Characterization of Opa interacting protein 5 as a new biomarker and therapeutic target for oral cancer

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Abstract. Oral cancer is a leading cause of cancer-related death worldwide. Current treatment for oral cancer includes surgery, radiotherapy, and chemotherapy; however, their effectiveness is still limited. To identify a new prognostic biomarker and therapeutic target for oral cancer, the Opa interacting protein 5 (OIP5), which plays an essential role in the proper segregation of chromosomes, was examined. Immunohistochemical staining using tissue microarrays indicated that OIP5 was expressed in 120 of 164 (73.2%) oral cancers but was minimally expressed in normal oral tissues. OIP5 expression was significantly associated with poor prognosis in patients with oral cancer. Overexpression of OIP5 enhanced the growth of oral cancer cells, whereas OIP5 knockdown using small interfering RNAs (siRNAs) significantly inhibited cell growth through cell cycle arrest at the G2/M phase. Suppression of OIP5 expression also induced senescence of oral cancer cells. Overall, the findings of the present study suggest that OIP5 may be a candidate prognostic biomarker and therapeutic target in oral cancer.

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Abbreviations: BSA, bovine serum albumin; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; H&E, hematoxylin and eosin; HJURP, Holliday junction recognition protein; HNSCC, head and neck squamous cell carcinoma; HOMKs, human oral mucosa keratinocytes; OIP5, opa interacting protein 5; SAHF, senescence-associated heterochromatic foci; SaS, StatView statistical

Key words: OIP5, oral cancer, biomarker, therapeutic target, oncoantigen

Introduction

Oral cancer is defined as a group of cancers originating from the oral cavity or lip and represents one of the most common malignancies worldwide (1). In 2020, a total of 377,713 new cases were diagnosed globally, and 177,757 patients succumbed to this disease (2). Oral squamous cell carcinoma is the most common pathological type of oral cancer; it represents more than 90% of the cases (3). Some modifiable risk factors have been reported to contribute to the development of oral cancer, including tobacco smoking, alcohol consumption, betel quid, human papillomavirus infection, and poor oral hygiene (4). The overall 5-year survival rate for oral cancer is 75-90% for stage I and 10-22% for stage IV (5). Currently, the standard treatment for oral cancer includes surgical resection, radiotherapy, and chemotherapy with molecular-targeted therapy and immunotherapy using immune checkpoint inhibitors. These treatments are associated with various adverse events, and many patients develop drug resistance during treatment. Thereby, local and regional recurrences are reported in 90% of oral cancers after surgery and radiotherapy (6,7). At present, clinical trials for head and neck squamous cell carcinoma (HNSCC) treated with immune checkpoint inhibitors are ongoing. Anti-PD-1 antibodies, such as nivolumab and pembrolizumab, have been approved by the US Food and Drug Administration for patients with relapsed or metastatic HNSCC, as well as cisplatin-resistant tumors (8). Anti-epidermal growth factor receptor (EGFR) antibodies, such as cetuximab, have been approved for use in combination with radiation therapy in patients with HNSCC (9). Despite these developments, efficacy remains limited, and adverse events have been reported. Therefore, further development of novel therapeutic strategies, such as personalized therapies based on cancer biomarkers and novel molecular-targeted therapies with no or low side effects in patients with oral cancer, are needed.

To identify molecular targets for the diagnosis and treatment of cancer, a genome-wide expression profile analysis and subsequent tissue microarray analysis with solid tumor tissues and normal tissues were performed. Several oncoantigens involved in the development and/or progression of solid

cancers were isolated (10-36). This systematic strategy identified Opa interacting protein 5 (OIP5) as a candidate molecular target overexpressed in the majority of oral cancers.

OIP5 encodes a 25-kDa protein and was initially identified in a yeast two-hybrid system (37). OIP5 contains a Yippee domain (38) and a coiled-coil domain (39), suggesting that it has a role in cell proliferation and mitosis-associated processes. OIP5 is a component of the Mis18 complex, which consists of three subcomplexes: Mis18α, Mis18β (OIP5), and Mis18BP1. OIP5 interacts with Holliday junction recognition protein (HJURP) and mediates the loading of newly synthesized centromeric protein A (CENP-A) on the centromere by the Mis18 complex, which is required for accurate chromosome segregation (40,41). Previous findings indicate that OIP5 is overexpressed in various types of human cancers, such as lung and esophageal carcinomas (29), bladder cancer (42), gastric cancer (43), and glioblastoma (44); however, its role in oral carcinogenesis and the clinical significance of OIP5 protein as a tissue biomarker for oral cancer has yet to be clarified.

In the present study, the aim was to determine whether OIP5 plays a significant role in the development of oral cancer and is a putative cancer biomarker and therapeutic target.

Materials and methods

Oral cancer cell lines and clinical tissue samples. The following oral cancer cell lines were used in this study: four tongue squamous cell carcinoma cell lines (SCC9, CAL 27, HSC3, and HSC4), one pharynx squamous cell carcinoma (FaDu), one mouth squamous cell carcinoma (HSC2), and one gingival squamous cell carcinoma (Ca9-22). The human oral mucosa keratinocyte (HOMK) cell line, used as a normal control, was purchased from the Cell Research Corporation (Singapore). The histology of the cell lines and resources are summarized in Table I. All cells were grown in monolayer in an appropriate medium. The oral cancer cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2 in a medium containing 10% fetal bovine serum (FBS) and antibiotics (Thermo Fisher Scientific, Inc.). HOMK cells were grown in a medium supplemented with EpiLife Defined Growth Supplement. In total, 13 oral squamous cell cancer tissue samples (5 females, 8 males; median age, 63 years; age range, 45-74 years) were obtained from ProteoGenex (Inglewood, USA), and a normal tongue tissue was obtained from Clontech (USA). A total of 164 existing formalin-fixed primary oral cancer tissue samples and adjacent normal tissue samples obtained from patients with oral cancer who had undergone surgery between 2004 and 2012 and treated with adjuvant or neoadjuvant chemotherapy at Kumamoto University were used in this study. The clinical stage of the tumor samples was judged according to the Union for International Cancer Control TNM classification (7th Edition of UICC; http://www.inen.sld.pe/portal/documentos/pdf/educacion/13072015_TNM%20Classification.pdf).

The study protocol and the use of existing clinical materials were approved by the Ethics Committees [Kumamoto University; Shiga University of Medical Science (no. G2009-163)] based on the national ethical guidelines for human subjects. It was confirmed that this study was fully ethically compliant, and that informed consent was waived by the ethics committee due to the retrospective nature of the study and the national ethical guidelines.

Real-time PCR. Total RNA was extracted from cultured oral cancer cells and oral cancer tissues using the Maxwell 16 LEV Simply RNA purification kit (Promega Corp.) according to the manufacturer's protocol. The RNA was reverse-transcribed into cDNA using the PrimeScript RT Master Mix (Takara Bio Inc.). Gene expression was measured by real-time PCR using TaqMan Universal Master Mix II and TaqMan assays on a StepOnePlus thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The reaction conditions were as follows: initial denaturation for 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 95˚C for 1 sec and 60˚C for 20 sec. Each experiment was performed in triplicate. *ACTB* (Hs01060665_g1) as an internal control and *OIP5* (Hs00944000_g1) primers (Applied Biosystems) were used for the PCR reaction. Relative *OIP5* mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (45).

Western blot analysis. To prepare whole cell lysates, the cells were lysed on ice in Pierce RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with 1% protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). After homogenization, the cell lysates were incubated on ice for 30 min and centrifuged at 14,000 x g for 15 min to separate the supernatant from the cellular debris. The concentration of total protein was estimated using the Qubit Protein Assay Kit (Thermo Fisher Scientific, Inc.), and the proteins were mixed with SDS sample buffer, boiled at 100˚C for 5 min, and incubated at room temperature for 5 min before loading into 12% Mini-Protean® TGX gels (Bio-Rad Laboratories). After electrophoresis at 200 V for 30 min, the proteins were transferred to Trans-Blot® Turbo 0.2 μm PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat milk in TBST at room temperature for 1 h. A commercially available rabbit polyclonal antibody to human OIP5 (dilution, 1:1,000; cat. no. 12142-1-AP; ProteinTech Group) was incubated with the membrane at room temperature for 1.5 h, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare, UK) for 1 h at room temperature. Protein bands were visualized by enhanced chemiluminescence using the Fusion Solo S system (Vilber Lourmat, France).

Immunocytochemistry. The cultured cells were seeded into Lab-Tek II chamber slides (Nalge Nunc Int.). After fixation in 4% formaldehyde solution for 15 min at room temperature, the cells were washed twice with PBS, and the upper part of the well was then removed. To render the cells permeable, PBS containing 0.1% Triton X-100 was added for 2 min at room temperature. The cells were covered with 3% bovine serum albumin (BSA) for 30 min at room temperature to block nonspecific binding. Then, the membrane was incubated with rabbit anti-OIP5 antibody (ca. no. 12142-1-AP; ProteinTech Group) in PBS containing 1% BSA and 0.1% Tween-20 at 4˚C overnight in a wet box. After washing with PBS, a goat anti-rabbit secondary antibody conjugated to Alexa 488

Table I. List of oral cancer cells and oral mucosa keratinocytes used in the present study.

Cell line	Histology	Resource	Catalog no.
FaDu	Squamous cell carcinoma of the pharynx	ATCC	HTB-43
SCC9	Squamous cell carcinoma of the tongue	ATCC	CRL-1629
CAL 27	Squamous cell carcinoma of the tongue	ATCC	CRL-2095
Ca9-22	Gingival squamous cell carcinoma	RIKEN BRC	RCB1976
HSC2	Squamous cell carcinoma of the mouth	RIKEN BRC	RCB1945
HSC3	Squamous cell carcinoma of the tongue	RIKEN BRC	RCB1975
HSC4	Squamous cell carcinoma of the tongue	RIKEN BRC	RCB1902
HOMK	Human oral mucosa keratinocyte	Cell Research Corporation Pte Ltd.	hOMK100

ATCC, American Type Culture Collection; RIKEN, BRC, RIKEN BioResource Center.

(Thermo Fisher Scientific, Inc.) was added for 1 h at room temperature in a wet box in the dark. Nuclei were stained with Vectashield Mounting Medium containing DAPI (Vector Laboratories). OIP5 antibody staining was visualized using fluorescence microscopy (BZ-X710, Keyence, Japan).

Immunohistochemistry and tissue microarray analysis. Tumor tissue microarrays were constructed according to previously published procedures using 164 formalin-fixed primary oral cancer tissues that were surgically resected (63 patients treated with curative surgery and neoadjuvant chemotherapy, and 101 patients treated with curative surgery and adjuvant chemotherapy). The tissue areas selected for sampling were determined by visual alignment with the corresponding hematoxylin and eosin (H&E)-stained sections on the slides. Three, four, or five tissue cores (diameter, 0.6 mm; height, 3-4 mm) taken from donor tumor blocks were placed into recipient paraffin blocks using a tissue microarrayer (Beecher Instruments Inc.). A core of normal oral epithelial tissue was punched in each case, and 5-μm sections of the resulting microarray blocks were used for immunohistochemical analysis.

Tissue microarray slides were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. For antigen retrieval, the slides were boiled in Target Retrieval Solution (pH 6.0) (Dako; Agilent Technologies, Inc.). A rabbit polyclonal anti-OIP5 antibody (cat. no. 12142-1-AP; ProteinTech Group) was added to each slide and incubated overnight after blocking endogenous peroxidase activity in a 0.3% hydrogen peroxide/methanol mixed solution and nonspecific protein binding sites in protein blocking buffer (cat. no. X0909; Dako Cytomation). After primary antibody incubation, sections were incubated with an HRP-labeled anti-rabbit IgG secondary antibody (Dako Cytomation) for 30 min. Chromogen substrate was added to visualize labeled protein, and the specimens were counterstained with H&E. Because the intensity of staining within each tumor tissue core was mostly homogeneous, positivity for OIP5 was assessed semi-quantitatively by three independent investigators without prior knowledge of the clinicopathological data, each of whom recorded staining intensity as positive or negative. Cases were accepted as positive if at least two investigators independently scored them as such.

Cell growth assay. The SCC9 and HSC2 cell lines that did not express endogenous OIP5 were plated at $0.8x10^6$ cells/100-mm dish and transfected with OIP5-expressing plasmid (pCMV6-Entry-Myc-DDK, OriGene, USA) or mock plasmids by FuGENE 6 (Promega Corp.). Cell viability was assessed using MTT assay (Cell Counting Kit-8; Dojindo Laboratories, Japan) 5 days after plasmid transfection.

RNA interference assay. To investigate the biological function of OIP5 in oral cancer cells, small interfering RNAs (siRNAs) (Sigma-Aldrich; Merck KGaA) were transfected into Ca9-22 and CAL 27 cells using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequences targeting each gene were as follows: si-*OIP5*-#1, 5'-GGU UCACUCAAAGGCAGUA-3'; si-*OIP5*-#2, 5'-CUAUUU ACCUUUAGGCUGA-3'; control 1: si-*LUC*, 5'-CGUACG CGGAAUACUUCGA-3'; and control 2: si-*EGFP*, 5'-GAA GCAGCACGACUUCUUC-3'. Cell numbers and viability were measured by colony formation and MTT assays using Cell Counting Kit-8 solution (Dojindo Laboratories) 6 days after transfection.

Flow cytometry. Cell cycle analysis was performed using a Cycletest Plus DNA reagent kit (BD Biosciences). Ca9-22 and CAL 27 cells, for measuring DNA ploidy, were harvested by trypsinization 72 h after si-OIP5-#2 or si-LUC transfection and suspended at a concentration of $1x10^6$ cells/ml. The sample was filtered through a 50-μm nylon mesh, kept on ice, and protected from light. Cell cycle analysis was conducted within 3 h using a flow cytometer (BD FACSCanto™ II. The DNA content was measured in cells selected from at least 20,000 ungated cells.

Live-cell imaging. The Ca9-22 and CAL 27 cells transfected with si-*OIP5*-#2 or si-*LUC* were seeded on 35-mm glass dishes in an appropriate culture medium containing 10% FBS. The Evos FL Auto Cell Imaging System (Life Technologies; Thermo Fisher Scientific, Inc.) time-lapse microscopy was used to monitor changes in cell morphology and death after transfection with OIP5 siRNA. Live-cell imaging was initiated 72 h after si-*LUC* and si-*OIP5* transfection, and images were captured every 15 min for 24 h up to 96 h.

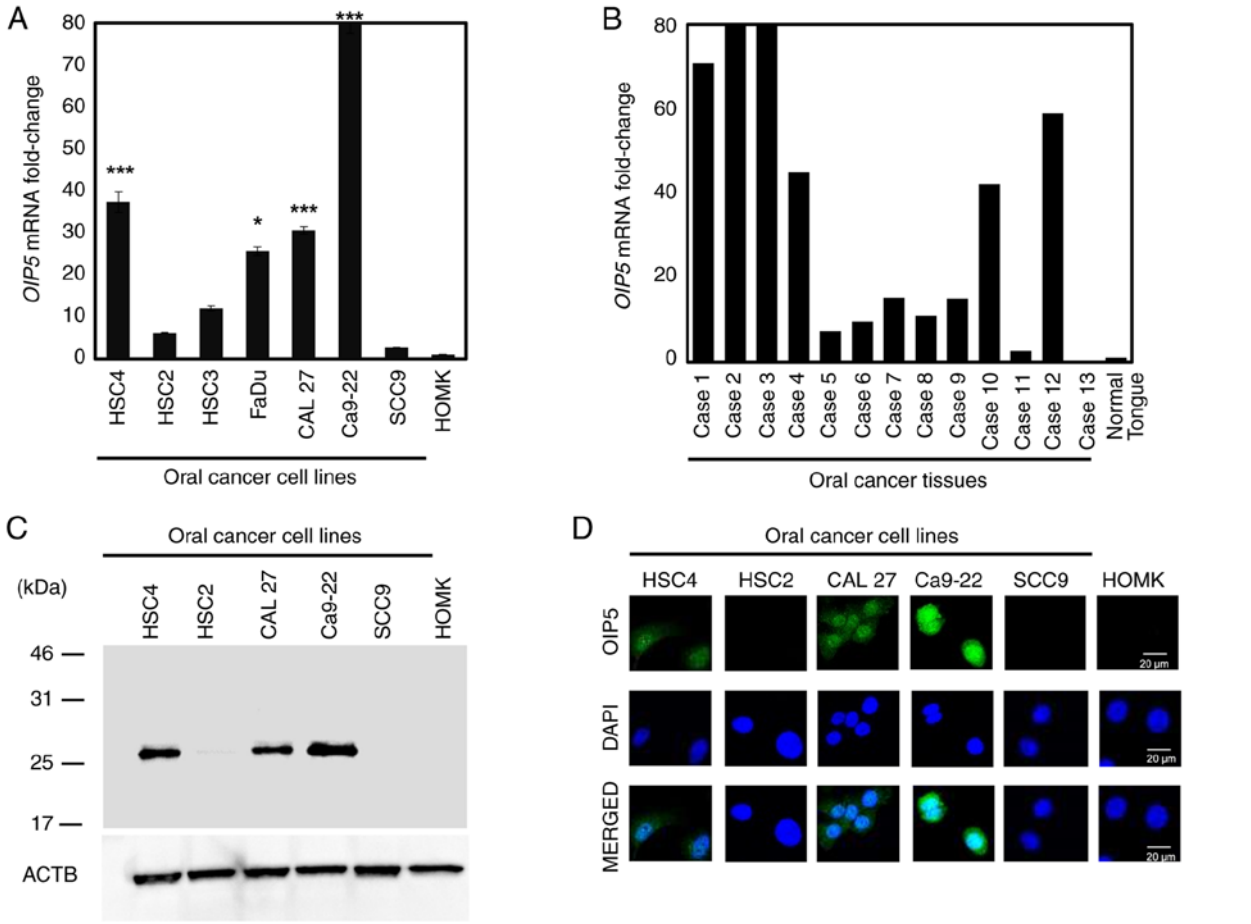

Figure 1. Expression of OIP5 in oral cancer cell lines and tissues. (A and B) *OIP5* mRNA expression was measured using RT-qPCR in oral cancer cell lines, a normal oral epithelial cell, human oral cancer tissues, and normal oral tissues. *P<0.05 vs. HOMK. ***P<0.001 vs. HOMK. (C) OIP5 protein expression was measured using western blot analysis in oral cancer cell lines. (D) Subcellular localization of endogenous OIP5 protein in oral cancer cell lines. Cells were stained with a rabbit polyclonal anti-OIP5 antibody (green) and DAPI (blue). HOMKs, human oral mucosa keratinocytes; OIP5, opa interacting protein 5.

Senescence assay. The cells were plated into 35-mm-diameter culture dishes up to 5 days after siRNA transfection into Ca9-22 and CAL 27 cells and stained with the senescence β-Galactosidase Staining Kit #9860 (Cell Signaling Technology, Inc.). The plates were incubated overnight at 37˚C in a non-CO_2 containing dry incubator. The percentage of senescence-associated β-galactosidase (SA-β-gal) positivity per 50 cells observed under a light microscope was calculated. Senescence-associated heterochromatic foci (SAHF) were detected by DAPI staining 96 h after siRNA transfection.

Statistical analysis. Statistical analyses were performed using the StatView 5.0 Statistical Program (SAS Institute, Inc.). The significance test analyzing the difference between two groups of cell-based assays was performed using the Student's t-test. One-way ANOVA followed by Tukey's post hoc test was performed to compare the means of each group with those of every other group when performing multiple comparisons. Results are presented as mean ± standard deviation. Each experiment was performed in triplicate. We used contingency tables to analyze the relationship between OIP5 expression and clinicopathological variables (e.g., sex, age, primary tumor region, and pathological TNM stage) in patients with oral cancer. Tumor-specific survival curves were generated on

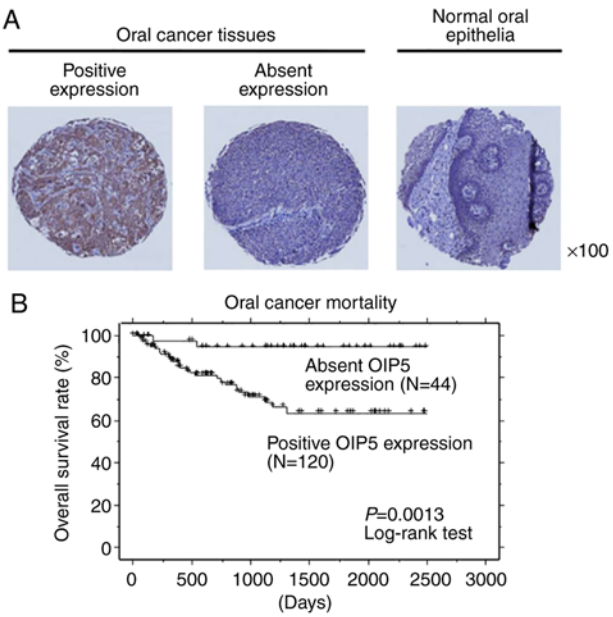

Figure 2. Correlation of OIP5 expression with poor clinical outcome in patients with oral cancer. (A) Immunohistochemical staining of OIP5 protein for positive and negative OIP5 expression in representative oral cancer tissues and a normal oral epithelial tissue (original magnification, x100). (B) Kaplan-Meier analysis of survival in patients with oral cancer with or without OIP5 expression. OIP5, opa interacting protein 5.

Table II. Association between OIP5 protein expression in oral cancer tissues and patient characteristics (N=164).

Parameters	Total	Positive OIP5 expression	Negative OIP5 expression	P-value (positive vs. negative)
Total number of specimens	164	120	44	
Treatment				0.8566
Neoadjuvant	63	47	16	
Adjuvant	101	73	28	
Sex				0.0202[a]
Male	94	62	32	
Female	70	58	12	
Age (years)				0.4733
<65	66	46	20	
≥65	98	74	24	
Region				0.0029[a]
Tongue	80	50	30	
Others[b]	84	70	14	
pT factor				0.2747
T1-T2	103	72	31	
T3-T4	61	48	13	
pN factor				0.3225
N0	120	85	35	
N1-N2	44	35	9	

[a]P<0.05, Fisher's exact test; [b]gingiva, buccal mucosa, etc. OIP5, opa interacting protein 5.

Table III. Cox proportional hazards model analysis of prognostic factors in the patients with oral cancer.

Variables	HR	95% CI	Unfavorable/Favorable	P-value
Univariate analysis				
OIP5 expression	7.482	1.773-31.577	Positive/Negative	0.0061[a]
Age (years)	1.979	0.904-4.331	≥65/<65	0.0877
Sex	1.831	0.893-3.755	Female/Male	0.0986
Region	1.538	0.745-3.176	Others[b]/Tongue	0.2443
T factor	1.461	0.709-3.011	T3-T4/T1-T2	0.3035
N factor	2.977	1.451-6.108	N1-N2/N0	0.0029[a]
Treatment	1.122	0.587-2.532	Neoadjuvant/Adjuvant chemotherapy	0.5957
Multivariate analysis				
OIP5 expression	6.907	1.634-29.204	Positive/Negative	0.0086[a]
N factor	2.699	1.315-5.542	N1-N2/N0	0.0068[a]

[a]P<0.05; [b]gingiva, buccal mucosa, etc. HR, hazard ratio; CI, confidence interval; OIP5, opa interacting protein 5.

the date of surgery to the time of death from oral cancer or last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and OIP5 expression in oral tumors. The differences in survival times among the patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazard regression model to determine the association between clinicopathological variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors, including OIP5 positivity, age, sex, primary region, pT factor, pN factor, and treatment (curative surgery with adjuvant chemotherapy vs. curative surgery with neoadjuvant chemotherapy). Second, a multivariate Cox analysis was performed in a stepwise fashion in which OIP5 positivity was forced into the model along with each significant variable. As significant prognostic factors were continually added to the model, P<0.05 was considered statistically significant and an independent factor.

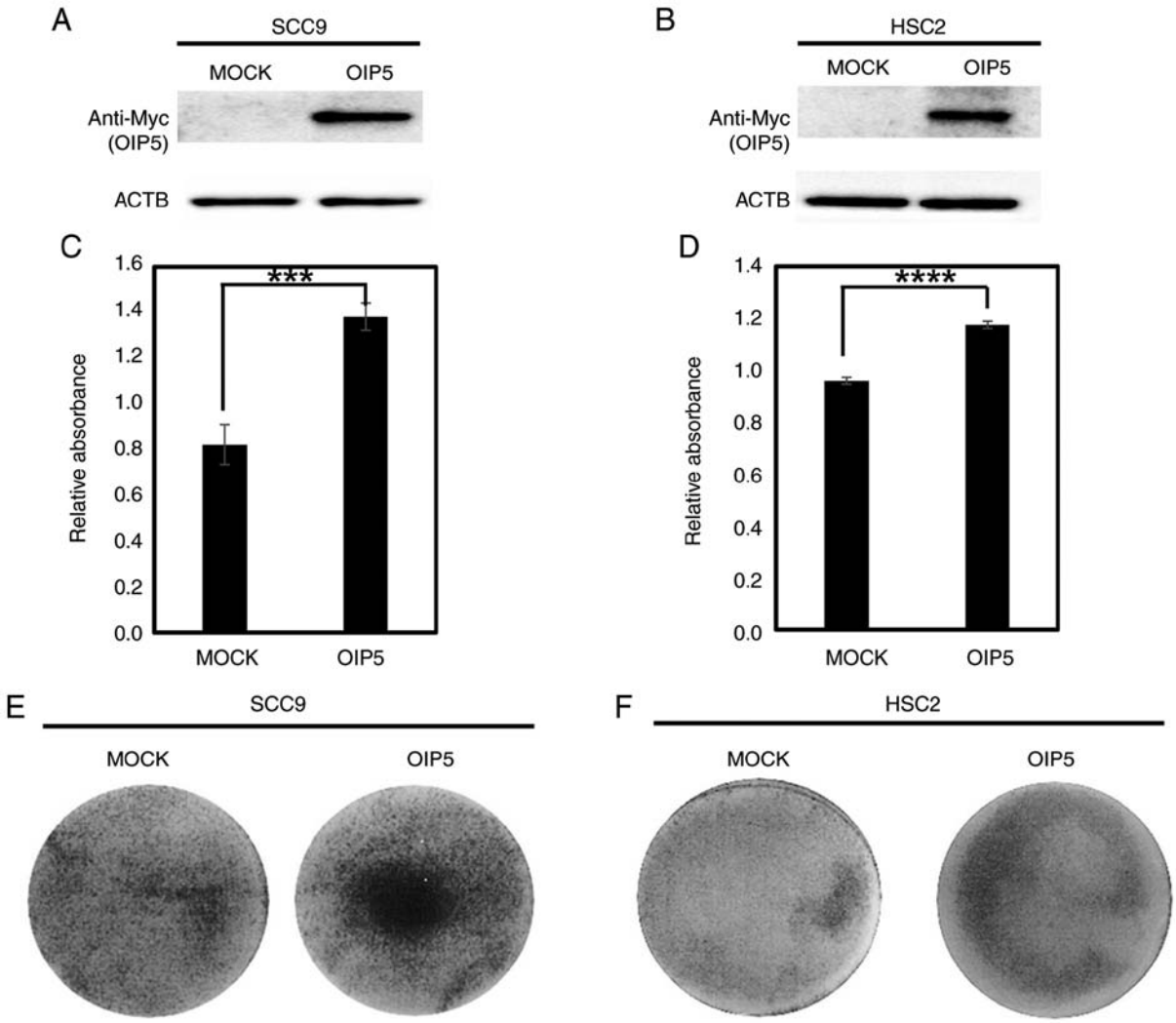

Figure 3. Promotion of growth in oral cancer cells after exogenous expression of OIP5. (A and B) Exogenous expression of Myc-tagged-OIP5 protein in OIP5-nonexpressing SCC9 and HSC2 cells after transfection with OIP5-expressing plasmids or mock plasmids as detected using western blot analysis. (C and D) Cell viability of SCC9 and HSC2 cells transfected with OIP5-expressing plasmids or mock plasmids as detected using MTT assay. (E and F) Colony formation assay of SCC9 and HSC2 cells after OIP5 overexpression. ****P<0.0001 vs. si-control group. ***P<0.001 vs. si-control group. OIP5, opa interacting protein 5.

Database analysis. The gene expression and signal pathways related to OIP5 were screened using the ONCOMINE database (https://www.oncomine.org/resource/login.html), Gene Set Enrichment Analysis (GSEA) database (https://www.gsea-msigdb.org/gsea/msigdb/search.jsp), BioGPS database (http://biogps.org/#goto=welcome), and UALCAN database (http://ualcan.path.uab.edu/). Mutational status of the *OIP5* gene was screened using cBioPortal for Cancer Genomics database (http://www.cbioportal.org/).

Results

Expression of OIP5 in oral cancer cells and tissues. We first confirmed that *OIP5* mRNA was frequently and significantly overexpressed in many of the oral cancer cell lines, while it was barely detectable in the HOMKs (Fig. 1A). Additionally, *OIP5* mRNA was frequently overexpressed in most of the oral cancer tissues, while its expression was hardly detectable in healthy tongue tissues (Fig. 1B). Western blotting revealed that OIP5 protein was expressed in many oral cancer cell lines; however, no expression was detected in the HOMK cells (Fig. 1C). Immunocytochemical analysis revealed that OIP5 protein was localized in the nucleus and cytoplasm of oral cancer cells (Fig. 1D).

Correlation of OIP5 expression with poor clinical outcome for patients with oral cancer. To verify the biological and clinicopathological significance of OIP5 in oral cancer tissues, we examined the expression of OIP5 protein using tissue microarrays containing oral cancer tissues from 164 patients who underwent radical resection. Immunohistochemical staining using anti-OIP5 antibody demonstrated that OIP5 expression was detectable in 120 of 164 (73.2%) oral cancer cases; however, it was barely detectable in the surrounding normal tissues (Fig. 2A). Next, we assessed the association between OIP5 protein expression and clinical parameters. Sex (higher in females; P=0.0202 using Fisher's exact test) and region (higher in the gingiva, buccal mucosa, and others; P=0.0029 using Fisher's exact test) were significantly associated with positive OIP5 expression (Table II). Furthermore, the positivity

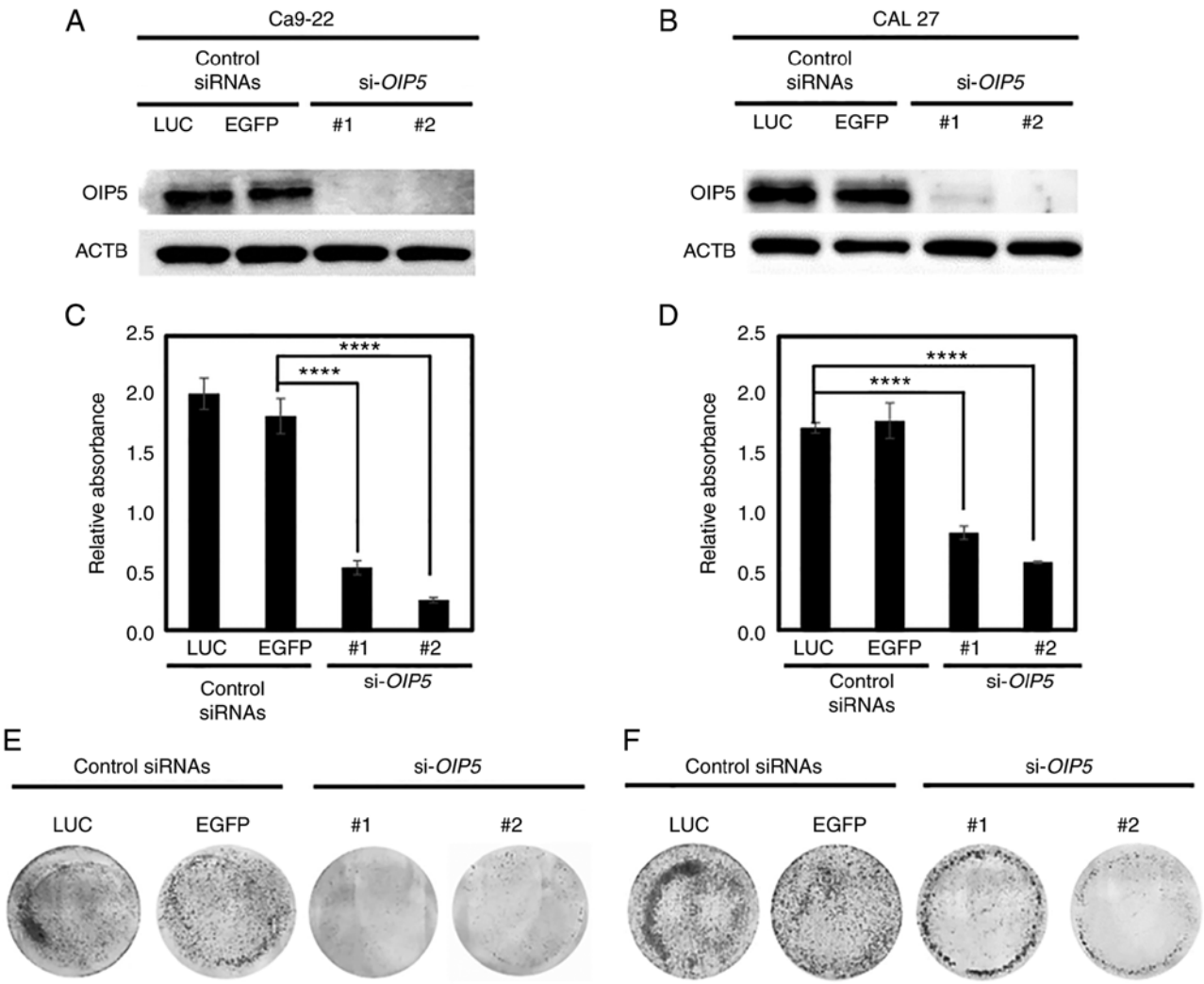

Figure 4. Inhibition of oral cancer growth by suppression of endogenous OIP5 expression. (A and B) Expression of OIP5 protein in Ca9-22 and CAL 27 cells after transfection of siRNAs for OIP5 or control siRNAs as detected using western blot analysis. (C and D) Cell viability of Ca9-22 and CAL 27 cells transfected with siRNAs for *OIP5* or control siRNAs as detected using MTT assay. (E and F) Colony formation assay for Ca9-22 and CAL 27 cells transfected with siRNAs for *OIP5* or control siRNAs. ****P<0.0001 vs. si-control group. OIP5, opa interacting protein 5; EGFR, epidermal growth factor receptor.

of OIP5 protein expression was also significantly related to poorer prognosis for patients with oral cancer (P=0.0013 using log-rank test; Fig. 2B). In addition, we also performed univariate analysis to determine the correlation between patient prognosis and other clinicopathological factors, including age (<65 vs. ≥65 years), sex (male vs. female), region (tongue vs. others), pT classification (T1-T2 vs. T3-T4), pN classification (N0 vs. N1-N2), treatment (curative surgery with adjuvant chemotherapy vs. curative surgery with neoadjuvant chemotherapy), and OIP5 expression status (absent vs. positive). Of these parameters, positive OIP5 expression (P=0.0061) and advanced pN stage (P=0.0029) were significantly associated with poor prognosis. Furthermore, multivariate analysis showed that positive OIP5 expression (P=0.0086) and advanced pN factor (P=0.0068) were independent prognostic factors (Table III).

Enforced OIP5 expression promotes oral cancer cell growth. To evaluate the potential role of OIP5 in tumorigenesis, plasmids expressing *OIP5* or mock plasmids were transfected into SCC9 and HSC2 cells that did not express endogenous OIP5. After confirmation of exogenous OIP5 expression by western blot analysis (Fig. 3A and B), cell viability was examined using MTT assay. It was found that the viability of OIP5-overexpressing cells was significantly higher compared than the viability of the cells transfected with the mock vectors (Fig. 3C and D). The colony formation assay also showed that OIP5 overexpression increased the numbers of SCC9 and HSC2 cells (Fig. 3E and F).

Inhibition of oral cancer cell growth by suppression of OIP5 expression. To further assess the role of OIP5 in oral cancer development and progression, siRNAs against *OIP5* (si-*OIP5*-#1 and si-*OIP5*-#2) along with control siRNAs (si-*LUC* and si-*EGFP*) were transfected into oral cancer cell lines (Ca9-22 and CAL 27). After confirming the reduction of OIP5 protein levels using western blot analysis (Fig. 4A and B), we continually performed an MTT assay and colony formation assay to examine the role of OIP5 in tumor proliferation. The MTT assay showed that the suppression of endogenous OIP5 expression by si-OIP5 effectively inhibited oral cancer cell viability (P<0.0001) in the si-OIP5-transfected Ca9-22

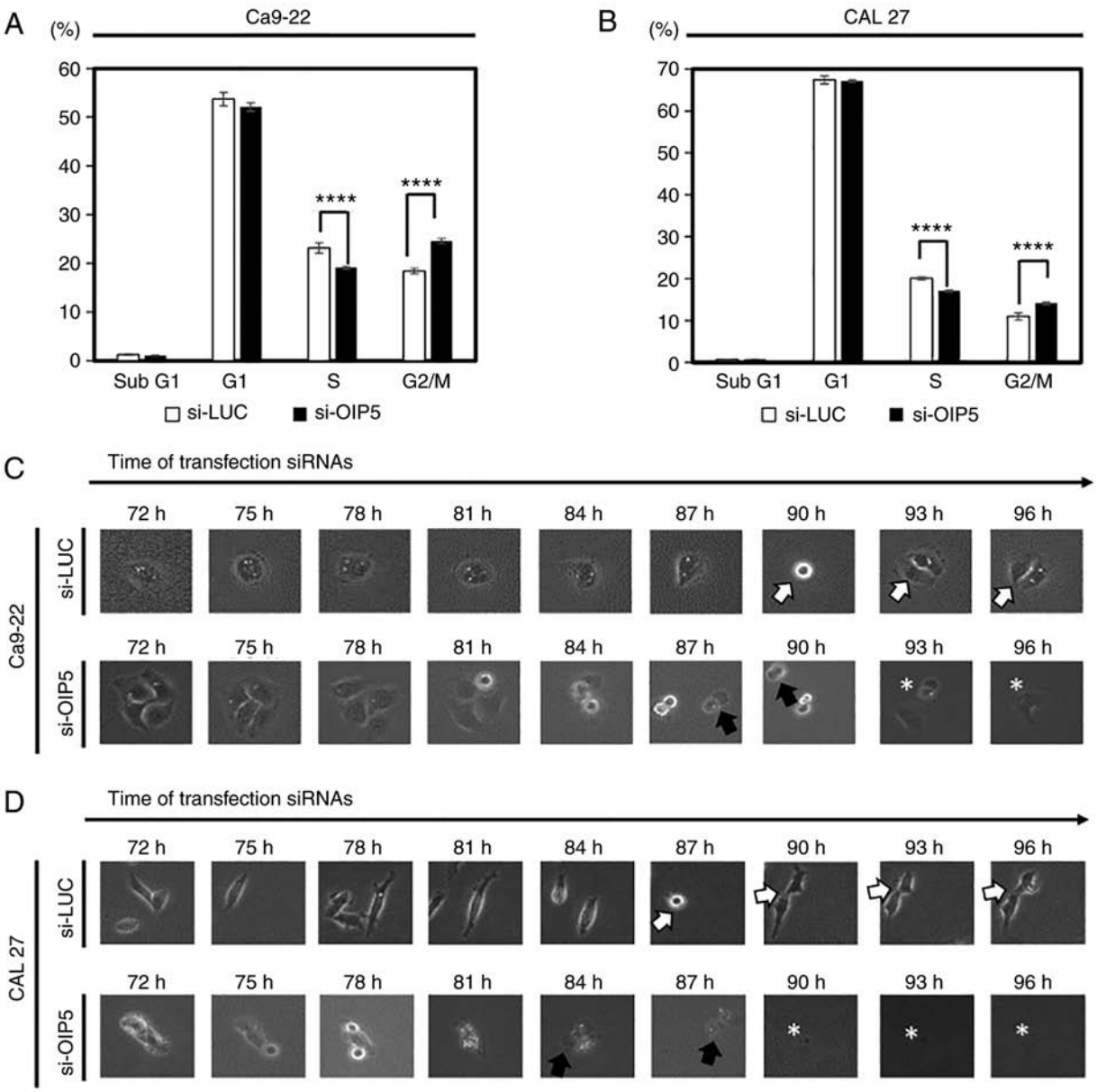

Figure 5. Cell cycle arrest and cell death of oral cancer cells by inhibition of endogenous *OIP5* expression. (A and B) Flow cytometric analysis of Ca9-22 and CAL 27 cells transfected with siRNAs for *OIP5*. The percentage of cells at each cell cycle phase was counted. ****P<0.0001 vs. si-control group. (C and D) Time-lapse imaging of Ca9-22 and CAL 27 cells after *OIP5* knockdown by siRNAs. Mitotic cell (black-white arrow), mitotic arrested cell (black arrow), and dead cell that peeled and subsequently disappeared from the microscopic field of view (*). OIP5, opa interacting protein 5.

and CAL 27 cells compared with si-controls (Fig. 4C and D). In addition, colony formation assays demonstrated that the suppression of OIP5 expression inhibited the growth of Ca9-22 and CAL 27 cells compared with controls (Fig. 4E and F).

Cell cycle arrest by inhibition of OIP5 expression. To examine the effect of OIP5 on cell cycle, flow cytometric analyses of Ca9-22 and CAL 27 cells 72 h after siRNA transfection was performed. Following OIP5 knockdown, we observed that the cell population at the G2/M phase was significantly increased (P<0.0001; Figs. 5A and B and S1). To further clarify the effect of OIP5 suppression on cellular morphology and cell cycle, live-cell imaging of the Ca9-22 and CAL 27 cells was carried out after si-*OIP5* or control siRNA transfection. Time-lapse microscopy revealed that the cells were normally divided in control siRNA-transfected cells, whereas the cells failed to divide and subsequently died in si-*OIP5*-transfected cells (Fig. 5C and D).

Suppression of OIP5 expression induces cellular senescence. To examine whether OIP5 regulates the immortality of oral cancer cells, senescence analysis was performed using acidic β-galactosidase (SA-β-Gal) staining. *OIP5* knockdown by si-*OIP5* significantly increased the number of cells stained with senescent cell markers of SA-β-gal compared with the controls (P<0.0001) (Fig. 6A-D). In addition, SAHF in the nucleus were also detected in si-*OIP5*-transfected oral cancer cells (Fig. 6E and F). The results indicate that OIP5 is likely an important molecule for the immortality of oral cancer cells.

Database analysis of OIP5 expression, mutation, and its related pathways. To validate the expression of *OIP5* in

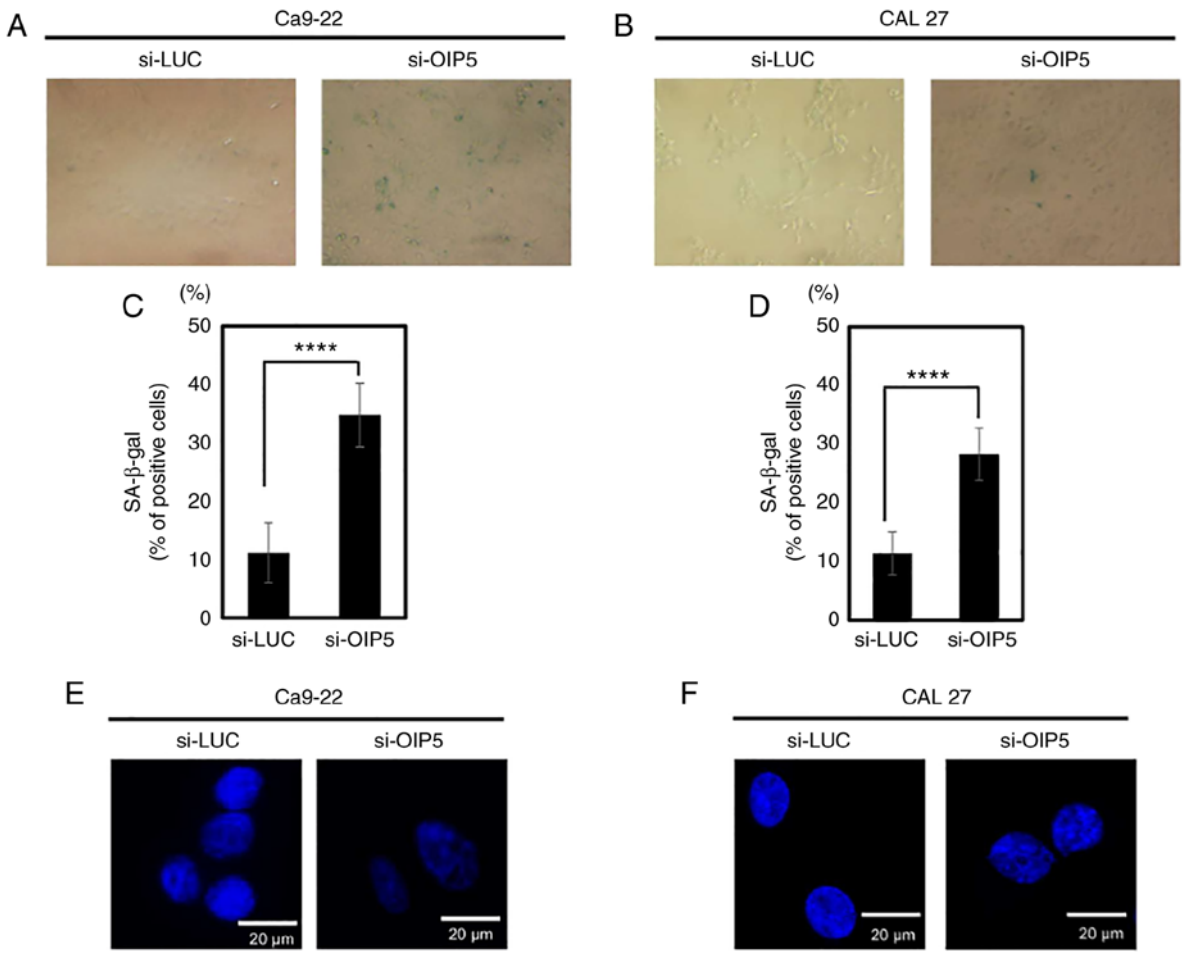

Figure 6. Induction of senescence in oral cancer cells by *OIP5* knockdown. (A and B) SA-β-gal staining in Ca9-22 and CAL 27 cells transfected with siRNAs for *OIP5* and control siRNAs. (C and D) The proportion of senescence cells in Ca9-22 and CAL 27 cells after *OIP5* knockdown. ****P<0.0001 vs. si-control group. (E and F) SAHF observed in the nuclei of cells stained with 4',6-diamidino-2-phenylindole (DAPI). OIP5, opa interacting protein 5; SAHF, senescence-associated heterochromatic foci.

HNSCC and normal tissues, we investigated the *OIP5* data using databases. BioGPS database analysis showed *OIP5* mRNA expression in testis is nearly 20 times higher than in other normal tissues. UALCAN database showed among 520 cases of primary HNSCC and 44 cases of normal tissues, OIP5 expression is significantly higher in tumors than in normal tissues, and the methylation levels of OIP5 promotor are likely to be lower in HNSCC tissues compare with that in normal tissues. To examine the mechanism of oncogenic *OIP5* activation in oral cancer, we investigated the *OIP5* data using the cBioPortal for Cancer Genomics database. Among 523 cases of HNSCC, mutations and deep deletions of *OIP5* were detected in only two cases (0.38%) and one case (0.19%), respectively; however, no amplification of *OIP5* was observed. To screen the biological role of OIP5, we referred the OIP5 data using pathway analysis. GSEA database revealed that OIP5 expression was associated with cell cycle and chromosome maintenance, as well as the deposition of new CENP-A-containing nucleosomes at the centromere. To examine the importance of the functional association of OIP5 with CENP-A in oral cancer, we confirmed the co-expression of OIP5 protein with CENP-A in HNSCC using the ONCOMINE database (GSE12452 and GSE9844 datasets with co-expression correlation coefficients of 0.818 and 0.663, respectively).

Discussion

Oral cancer is highly malignant and shows resistance to anticancer treatment. Despite progression following oral cancer treatment, only a 5% improvement in overall survival has been achieved over the last 2 decades (46). Targeting specific molecules involved in oral cancer development may be an effective therapeutic approach that exhibits high efficacy and less toxicity if the molecular mechanisms of oral cancer proliferation and survival are well defined. In the present study, a high level of OIP5 expression was detected in most oral cancer cells and tissues, but low or no detectable expression of OIP5 was observed in normal oral cells and tissues. A gene expression database suggested that, except for the testes, *OIP5* is expressed at minimal levels in normal tissues and organs, indicating that it is a candidate target for the development of new diagnostics and therapeutics. In addition, *OIP5* expression is significantly increased in HNSCC, and the methylation levels of *OIP5* promotor are likely to be lower in HNSCC tissues compare with that in normal tissues. Genetic mutation and amplification of *OIP5* are not frequent in HNSCC. Therefore, *OIP5* overexpression may be caused by an epigenetic mechanism in oral cancer cells. Further analysis of the transcriptional regulation of *OIP5* is warranted.

Tissue microarray analysis showed that OIP5 expression was associated with poor clinical outcomes for patients with oral cancer. We found that there was no significant association between the type of oral cancer treatment (neoadjuvant cases and adjuvant cases) and OIP5 protein expression level in oral cancer tissues (positive vs. negative; P=0.8566), thus confirming that neoadjuvant chemotherapy did not affect OIP5 expression. In addition, multivariate analysis showed that positive OIP5 expression was an independent prognostic factor, indicating that OIP5 expression in oral cancer tissues may represent a clinical prognostic indicator that warrants further study in patients receiving chemotherapy for oral cancer.

The exogenous expression of OIP5 in oral cancer cell lines that did not normally express endogenous OIP5 enhanced cell growth. In contrast, OIP5 knockdown led to G2/M cell cycle arrest and subsequent cell death and/or cellular senescence in oral cancer cells, as monitored by live-cell imaging and senescence assays; this suggests that OIP5 may play a role in cell cycle and cellular senescence. Therefore, targeting OIP5 and its pathway with selective small molecule inhibitors and/or gene therapy may be a therapeutic strategy for oral cancer.

Using the database, we found that OIP5 expression was associated with cell cycle and chromosome maintenance as well as the deposition of new CENP-A-containing nucleosomes at the centromere. The old CENP-A nucleosome recruits CENP-C through their interaction. In addition, CENP-C recruits the Mis18 complex containing OIP5 as a component at late anaphase when CDK1 activity is markedly reduced. Thereby, the Mis18 complex recruits the prenucleosomal CENP-A complex through the direct binding of Mis18β and HJURP (41). Previous reports have demonstrated that the centromere recruitment of newly synthesized CENP-A was rapidly abolished by the suppression of OIP5 expression in HeLa cells, followed by defects, such as misaligned chromosomes, anaphase missegregation, and interphase micronuclei (40). Those cell defects could support our results that depletion of OIP5 inhibits oral cancer cell proliferation. To examine the importance of the functional association of OIP5 with CENP-A in oral cancer, we confirmed the co-expression of OIP5 protein with CENP-A using the database. To identify novel pathways related to OIP5 overexpression, further investigation of downstream signaling of OIP5 is important for clarifying the role of OIP5 in oral cancer development.

In summary, OIP5 is likely to function as an oncoprotein in oral cancer and may play an important role in oral cancer proliferation and survival. OIP5 is a putative biomarker that may predict the prognosis of patients with oral cancer. Therefore, targeting OIP5 may be useful for developing novel treatments, including immunotherapies and molecular-targeted therapies, which may exert strong biological effects with minimal adverse effects.

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Availability of data and materials

All data generated or analyzed in this study are included in the published paper.

Authors' contributions

MZ, AT, and YD conceptualized and designed the study. MZ, AT, and YD developed the study methodology. YY, MS, and YD acquired the data (acquiring and managing patients and providing facilities). MZ, AT, BT, and YD analyzed and interpreted the data (statistical analysis, biostatistics, and computational analysis) and confirmed the accuracy. MZ, AT, and YD wrote, reviewed, and/or revised the manuscript. MZ, AT, and YD were involved in administrative, technical, or material support (reporting or organizing data and constructing databases). YD supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees (Kumamoto University; Shiga University of Medical Science).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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