analysis of SDCCAG8 gene expression with clinicopathological parameters of oral/head and neck cancer patients. The results show that high expression of SDCCAG8 is significantly associated with overall survival, tumor size and stage of the cancer patients (**Table 3**).

3.2.9 SDCCAG8 protein expression in various types of cancers

We examined the protein expression levels of SDCCAG8 in different types of cancer from the Human Protein Atlas database. **Figure 23** shows the immunohischemistry data of various human cancers expressing moderate to high levels of SDCCAG8. HNSCC showed significant high expression levels of SDCCAG8 as well as breast cancer, melanoma, lung cancer, and urothelial cancer when compared to normal tissues.

3.2.10 Expression of SOX 11 and SDCCAG8 in pancreatic and lung cancer cells

To further identify the characteristics of SOX11 and SDCCAG8 expression levels in cancer cells, we performed Western blot analysis of SOX11 and SDCCAG8 expression in pancreatic cancer cells. As shown in **Figure 24**, SOX11 and SDCCAG8 are over-expressed in both BxPC-3 and MiaPaCa-2 pancreatic cancer cells over HPDE cells. We further investigated SOX11 and SDCCAG8 protein expression in lung cancer cells. As shown in **Figure 25**, both SOX11 and SDCCAG8 proteins are significantly upregulated in AST and A549 lung cancer cells.

4. DISCUSSION

There are several studies that have investigated the functional processes of mutated SDCCAG8 in diseases, however, thorough investigation of SDCCAG8 in cancer cells, especially with its transcription factor SOX11, has not yet been performed. Although SDCCAG8 has been identified as a potential cancer antigen and proclaimed to be associated with a variety of cancerous tumors, it was inconclusive whether SDCCAG8 influences tumor formation by acting through recruiting binding partners, or if it is due to SDCCAG8 undergoing deletion/mutation. Further studies are needed to determine what the exact molecular mechanisms pertaining to SDCCAG8 and SOX11 in human cancer, including HNSCC.

In this study, we have demonstrated for the first time that SOX11 is a novel transcription regulator of SDCCAG8 in HNSCC. We have performed comparative proteomic analysis, Western blot analysis, chromatin Immunoprecipitation assay (ChIP assay), and qPCR analysis. From these experiments, we came across a discovery that was portrayed consistently throughout, such that, highly invasive oral/head and neck cancer cells display an elevated SOX11 and SDCCAG8 expression level when compared to low invasive cancer cells. We have also performed knockdown experiments of SOX11, as well as experiments that examined the overexpression of SOX11, concurrently with similar knockdown and overexpression experiments aimed towards SDCCAG8, so that we can have high confidence and verification of expression level correlation of SDCCAG8, relative to SOX11. Our data provide evidence that SDCCAG8 is regulated by SOX11 in HNSCC cells, and highlight the functional significance of SOX11-SDCCAG8 in HNSCC and how it may affect head and neck tumor phenotypes.

The first goal of this study was to examine a possible role of SOX11 in HNSCC, in regards to how it regulates SDCCAG8 gene expression. SOX11 is strongly up-regulated in some malignancies and have a functional role in tumorgenesis [48]. We investigated the role of

SOX11, as a transcriptional regulator, that regulates a downstream gene which may be directly or indirectly involved oral/head and neck cancer development. In order to achieve temporal and tissue specificity, an interaction between transcription factor proteins and DNA must occur, due to the interaction playing an important role in regulating transcription [51]. Since identifying the transcription factor binding sites gives us a better understanding of gene regulation [51], we conducted ChIP assay on oral/head and neck cancer cells. Our ChIP assay results suggested a potential target gene, SDCCAG8, of which the promoter site may be directly bound by the transcription factor, SOX11, in oral/head and neck cancer cells. We detected a significant difference in transcriptional activity when SOX11 interacted with SDCCAG8 promoter, which indicates that SOX11 contributes to the transcription factor-DNA binding on SDCCAG8 promoters. Based on protein and mRNA expression levels of SOX11 and SDCCAG8, which shows similar patterns in oral/head and neck cancer cells, we predicted that there might be a functional regulatory role with SOX11 when associated with SDCCAG8 such that, enhance proliferation could occur, as well as migration, and invasion of HNSCC cells. In our studies, both mRNA and protein expression levels of SOX11 and SDCCAG8 were significantly upregulated in invasive oral/head and neck cancer cells, UM1 and UM5, when compared to low-invasive oral/head and neck cancer cells, UM2 and UM6. As we revealed in our previous studies, UM1 and UM5 cells possess a more aggressive metabolic phenotype and express significantly higher levels of metabolites, which can also contribute towards tumorogenesis, than UM2 and UM6 cells [52].

Our luciferase assay studies also confirmed that SOX11 regulates SDCCAG8 in oral/head and neck cancer cells. This was achieved by performing luciferase assay with plasmids containing SOX11F or SOX11FΔTAD. Wild type SOX11F, when overexpressed in UM1 and UM5 cancer cells, were found to induce the promoter activity of SDCCAG8, whereas mutant SOX11FΔTAD plasmid, when overexpressed in UM1 and UM5 cells, showed lower

activity of SDCCAG8 promoter. This further verify that SOX11 binds to the promoter site of SDCCAG8. Identifying and investigating transcription factor binding sites can provide results that can lead to the understanding of functional outcomes such as, the transcription factor binding for biological network or phenotype in different cell lines [51]. From previous studies, SOX11 was found to bind in the minor groove of the DNA helix and cause bending of the backbone, allowing DNA complexes to remain stabilized thus, making it more efficient for a pursuant to gain access and initiate transcriptional activity [53]. Our luciferase assay produced results indicating that SOX11 might play an important role in relation to SDCCAG8 gene activation in head and neck cancer cells. The previous study has shown that overexpression of SOX11 in Neuro2a cells increased the brain-derived neurotrophic factor (BDNF) gene expression and an exon promoter activity to enhance neuronal regeneration after nerve injury [54]. Also, SOX11 in mesenchymal stem cells, showed transcription activity with runtrelated transcription factor 2 and CXC chemokine receptor-4 expression to regulate differentiation and migration of bone fracture site for bone healing [55]. In addition, when SOX11 gene is functionally knocked down during embryonic activity of mice, lack of SOX11 hinders the embryonic axon growth and inhibits neuron survival, proliferation, and axon outgrowth [53]. Previous studies also reported controversial results on the role of SOX11 in human cancers. For instance, in mantle cell lymphoma (MCL), knocking down SOX11 promotes MCL cell proliferation and regulates the tumor cell growth. Among gastric cancer patient, patients who expressed SOX11 highly, had higher survival rates when compared to patients with lower expressed SOX11 [26]. In contrast, we found that suppressing SOX11 expression inhibits invasion, proliferation, and migration abilities of oral/head and neck cancer cells. When we silenced SOX11, protein expression levels of SDCCAG8 were downregulated, whereas when we overexpressed SOX11, protein expression levels of SDCCAG8 were upregulated in oral/head and neck cancer cells. However, when we silenced the expression of SDCCAG8, SOX11 expression levels did not change thus sugesting

that SDCCAG8 may not regulate the expression of SOX11 in oral/head and neck cancer cells. Together, these findings indicate that there would be a close correlation between SOX11 and SDCCAG8 expression, which affects invasiveness and metastatic characteristics of oral/head and neck cancer cells.

The second goal of this study is to conduct phenotypic studies and investigate if silencing SOX11 or SDCCAG8 affects the phenotypes of oral/head and neck cancer cells. We silenced SOX11 and SDCCAG8, individually, and performed proliferation, invasion and migration assays. Our results suggested that SOX11 and SDCCAG8 promote invasion, migration, and proliferation of oral/head and neck cancer cells and the silencing SOX11 and SDCCAG8 showed very similar results. This might be because SOX11 regulates SDCCAG8 in oral/head and neck cancer cells. Similar to our study, when PFTK1 was overexpressed, which caused enhancement of gastric cancer migration and invasion, they stated that it might have been because of the PFTK1 signaling pathway changes [56]. RNA interference is a powerful approach that can be harnessed to engineer gene-specific silencing in mammalian cells and tissues. It has become the technique of choice for analysis of gene function in oncological research [57]. When SDCCAG8 is depleted in zebrafish, it causes kidney cysts, body axis defects, and renal cell defects [35]. The previous study found that transfecting embryos with siSDCCAG8 caused multiple developmental defects in zebrafish [35]. Such a RNA interference approach may be used to target SOX11 or SDCCAG8 for potential treatment studies.

SOX11 is known to be present at specific stages during embryo development with a very restricted expression in adult tissue, indicating precise regulation of transcription. SOX11 is also strongly up-regulated in some malignancies and has a functional role in tumorigenesis. Regarding SDCCAG9, it has been reported that not only is SDCCAG8 related to various diseases when it is mutated, but also when it interacts with certain binding proteins [35,39]. The protein is also known to be associated with various cancers [33,41,42,44]. Therefore, the third goal of

our study is to explore whether SDCCAG8 have clinical significance in oral/head and neck cancer or other cancer types. Protein expression levels of SOX11 and SDCCAG8 have been compared among 20 different types of cancer and normal tissue cells, individually [3]. In oral/head and neck cancer, both SOX11 and SDCCAG8 were found to be over-expressed in cancer tissues compared to normal tissues based on the Human Proteome Atlas Database. These findings agree to our results and confirm the high expression of SOX11 and SDCCAG8 proteins in head and neck cancer cells.

To better understand the clinical significance of SDCCAG8 in oral/head and neck cancer, we investigated the overall survival rate and mRNA expression level of SDCCAG8 in head and neck cancer patients. Cox regression multivariate analysis shows that SDCCAG8 can be a factor for survival of head and neck cancer patients. High expression level of SDCCAG8 is significantly correlated with poor survival in head and neck patients. To explore SDCCAG8 expression in other types of cancer, we further measured the protein expression of SDCCAG8 in cultured pancreatic and lung cancer cells. It is well known that pancreatic cancer is one of the most invasive and aggressive cancers with very low survival rates [58]. Unfortunately, when patients get diagnosed with pancreatic cancer, most of the time the patients exhibits metastasis of the tumor mass due to the poor prognosis of this disease. [58]. Miapaca-2 is more aggressive than BxPc3, and both are derived from the primary tumors [59]. Although overall survival rate of pancreatic cancer cell patients with high levels of SDCCAG8 expression has less chance to live than the patients with the low SDCCAG8 expression level, the data is not significant and is not shown in this paper. However, based on our Western blot data, we were able to observe significant overexpressed levels of SDCCAG8 in the two pancreatic cancer cell lines, MiaPaCa-2 and BxPc3 when compared to HPDE. Lung cancer is the leading malignancy to cause mortality [60]. SOX11 protein expression was found to be upregulated in AST and A549 lung cancer cell lines, as well as SDCCAG8 protein expression level, when compared to normal

BEAS-2B cells. These results indicate that SOX11 and SDCCAG8 are not only highly expressed in oral/head and neck cancer cells but also shows significant expression levels in pancreatic and lung cancer cells. A previous study stated that SDCCAG8 is one of the genes that plays important role in the invasion of lung cancer cells[41]. Also, Miapaca-2 and BxPc3 are often used to test invasion assay due to their mobility with different targeted gene [58]. Therefore, we believe we should further investigate the role of SOX11 and SDCCAG8 in pancreatic and lung cancers in the future.

5. CONCLUSION

In conclusion, based on our data, we have confirmed that SOX11 regulates the expression of SDCCAG8 in oral/head and neck cancer cells. As a transcription factor, SOX11 directly binds to the promoter of SDCCAG8 and induces its promoter activities. Our data have demonstrated that both SOX11 and SDCCAG8 may promote the proliferation, migration, and invasion of oral/head and neck cancer cells. Furthermore, clinical analysis has revealed that high expression of SDCCAG8 is significantly correlated with poor overall survival rate, larger tumor size and more advanced tumor stage in oral/head and neck cancer patients. We have also shown the expression levels of SDCCAG8 are elevated in pancreatic and lung cancer cells. Our results indicate that SDCCAG8 is valuable prognostic biomarker of oral/head and neck cancer and SOX11 may promote oral/head and neck cancer progression via the regulation of SDCCAG8. These two proteins may also serve as target molecules for therapeutic intervention in oral/head and neck cancer.

6. TABLES AND FIGURES

Accession	MW [kDa]	calc. pI	129:128	131:130	Description
IPI:IPI00004859.1	158.9	7.49	0.4539	0.882	DNA helicase, RecQ-like type 2
IPI:IPI00719649.1	6.7	9.44	0.7247	0.705	Transcription factor E 4F1 protein
IPI:IPI00439925.2	54.2	8.38	0.7664	0.879	ETS-related transcription factor Elf-2
IPI:IPI00307829.7	149.0	5.67	0.8613	0.690	Cingulin-like protein 1 (cell junction)
IPI:IPI00304639.2	425.3	5.92	0.8459	0.754	Dystrophin (ECM protein)
IPI:IPI00063925.1	35.4	9.85	0.7723	0.697	Epithelial-stromal interaction protein 1
IPI:IPI00556609.1	59.2	6.05	0.7224	0.881	Integrin alpha-IIb variant
IPI:IPI00922108.1	111.1	5.62	0.8051	0.882	integrin alpha-V isoform 2
IPI:IPI00017513.1	38.4	5.16	0.8445	0.829	Integrin beta-1-binding protein 2
IPI:IPI00220297.1	66.8	5.43	0.6551	0.519	Kelch repeat and BTB domain-containing protein 10 (Sarcosin)
IPI:IPI00185325.2	47.8	8.43	0.8546	0.867	Stromal membrane-associated protein 1
IPI:IPI00792295.2	102.7	6.54	0.7607	0.891	Tight junction protein ZO-3
IPI:IPI00298971.1	54.3	5.80	0.4968	0.956	Vitronectin
IPI:IPI00514234.4	57.1	9.67	0.8145	0.887	Ladinin-1 (ECM protein)
IPI:IPI00296922.4	195.9	6.52	0.7815	0.946	Laminin subunit beta-2
IPI:IPI00220572.2	121.5	6.38	0.6013	0.580	Laminin subunit gamma-2
IPI:IPI00456834.2	12.5	7.39	0.8674	0.729	Notch-regulated ankyrin repeat-containing protein (cell adhesion)
IPI:IPI00399180.3	46.2	9.10	0.6178	0.790	Serine/threonine-protein kinase SBK1
IPI:IPI00792792.2	169.4	5.29	0.7845	0.896	Latent-transforming growth factor beta-binding protein 4
IPI:IPI00021780.1	47.3	8.03	0.7386	0.828	Transforming growth factor beta-3
IPI:IPI00304865.3	93.4	5.71	1.0299	0.944	Transforming growth factor, beta receptor III
IPI:IPI00398090.1	53.6	7.66	0.3740	0.504	Tripartite motif-containing protein 75 (TRIM 75)
IPI:IPI00171205.3	41.1	6.13	0.3815	0.578	Usher syndrome 1C binding protein 1
IPI:IPI00386208.1	14.9	7.50	0.8623	0.732	Gastric-associated differentially-expressed protein YA61P
IPI:IPI00657884.3	78.1	5.94	0.7968	0.838	Serologically defined colon cancer antigen 8
IPI:IPI00892901.1	4.0	4.60	0.8010	0.817	Ha-Ras1 proto-oncoprotein variant
IPI:IPI00020956.1	26.8	4.73	0.6667	0.997	Hepatoma-derived growth factor
IPI:IPI00550900.1	19.6	4.93	0.7959	0.949	Translationally-controlled tumor protein
IPI:IPI00844287.1	175.8	4.41	0.5299	0.828	Nestin (brain development)

Table 1. Proteomic data analysis of UM1 and UM17B cells after silencing SOX11. UM1 cells were transfected with siCTRL (TMT128) or siSOX11 (TMT129). Similarly, UM17B cells were transfected with siCTRL (TMT130) or siSOX11 (TMT131).

Sample	SOX11F	SOX11FΔTAD
DNA concentration(ng/ul)	1250.3	880

Table 2. Purified SOX11 plasmid DNA concentration. SOX11F contained 1250.3 ug/ul and SOX11FΔTAD contained 880ng/ul.

Clinicopathological feature	*SDCCAG8* mRNA expression		*P*
	Low	High	
Age	59.77±12.96	61.98±12.96	0.0134
Gender			0.0693
Female	71	57	
Male	176	205	
T stage			**0.0107**
T1-T2	107	79	
T3-T4	145	172	
Lymph node metastasis			0.6856
N0	124	119	
N1	125	129	
Distant metastasis			0.9841
M0	246	242	
M1	3	3	
Stage			**0.0067**
I-II	72	46	
III-IV	181	206	
Angiolymphatic invasion			0.0854
No	121	105	
Yes	54	69	

Table 3. The association between SDCCAG8 gene expression and clinicopathological parameters of HNSCC. T stage and Stage parameters show the significant data.

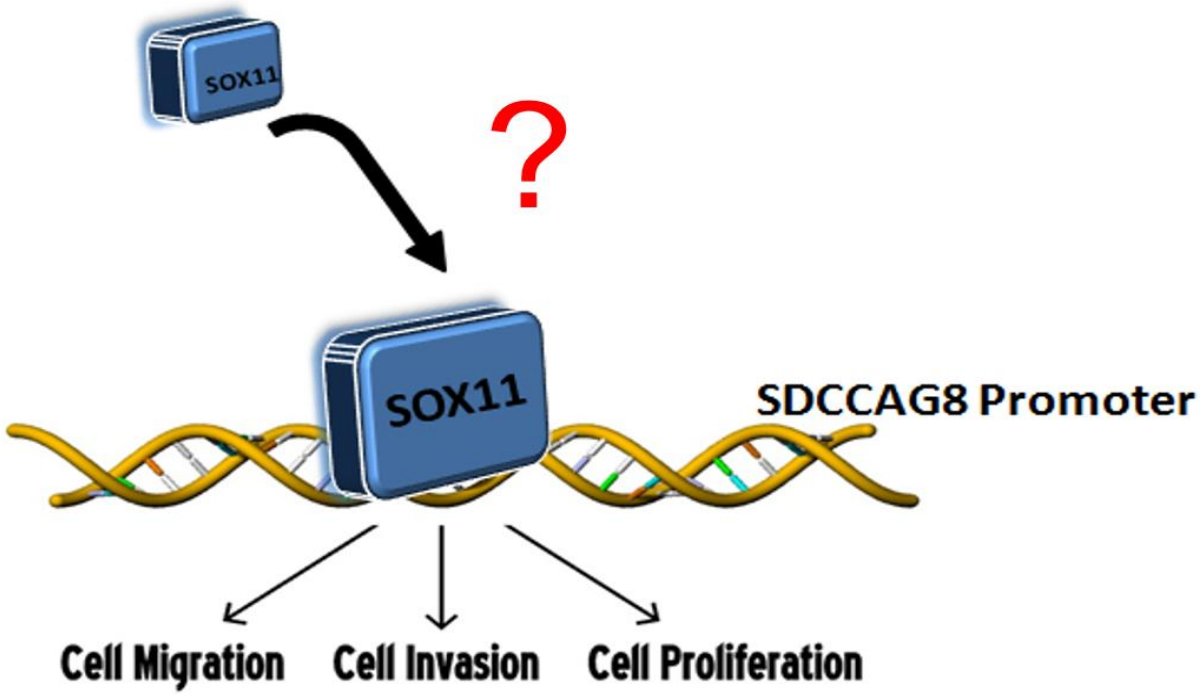

Figure 1. Schematic diagram of expected relationship between SOX11 and SDCCAG8. We hypothesized that SOX11 regulates the expression of SDCCAG8 and consequently these two proteins could be constituents of a regulatory pathway in oral/head and neck tumorigenesis.

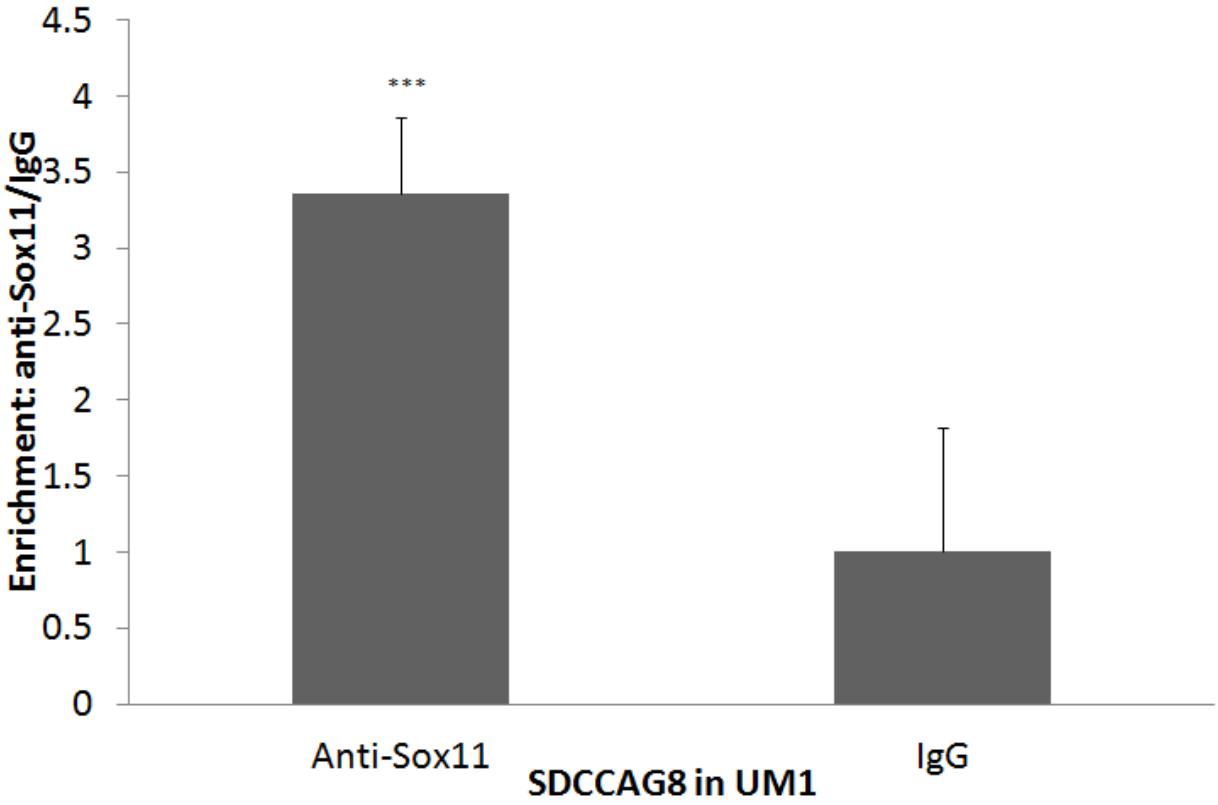

Figure 2. Relative DNA binding activity on SDCCAG8 promoter region. The figure shows the ChIP assay result of SOX11 binding to SDCCAG8 promoter in UM1 cancer cells quantified by qPCR. Note that averages of the results from four experiments are plotted (n=4). Significance is indicated by asterisks: *, *P*<0.001.**

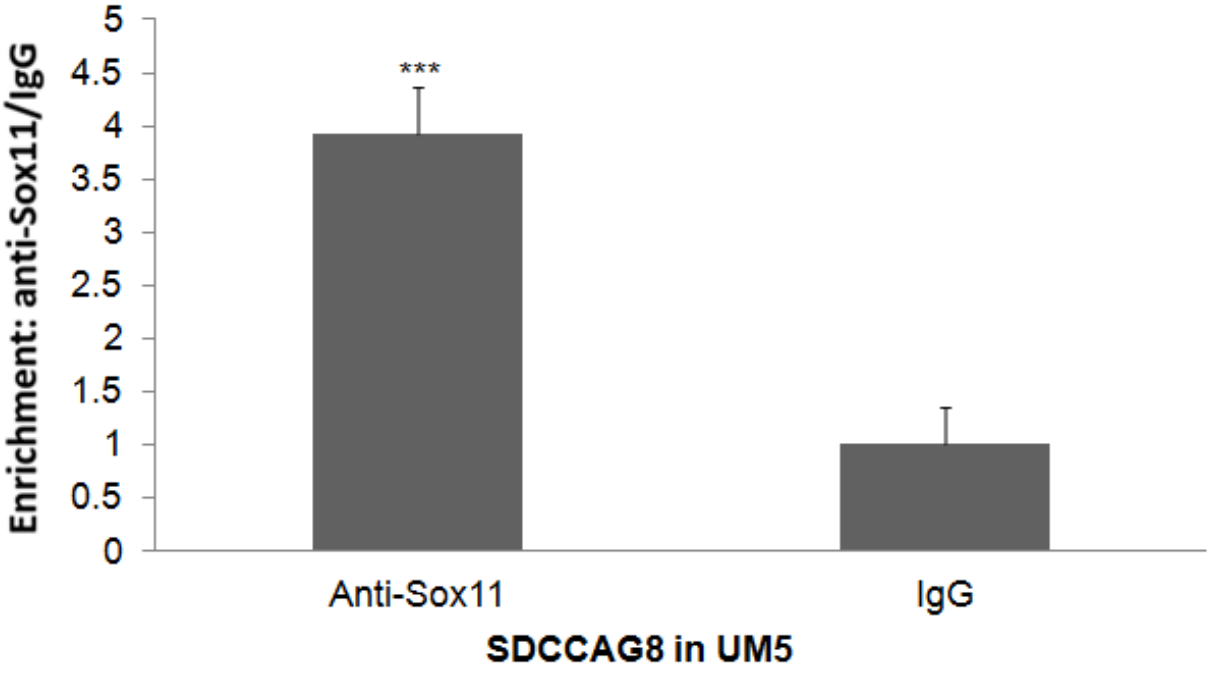

Figure 3. Relative DNA binding activity on SDCCAG8 promoter region. The figure shows the ChIP assay result of SOX11 binding to SDCCAG8 promoter in UM5 cancer cells as quantified by qPCR. Note that averages of the results from four experiments are plotted (n=4). Significance is indicated by asterisks: *, *P*<0.001.**

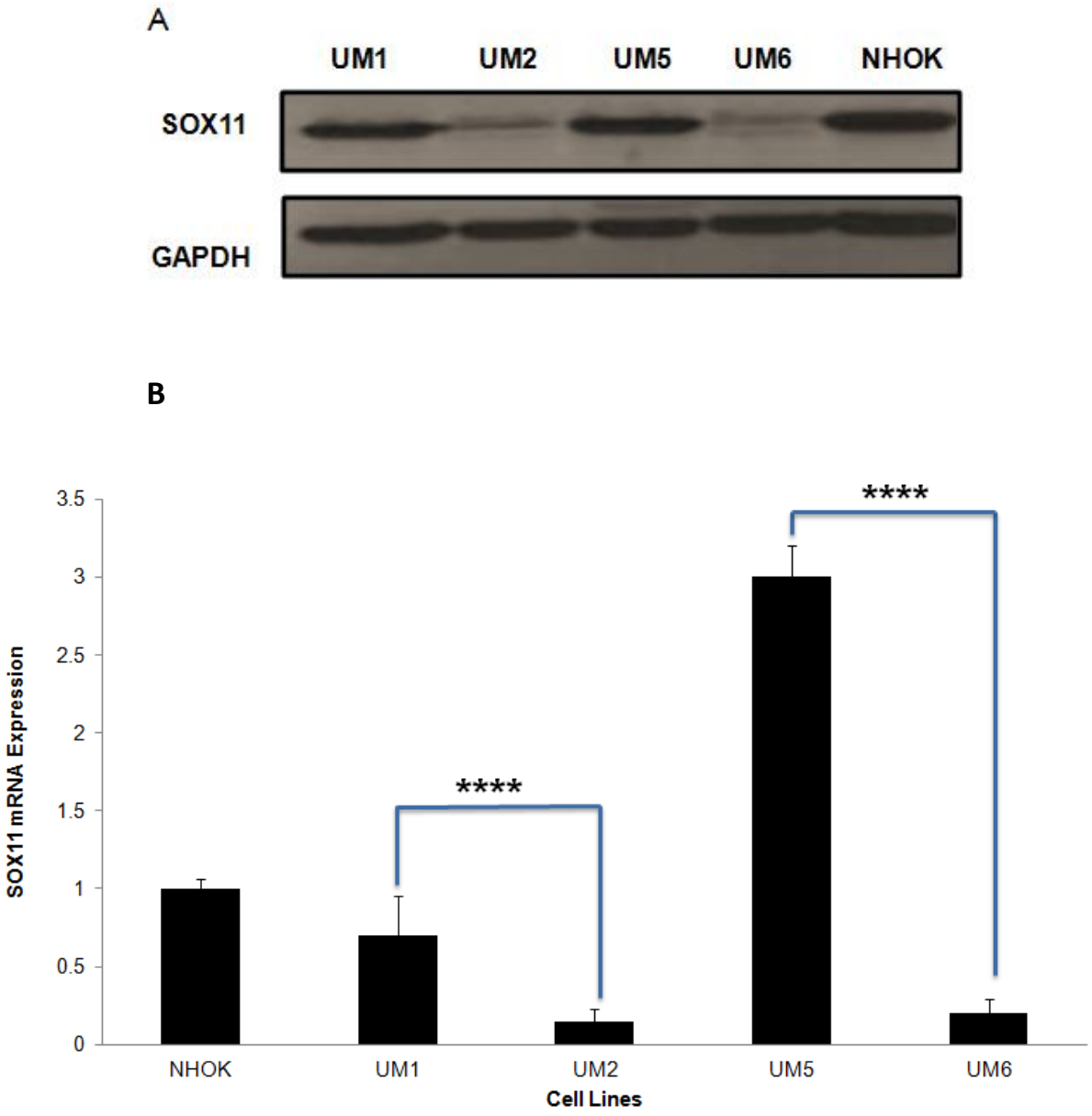

Figure 4A and 4B. SOX11 protein/gene expression levels in NHOKs, UM1, UM2, UM5 and UM6 cancer cells. (A) Western blot analysis of cell lysates which showed SOX11(47 kDa) over-expression in invasive oral/head and neck cancer cells (UM1 and UM5 cells). (B) Relative mRNA expression levels of SOX11 in NHOK, UM1, UM2, UM5 and UM6 cells. Results from four replicates are plotted. Significance is indicated by asterisks: ****, P<0.0001; *****, P<0.00001.

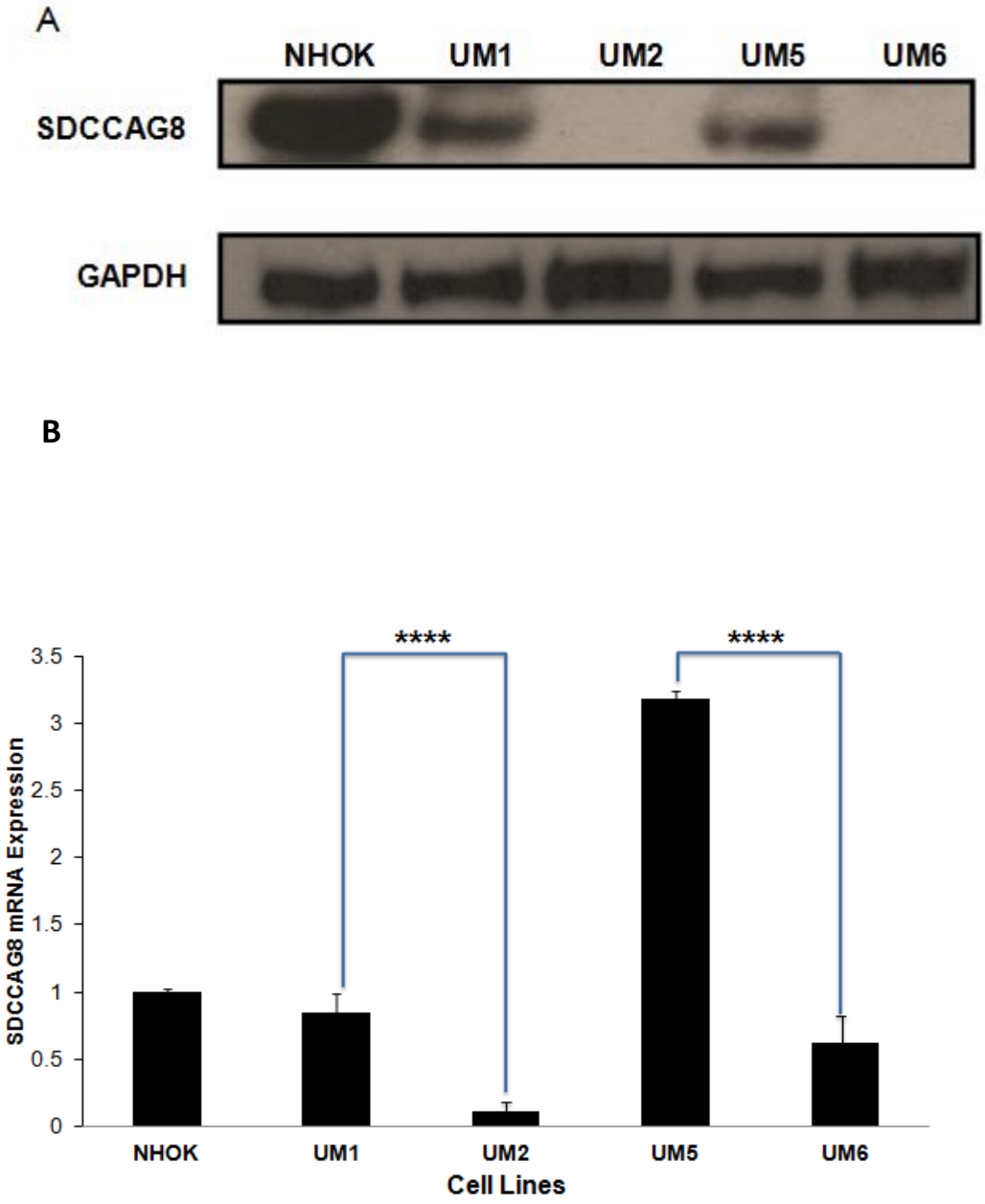

Figure 5A and 5B. SDCCAG8 protein expression levels in NHOK, UM1, UM2, UM5 and UM6 cancer cells. (A) Western blot analysis of cell lysates showing SDCCAG8 (82 kDa) over-expressions in invasive oral/head and neck cancer cells (UM1 and UM5 cells) versus low invasive cancer cells (UM2 and UM6 cells). (B) Relative mRNA expression levels of SDCCAG8 in NHOKs, UM1, UM2, UM5 and UM6 cancer cells. Results from four replicates are plotted. Significance is indicated by asterisks: ****, P<0.0001.

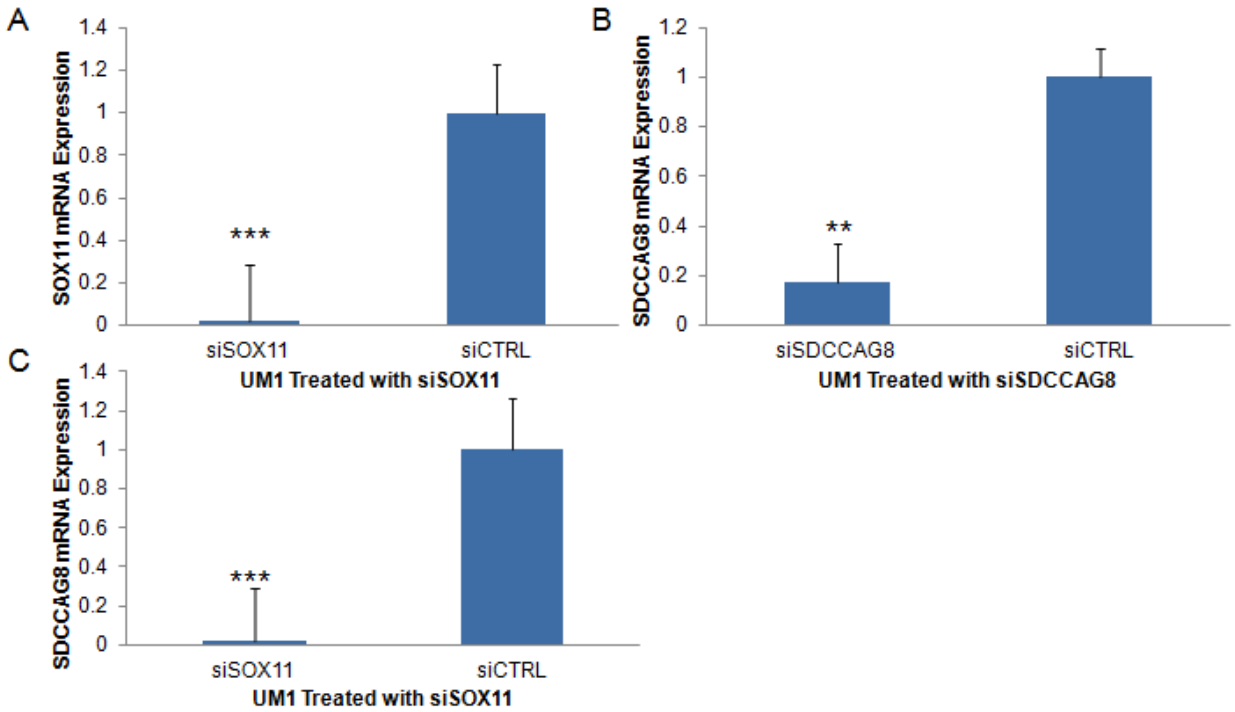

Figure 6. mRNA expression of SDCCAG8 and SOX11 after transfecting UM1 cancer cells. (A) Relative SOX11 mRNA expression level in UM1 cells transfected with siSOX11#1 and #2. (B) Relative SDCCAG8 mRNA expression level in UM1 transfected with siSDCCAG8. (C) Relative SDCCAG8 mRNA expression level in UM1 cells transfected with siSOX11. Significance is indicated by asterisks: **, P<0.01; ***, P<0.001.

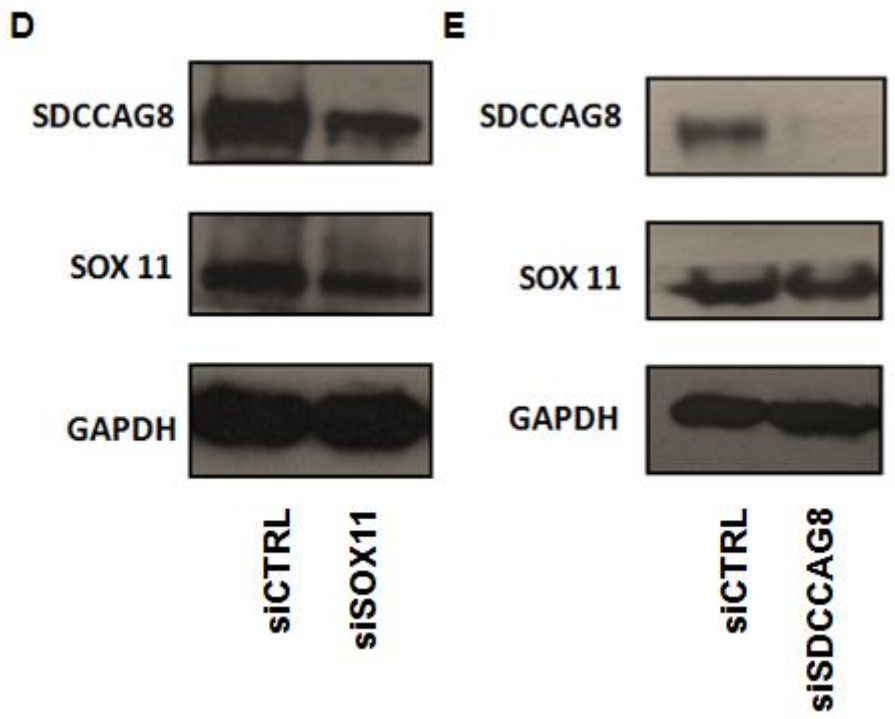

Figure 6. Protein expression of SDCCAG8 and SOX11 after transfecting UM1 cancer cells. (D) Western blot analysis which shows the inhibited expression of SDCCAG8 and SOX11 by administration of siSOX11. (E) Western blot analysis showing the inhibited expression of SDCCAG8 by administration of siSDCCAG8 in UM1 cells; however, SOX11 expression level did not change after transfecting UM1 cells with siSDCCAG8.

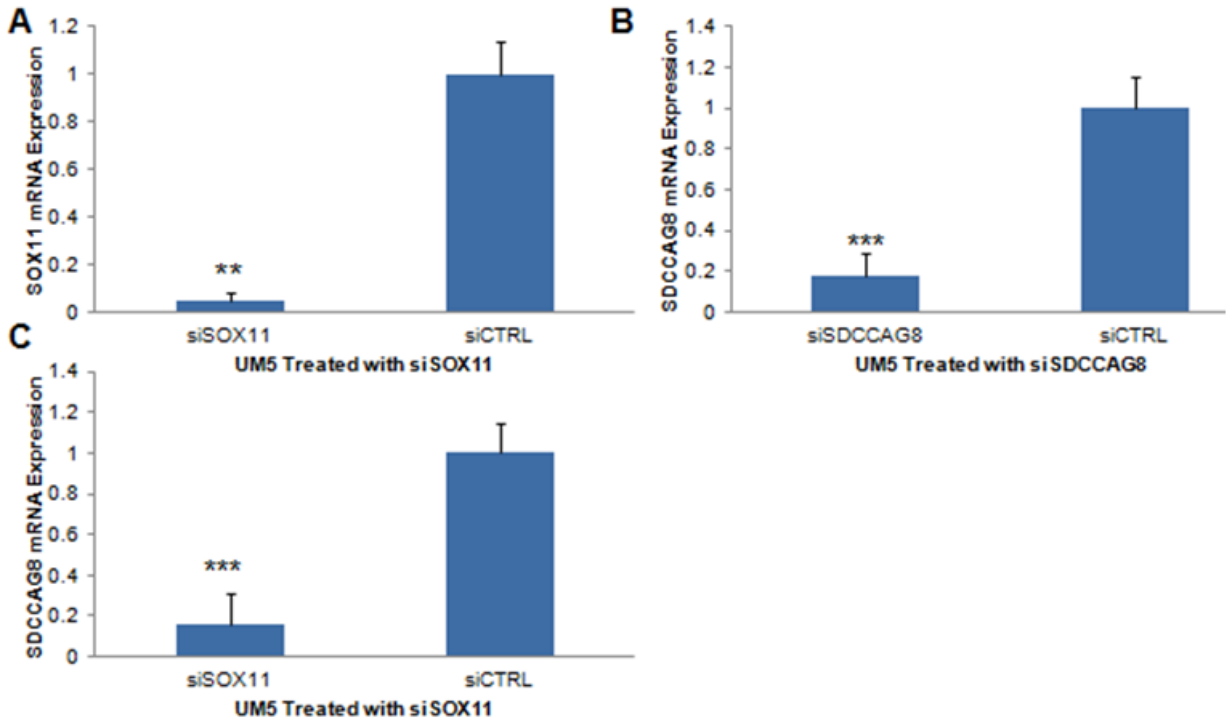

Figure 7. mRNA expression of SDCCAG8 and SOX11 after transfecting UM5 cancer cells. (A) Relative SOX11 mRNA expression level in UM5 cells transfected with siSOX11 or siCTRL. (B) Relative SDCCAG8 mRNA expression level in UM5 cells transfected with siSDCCAG8 or siCTRL. (C) Relative SDCCAG8 mRNA expression in UM5 cells transfected with siSOX11. All mRNA expression levels were compared with siCTRL (n=3). Significance is indicated by asterisks: **, P<0.01; ***, P<0.001.

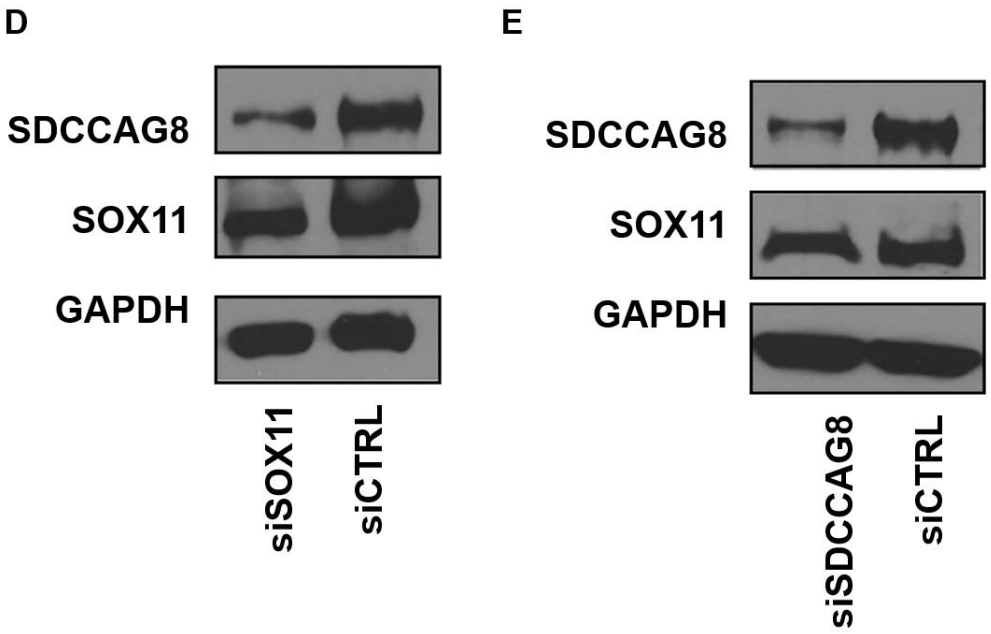

Figure 7. Protein expression of SDCCAG8 and SOX11 after transfecting UM5 cancer cells. (D) Western blot analysis showing the inhibited expression of SDCCAG8 and SOX11 by administration of siSOX11. (E) Western blot analysis showing the inhibited expression of SDCCAG8 by administration of siSDCCAG8 in UM5; however, SOX11 expression level did not change after transfecting UM5 with siSDCCAG8.

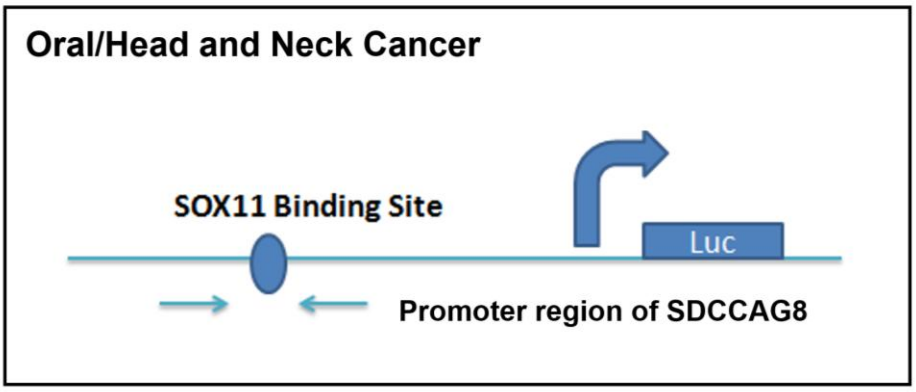

Figure 8. Schematic diagram of SOX11 binding site on SDCCAG8 gene promoter region. We expect SOX11 binds to and upregulates SDCCAG8 promoter activity in oral/head and neck cancer cells.

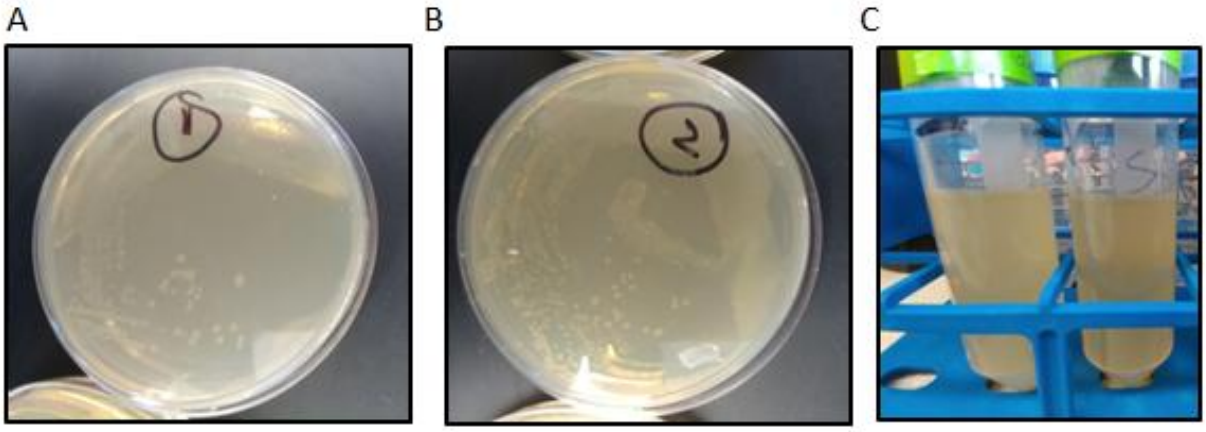

Figure 9. SOX11 plasmid colonies grown in baterial cultural medium. (A) Colonies containing FLAG-tagged SOX11 (SOX11F). (B) Colonies containing a mutant lacking the transactivation domain (SOX11FΔTAD). (C) SOX11F (left) and SOX11FΔTAD (right) colonies in growth medium.

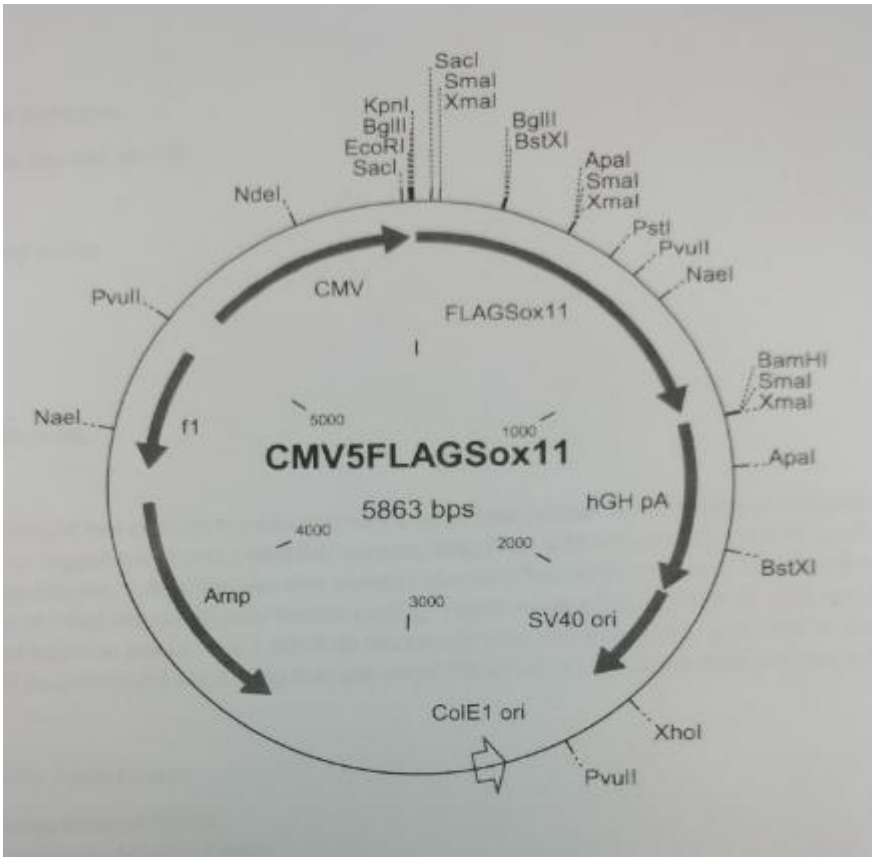

Figure 10. CMV5FLAGSOX11 MAP. Various restriction enzymes are shown on the map. We selected EcoRI, XhoI and NotI restriction enzymes for site specific cleavage.

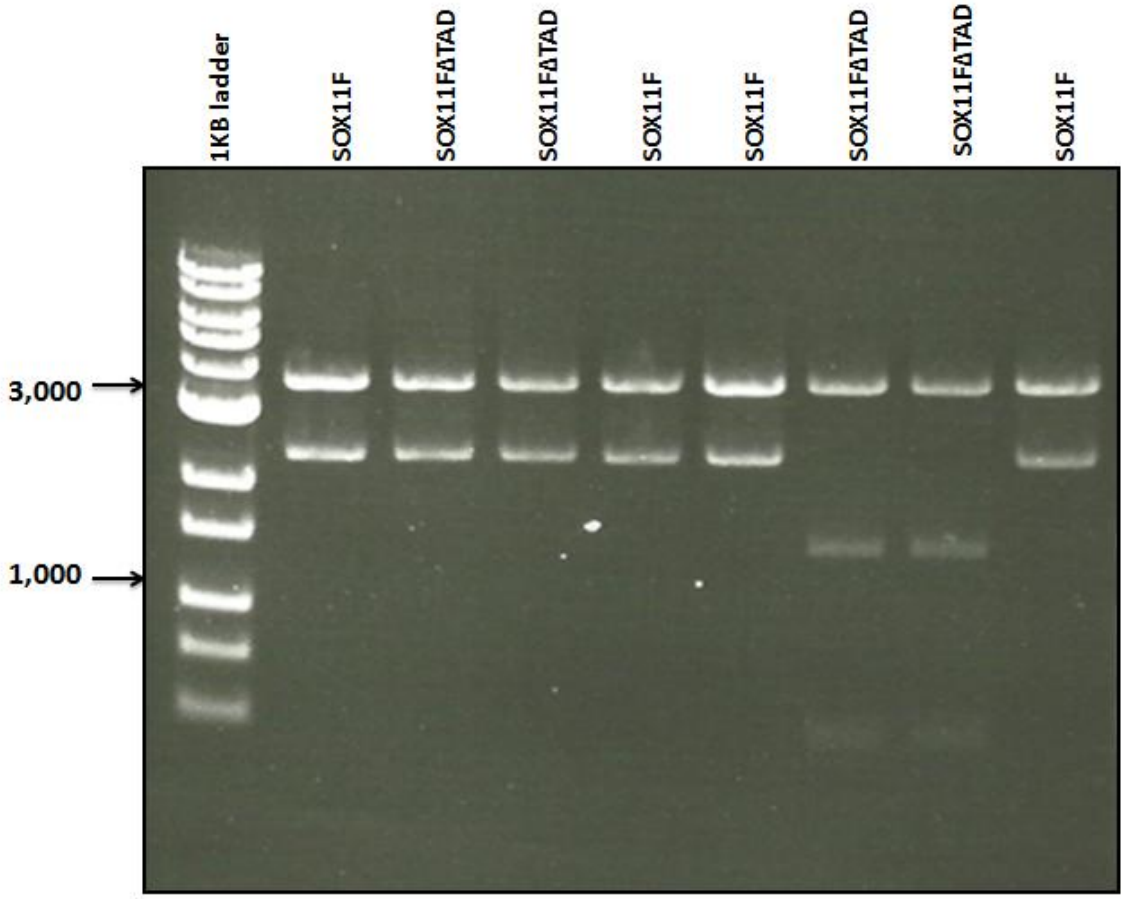

Figure 11. Agarose gel image (1% agarose gel) of DNA fragments derived from purified SOX11 plasmids: SOX11F and SOX11FΔTAD. In lanes 7 and 8, SOX11FΔTAD showed 3 bands because it was treated with three restriction enzymes including EcoRI, XhoI and NotI.

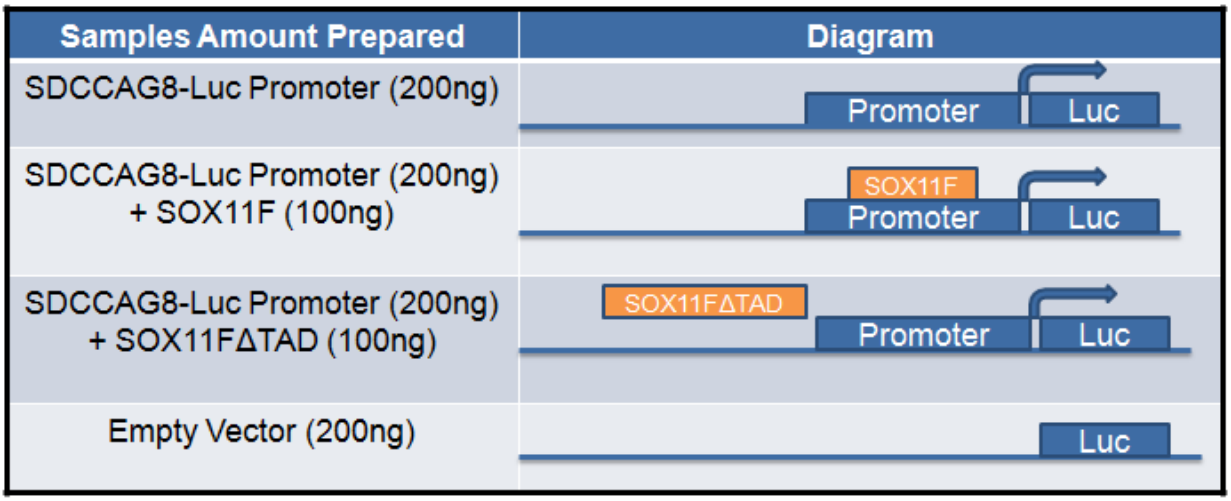

Figure 12. Luciferase assay of SDCCAG8 promoter constructs. The figure shows a schematic diagram of SOX11 binding to SDCCAG8 promoter site and the amount of SDCCAG8 promoter construct and SOX11F/SOX11FΔTAD plasmids were used in the experiments.

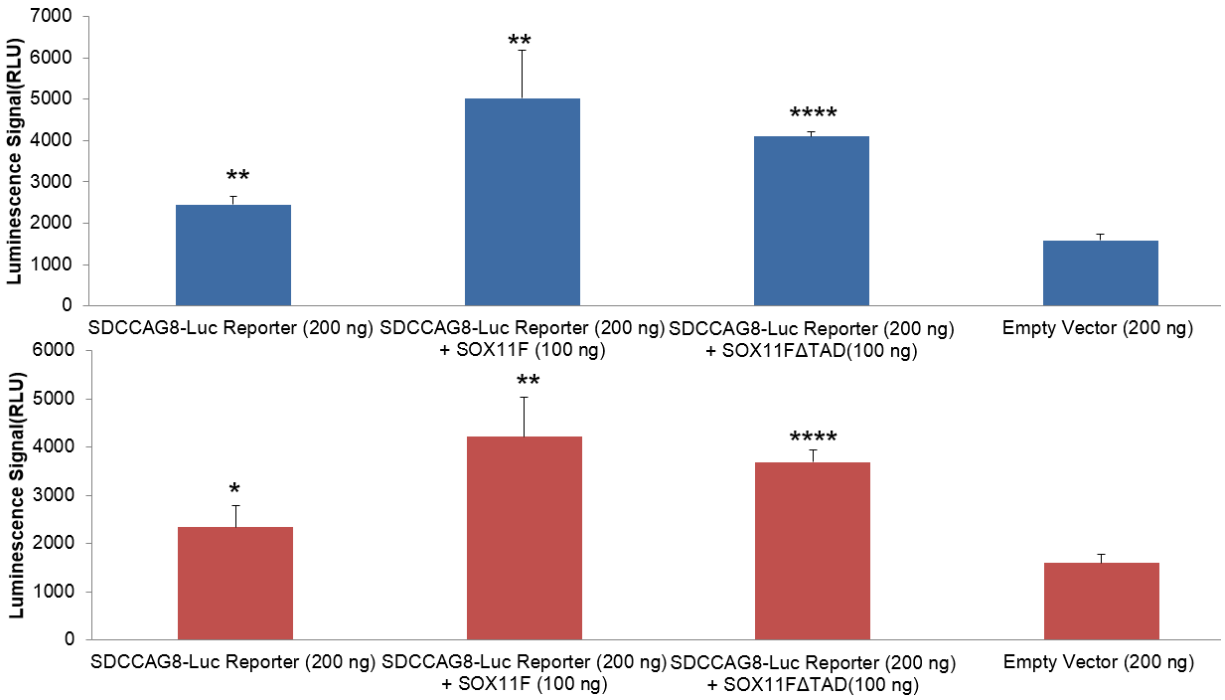

Figure 13. The measured luminescenece signal activities in (A) UM1 and (B) UM5 transfected cells. Significance is indicated by asterisks: *, P<0.05; **, P<0.01; ****, P<0.0001.

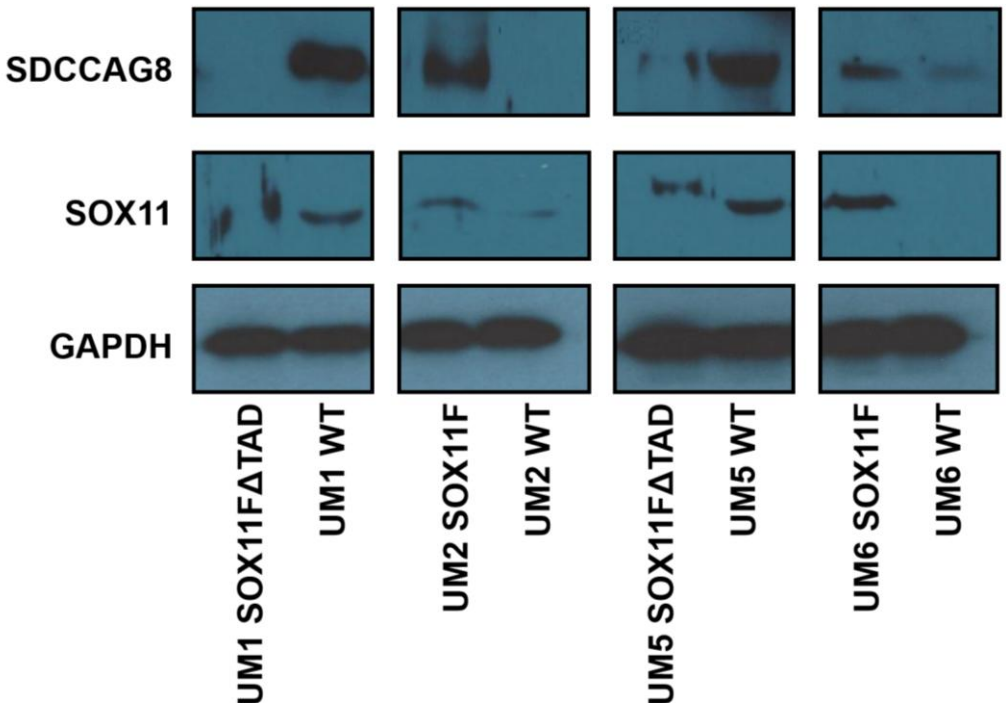

Figure 14. Western blot analysis of SDCCAG8 and SOX11 protein expression levels in UM1, UM2, UM5 and UM6 cells transfected with SOX11F or SOX11FΔTAD. Western blot analysis shows overexpressed levels of SOX11 and SDCCAG8 in UM2 and UM6 cells by administration of SOX11F plasmid as well as under-expressed levels of SDCCAG8 in UM1 and UM5 cells by administration of SOX11F FΔTAD.

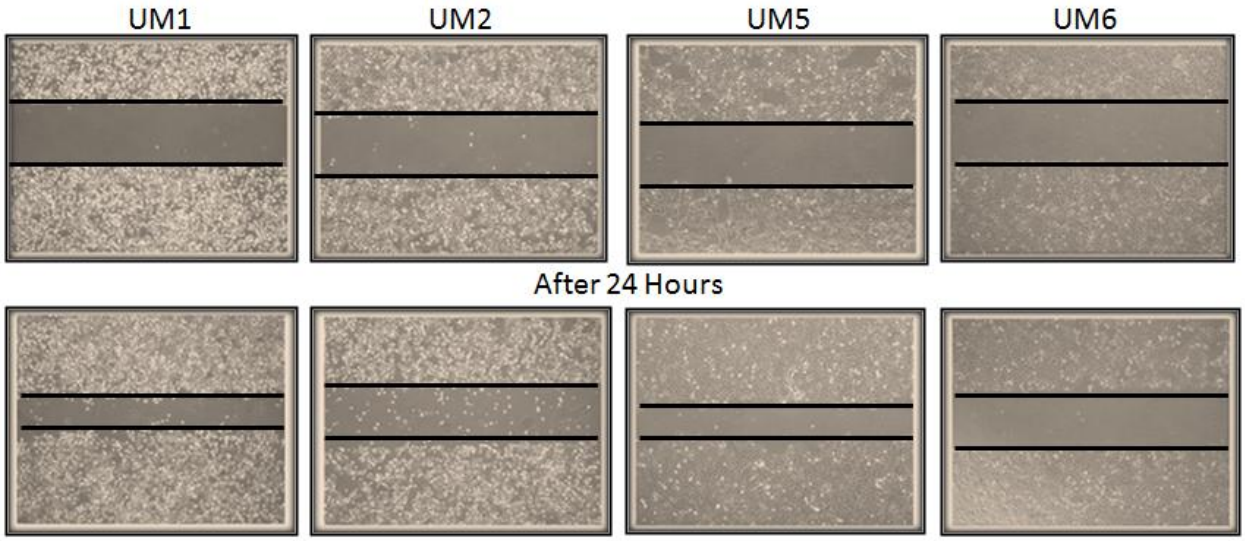

Figure 15. Different invasion ability among the 4 HNSCC cell lines. (A) UM1 and UM5 displayed a faster migration speed in wound healing experiments than UM2 and UM6 which did not migrate obviously after 24 hours. (B) Invasion assay showed a lot of UM1 and UM5 cells but not UM2 and UM6 cells invaded through the transwell.

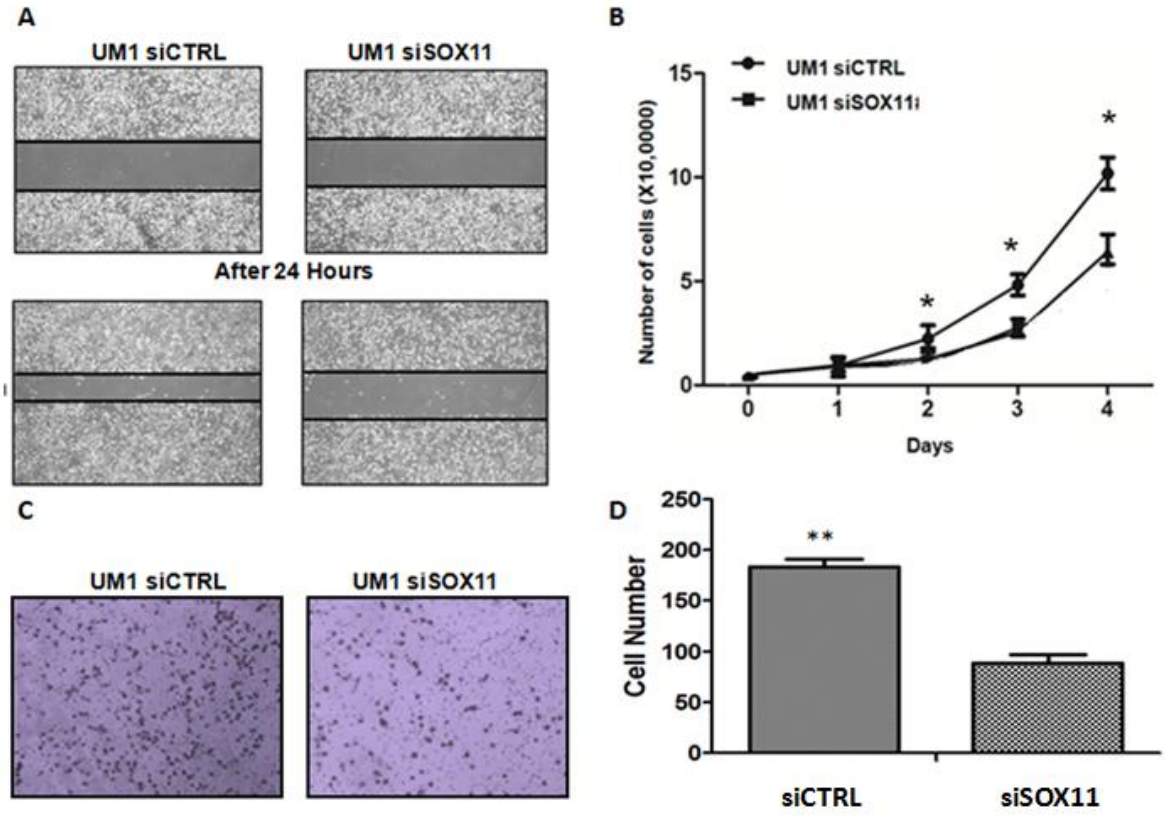

Figure 16. Down-regulated SOX11 expression in transfected UM1 cell with siSOX11. (A) Different migration gaps 24 hours after transfecting UM1 cells. (B) Proliferation curves of the transfected UM1 cells. (C)The transfected UM1 cells on the surface of the transwell chamber after staining. (D) Numbers of the invaded UM1 cells after transfection with siSOX11 (n=3). Significance is indicated by asterisks: *, P<0.05; **, P<0.01.

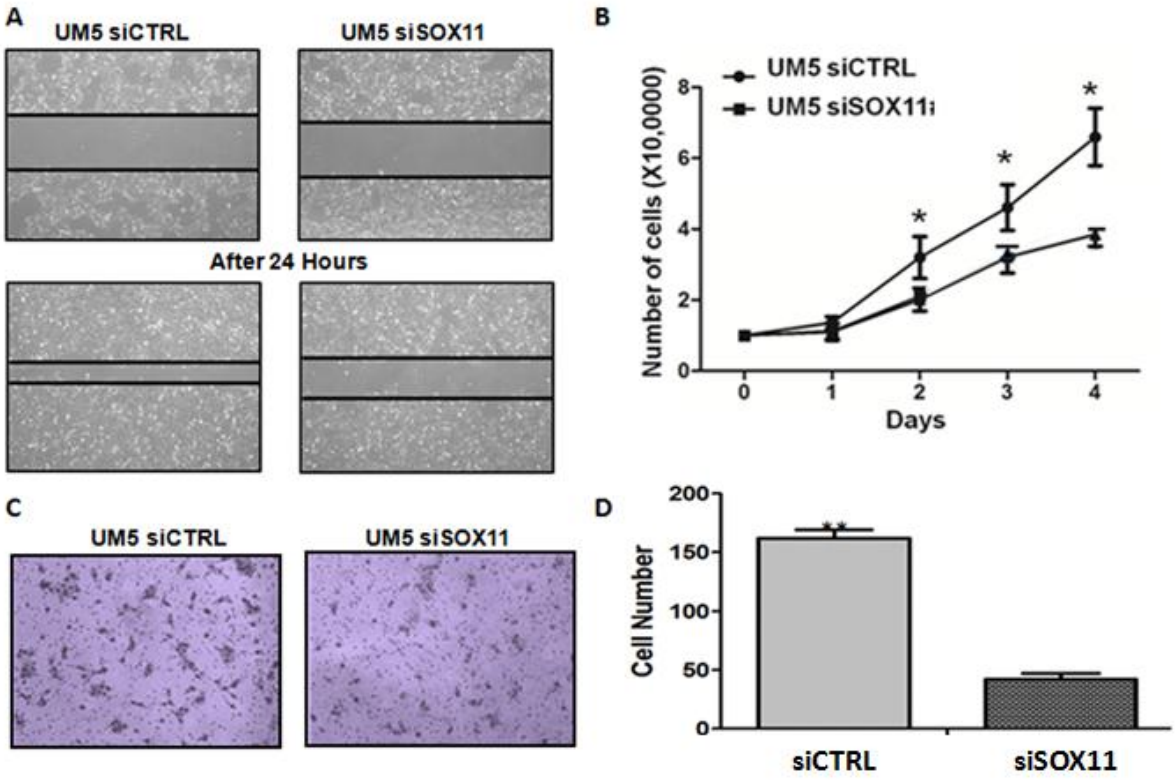

Figure 17. Down-regulated SOX11 expression in transfected UM5 cells with siSOX11. (A) Different migration gaps 24 hours after transfecting UM5 cells. (B) Proliferation curves of the transfected UM5 cells. (C) The transfected UM5 cells on the surface of the transwell chamber after staining. (D) Numbers of the invaded UM5 cells after transfection with siSOX11 (n=3). Significance is indicated by asterisks: *, P<0.05; **, P<0.01.

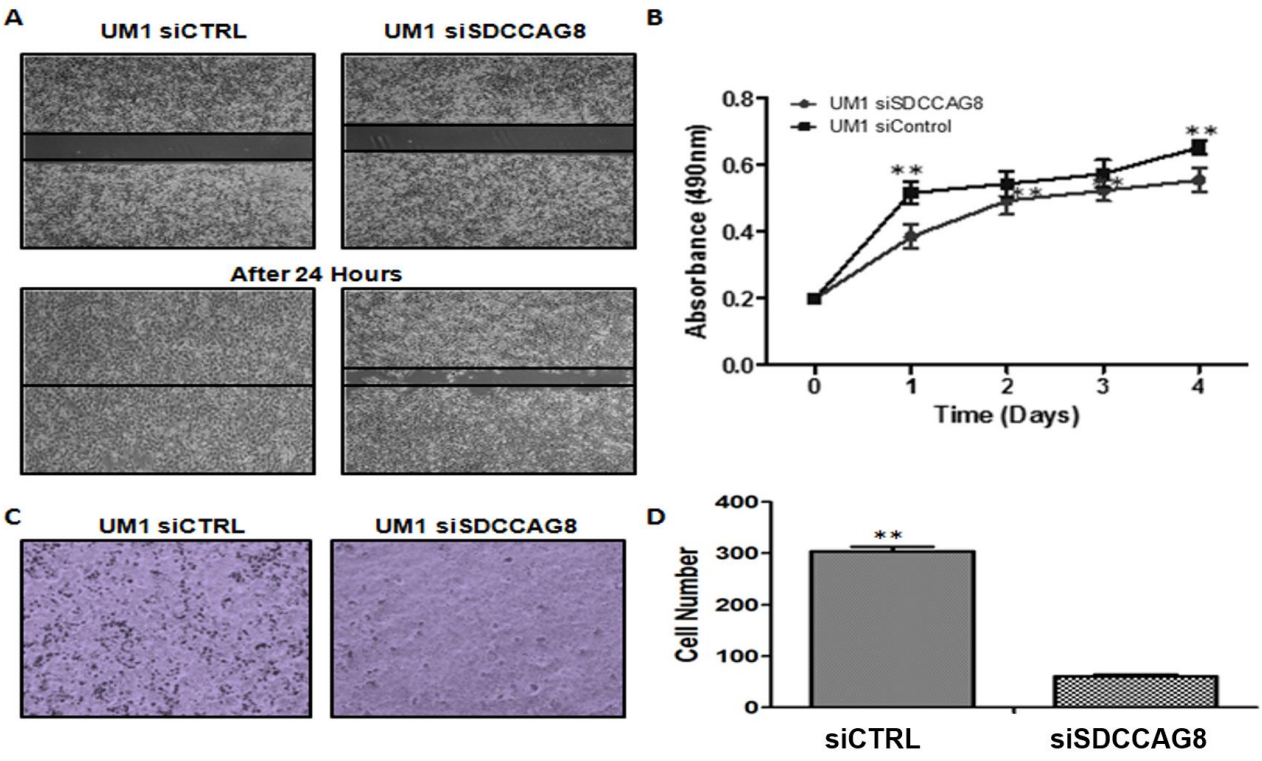

Figure 18. Down-regulated SDCCAG8 expression in transfected UM1 cells with siSDCCAG8. (A) Different migration gaps 24 hours after transfecting UM1 cells. (B) Proliferation curves of the transfected UM1 cells. (C) The transfected UM1 cells on the surface of the transwell chamber after staining. (D) Numbers of the invaded UM1 cells after transfection with SDCCAG8 (n=3). Significance is indicated by asterisks: *, P<0.05; **, P<0.01.

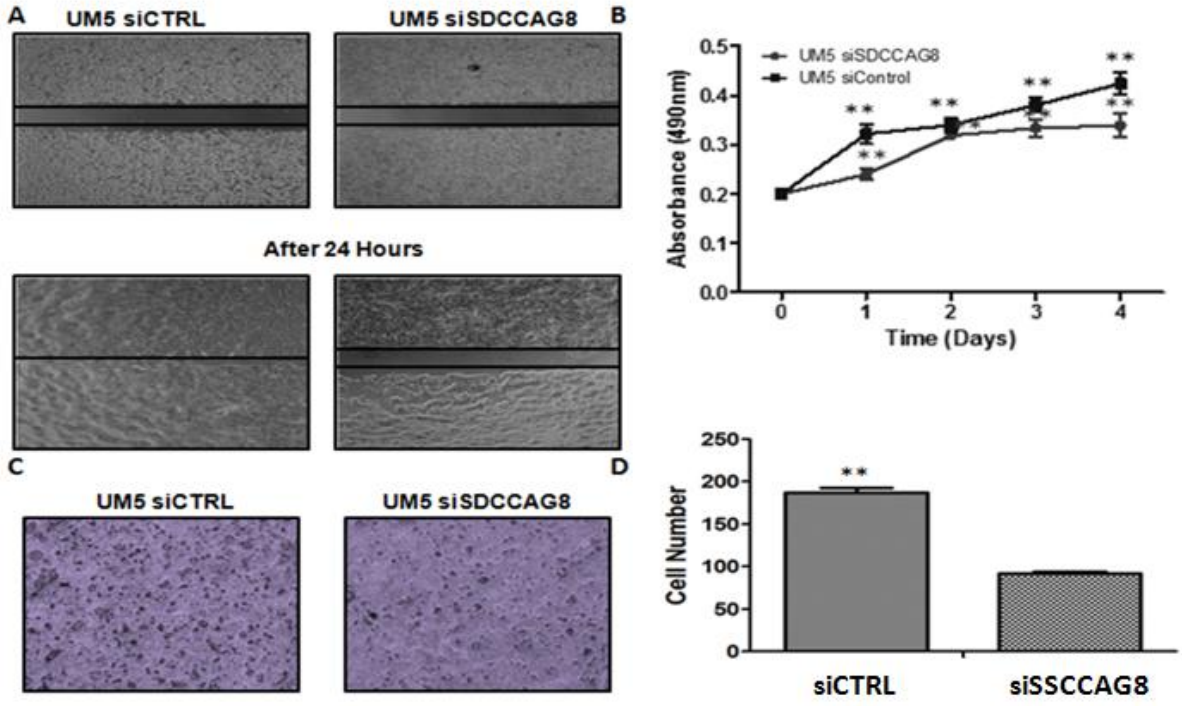

Figure 19. Down-regulated SDCCAG8 expression in transfected UM5 cells with siSDCCAG8. (A) Different migration gaps 24 hours after transfecting UM5 cells. (B) Proliferation curves of the transfected UM5 cells. (C) The transfected UM5 cells on the surface of the transwell chamber after staining. (D) Numbers of the invaded UM5 cells after transfection with SDCCAG8 (n=3). Significance is indicated by asterisks: *, P<0.05; **, P<0.01.

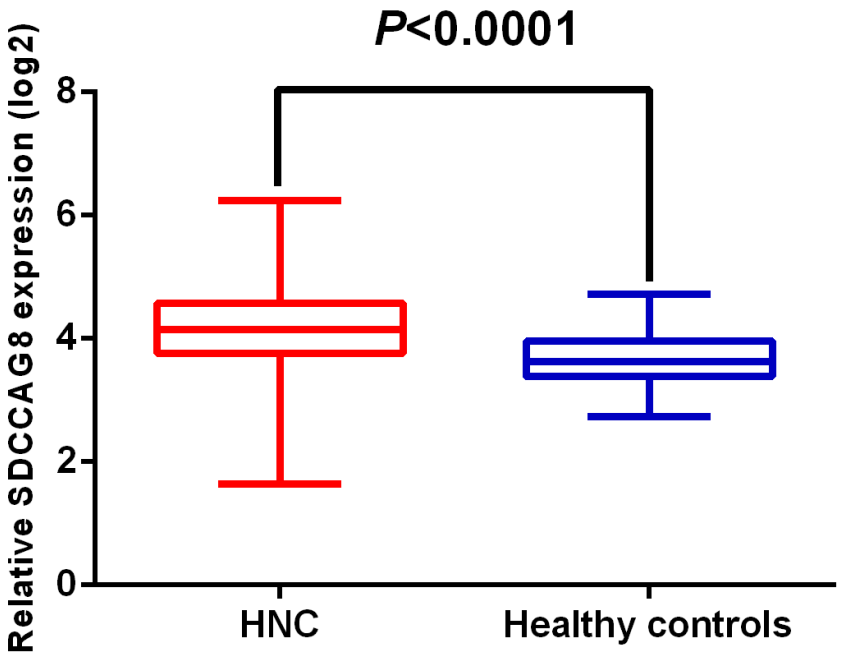

Figure 20. The gene expression level of SDCCAG8 in HNSCC patients compared to healthy controls. Head and neck cancer cell patients show significantly higher mRNA expression levels of SDCCAG8.

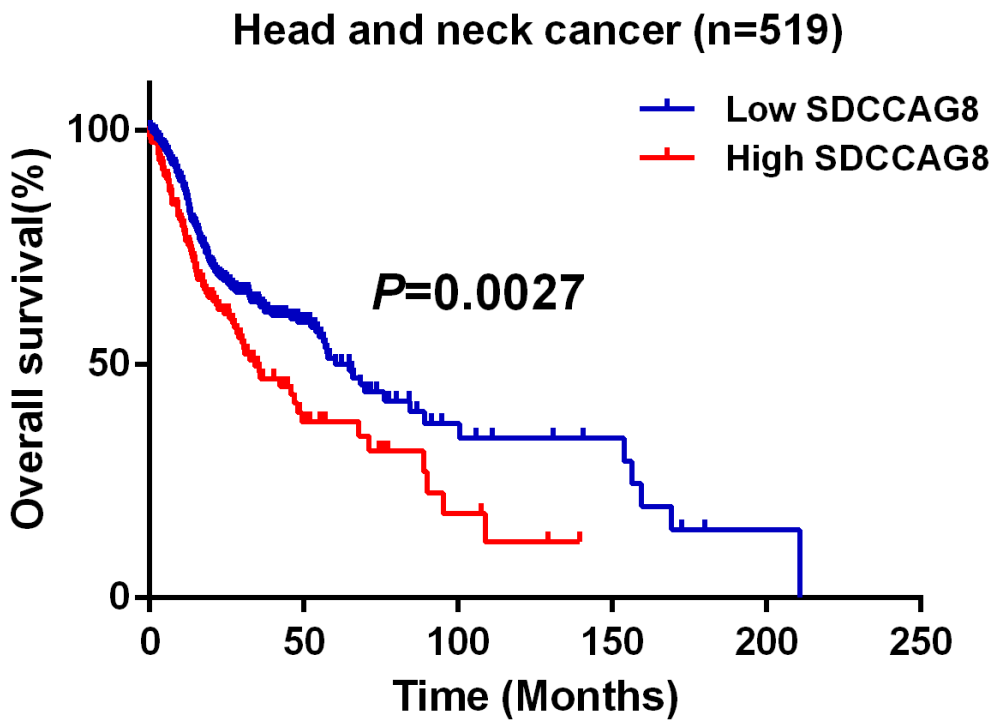

Figure 21. Overall survival rate of HNSCC patients with higher expression level of SDCCAG8. Head and cancer patients with high expression levels of SDCCAG8 show lower survival rate than the patients with low expression levels of SDCCAG8.

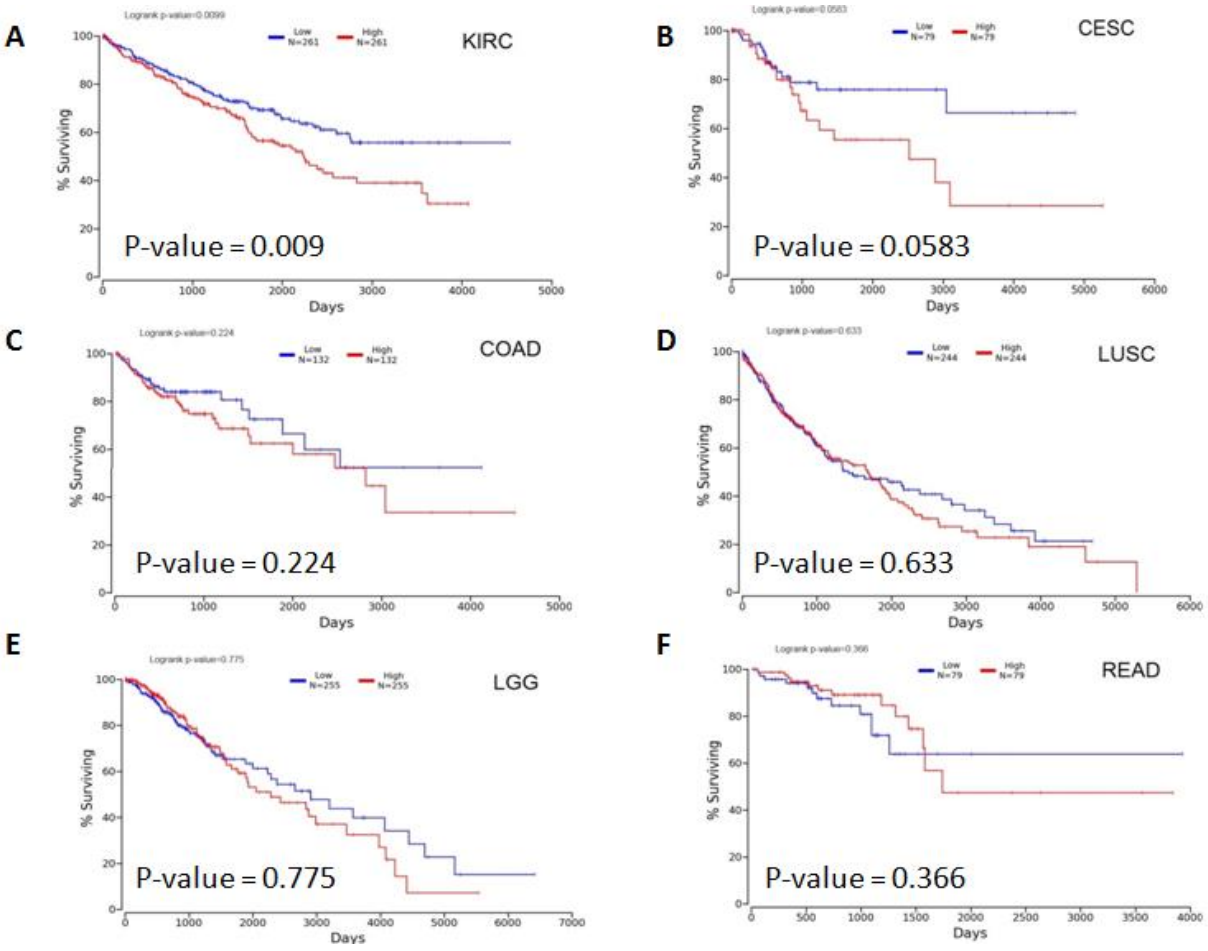

Figure 22. Overall survival rate of 6 different types of cancer patients with higher expression levels of SDCCAG8. Clinical data are for KIRC, CESC, COAD, LUSC, LGG, and READ cancer patients. KIRC and CESC patients showed association of high SDCCAG8 expression level with low survival rate; however, the association for other 4 types of cancer patients was insignificant. Data are based on the TCGA database.

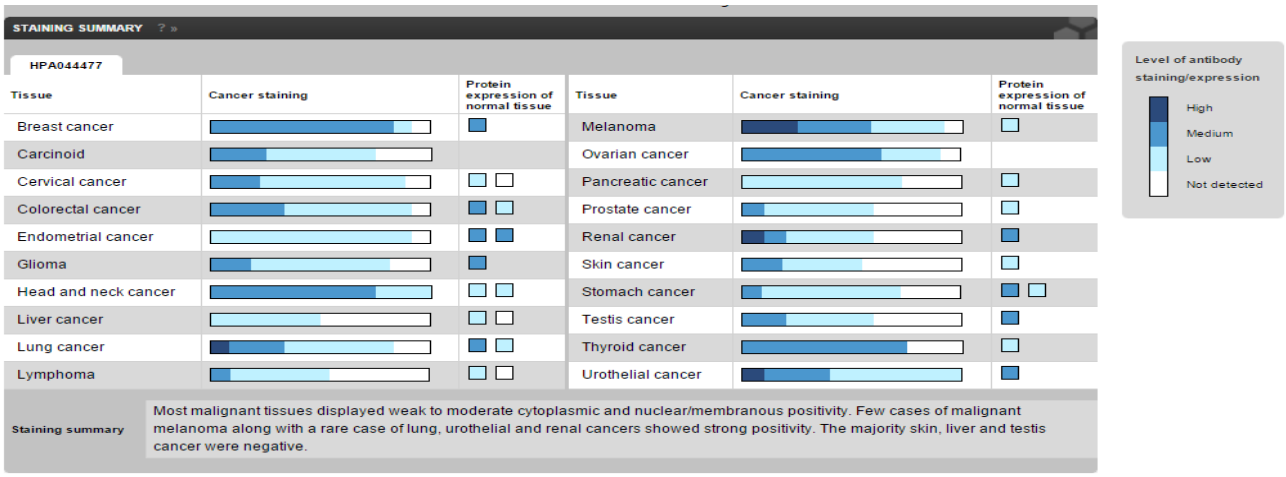

Figure 23. SDCCAG8 protein expression levels in different types of cancer as measured by immunohistochemistry (IHC). Many cancer types showed high protein expression levels of SDCCAG8 compared to normal tissues. Data was imported from the Human Protein Atlas Website.

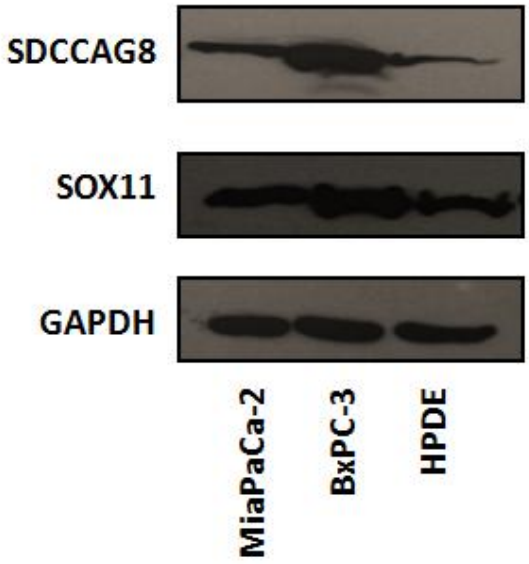

Figure 24. Protein expression of SOX11 and SDCCAG8 in pancreatic cancer cells, MiaPaCa-2 and BxPC-3. Western blot analysis showed that SDCCAG8 and SOX11 expression levels were upregulated in pancreatic cancer cells, MiaPaCa-2 and BxPC-3, compared to normal HPDE cells.

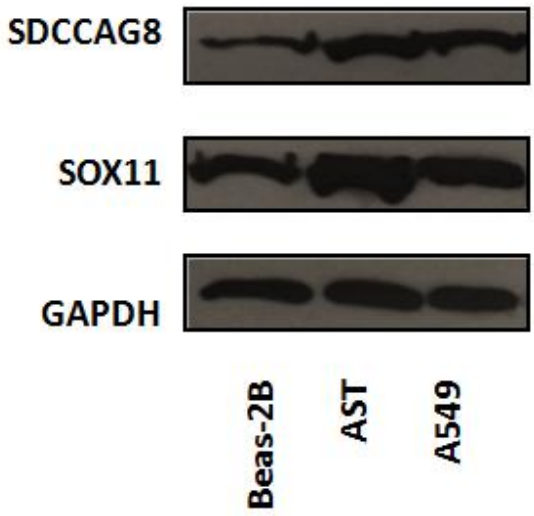

Figure 25. Protein expression of SOX11 and SDCCAG8 in lung cancer cells, AST and A549. Western blot analysis showed that SDCCAG8 and SOX11 protein expression levels were upregulated in lung cancer cells, AST and A549 when compared to normal lung cells.

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