The E3 Ubiquitin Ligase SIAH2 Is a Prosurvival Factor Overexpressed in Oral Cancer

SHU-CHEN HSIEH[1], SHENG-NAN KUO[2], YI-HAN ZHENG[1], MING-HSUI TSAI[3,4], YU-SHENG LIN[5] and JU-HWA LIN[5]

[1]*Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan, ROC;*
[2]*Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan, ROC;*
[3]*Department of Otolaryngology, China Medical University Hospital, Taichung, Taiwan, ROC;*
[4]*Graduate Institute of Clinical Medical Science, and*
[5]*Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, R.O.C.*

Abstract. *Background: This study aimed at investigating the expression and functional significance of seven in absentia homologues 2 (SIAH2), an E3 ubiquitin ligase, in oral squamous cell carcinoma (OSCC). Materials and Methods: Comparative genomic hybridization and real-time PCR were performed to examine the amplification of SIAH2 gene in clinical specimens of OSCC tissues. Expression of SIAH2 mRNA and protein was examined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical assays, respectively. Apoptosis was examined by annexing V staining and Poly (ADP-ribose) polymerase (PARP) cleavage, with or without sienna-mediated SIAH2 knockdown. The expression of p53 was analyzed by immunoblotting. Results: Levels of SIAH2 DNA and mRNA were significantly greater in clinical OSCC specimens and in cultured OSCC cells, which also stained positively for the SAIH2 protein. Knockdown of SIAH2 led to growth suppression and apoptosis induction in a p53-independent mechanism. Conclusion: These results revealed a tight correlation of SIAH2 overexpression with OSCC and suggest an oncogenic role of SIAH2 in oral cancer.*

Oral cancer is one of the leading causes of cancer death worldwide (1). A rise in the incidence of oral cancer in Europe, as well as an increased mortality of the disease in Asia, have been observed. Indeed, a World Health Organization estimation indicates that a continuous increase in the prevalence of oral cancer is expected in the decades to come (2). Risk factors of oral cancer include tobacco smoking and alcohol abuse. In addition, use of areca nut (betel nut) in oriental countries is tightly linked to the development of oral squamous cell carcinoma (OSCC). Arecoline, the major alkaloid of the areca nut, has been shown to be a carcinogen (3). Analyses of oral cancer tissues by comparative genomic hybridization (CGH) have revealed frequent amplification in chromosomes 3q, 8q, 9q, 11q and 20q, as well as deletion in the 4q, 3p, 5q, and 15q chromosome regions (4, 5). Mechanisms underlying oral cancer development remain to be fully-explored, identification of molecules contributing to the progression of oral cancer will be vital for improving the detection and treatment of the disease.

Protein ubiquitination is a versatile post-translational modification that targets proteins to proteasomal degradation. The ubiquitination-dependent regulation of protein stability affects all aspects of cell physiology, including cell-cycle progression, senescence, differentiation and apoptosis (6, 7). Ubiquitination consists of three consecutive enzymatic reactions: E1 ubiquitin-activating protein, E2 ubiquitin-conjugating protein and E3 ubiquitin ligase. The seven in absentia gene is an E3 ligase first identified in Drosophila shown to be required for the cell fate specification of the R7 photoreceptor (8). Two seven in absentia homologues (Siah), Siah1 and -2, exist in mammals. The SIAH proteins are really interesting new gene (RING) domain-containing ubiquitin ligases that regulate the stability of a diverse group of substrates (9), including nuclear corepressor, β-catenin, TNF receptor-associated factor 2 (TRAF2), α-ketoglutarate dehydrogenase, and prolyl hydroxylase 3 (PHD3). The SIAH2 proteins have been implicated in a range of cellular processes, including hypoxia response, survival and mitochondrial biogenesis (10-14). The SIAH2 proteins share a highly conserved substrate-binding domain and are known to interact with overlapping protein targets. Nonetheless,

Correspondence to: Dr. Ju-Hwa Lin, Department of Biological Science and Technology, China Medical University, 91 Hsueh-Shih Rd., Taichung 404, Taiwan, R.O.C. Tel: +886 422053366 ext 2513, Fax: +886 422071507, e-mail: linjh@mail.cmu.edu.tw

Key Words: Oral cancer, Siah2, ubiquitin ligase, apoptosis.

SIAH1 and SIAH2 each has isoform-specific substrates, and can play distinct roles in cellular functions (15). SIAH2 has been implicated in the progression of cancer of various cell types. Depletion of SIAH2 in cancer cells is reported to suppress tumorigenesis, including of lung, pancreas, breast, prostate and melanoma (16-19). The role of SIAH2 in oral cancer development, however, has not been investigated to us knowledge.

In this study, we employed array CGH assays and PCR analyses to examine for chromosomal aberrations in the clinical specimens from patients with oral cancer.

Materials and Methods

Patients and clinicopathological data. Oral tissue specimens for array CGH assays were obtained from 10 patients who underwent surgical resection for oral cancer at the China Medical University Hospital (Taichung, Taiwan, ROC) in 2009. An additional 25 tissue specimens were collected from patients with OSCC who underwent primary surgical resection at the same hospital between 2009 and 2010; age of these patients ranged between 35-69 years (average 50 years). Clinicopathological information, obtained retrospectively from medical records, including primary tumor size, nodal status, and histological grade, is shown in Table I. The collection and use of tissue samples were in compliance with the guidelines of the Ethics and Clinical Research Committee of China Medical University. Informed consent was obtained from all patients or their legal guardians.

Cell culture. The OSCC cell lines, Ca922, SAS and HSC-3 (all were from Japanese Collection of Research Bioresources, Tokyo, Japan) and CAL27 from American Type Culture Collection (ATCC, Manassas, Virginia, USA) are frequently used model of OSCC cell systems. OECM-1 cells were provided by Dr. Kuo-Wei Chang (20). The TW206 cell line, cloned from a Taiwanese patient with oral cancer from chewing betel nut, was from Dr. Pei-Jung Lu (National Cheng Kung University, Taiwan, ROC). All cells were maintained and passaged according to the guidelines provided by the cell banks. The Ca922 cells were cultured in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA, USA), SAS, HSC-3, TW206 and CAL27 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen), OECM-1 cells were cultured in RPMI-1640 (Life Tech, Gaithersburg, MD, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured in a humidified chamber at 37˚C containing 5% CO_2.

Array-based CGH (aCGH) assays. Ten pairs of human oral cancer samples and the adjacent morphologically-normal tissues were analyzed by aCGH using the Human Genome CGH Microarray Kit 244A (Agilent Technologies, Santa Clara, CA, USA) which detects more than 236,000 coding and non-coding human loci. The probes used were as suggested by the instructions of NCBI build 36 (http://www.ncbi.nlm.nih.gov/projects/genome/guide/human/index.shtml). Data analysis was performed by the CGH Analytics 3.4.40 software (Agilent Technologies), and statistical significance was analyzed by the Student's *t*-test ($p<0.05$ indicates significant difference). The ADM-2 algorithm was used, with a threshold of 6.0 to detect significantly altered genomic regions.

Table I. *Clinicopathological features of oral cancer patients*.

Factor	Number (%)
Age (ave=50 years)	
≤40 years	6 (24)
>40 years	19 (76)
Gender	
Male	2 (8)
Female	23 (82)
Smokers	19 (76)
Areca users	16 (64)
Clinical stage	
I	10 (40)
II	10 (40)
III	5 (20)
Differentiation	
Poor	10 (40)
Moderate	12 (48)
Well	3 (12)

DNA extraction. Tissues from patients with oral cancer were removed from liquid nitrogen and ground into powder. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Annexin V-fluorescein isothiocyanate (FITC) binding assay. Apoptosis was detected by the annexin V/FITC binding assays. The normal, early apoptotic, late apoptotic and necrotic cells were discerned using the Annexin V-Propidium Iodide (PI) kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). After washing with PBS, the cells were resuspended and incubated in the staining solution for 10 min. At least 100,000 cells were analyzed by flow cytometry for apoptotic cells.

Real time reverse transcription-polymerase chain reaction (real time RT PCR). Total tissue RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The first-strand cDNA was synthesized using the NCode™ miRNA kit (Invitrogen) with 2 μg of RNA according to the manufacturer's protocol. PCR amplification was performed using the PRISM 7900HT Sequence Detection System (ABI, Foster City, CA, USA). Primers used for amplifying *SIAH2* gene were: AGGTTGCC CTCTGCCGATA (forward), and ACATAGGTGAGTGGCCAA ATCTC (reverse). Relative gene expression was analyzed using the $-2^{-\Delta\Delta CT}$ method (21).

Immunohistochemistry and immunocytochemical analyses. The tissue sections or cells (2×10^4) were washed with PBS and fixed in PBS containing 4% w/v paraformaldehyde at 22˚C for 30 min. The samples were rinsed in PBS, permeabilized with 0.3% Triton X-100, and incubated with an antibody against SIAH2 (Santa Cruz, Dallas, Tx, USA) at a dilution of 1:1000 for 2 h at 25˚C in a humidified chamber. After PBS washing, immunoreactivity was detected by the streptavidin-biotin complex system (Dako, Carpinteria, CA, USA). The sections were counterstained with hematoxylin, dehydrated and mounted with Clearmount (Zymed, San Francisco, CA, USA).

Immunoblot analysis. Cells were lysed in buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, and 0.02% NaN_3) containing 1% NP40 and protease inhibitors (Roche, Indianapolis, IN, USA) for 30 min on ice. The lysates were centrifuged at 12,054 *g* for 30 min at 4˚C and the concentrations of proteins were determined by the Bradford assay kit (Bio-Rad, Hercules, CA, USA). Approximately 40 μg of lysates were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and analyzed by immunoblotting using specific antibodies for SIAH2 (Santa Cruz, Dallas, Texas) and actin (Millipore Corporation, Billerica, MA, USA).

Plasmid construction and transfection. Short hairpin RNA (shRNA) expression plasmids obtained from The RNAi Consortium (TRC, Taiwan, ROC) were employed to knockdown the expression of SIAH2. Two pLKO.1-shRNA constructs: SIAH2 sh-1 (TRCN0000007416) and SIAH2 sh-2 (TRCN0000007418), were obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan, ROC). For transfection, cells were seeded at 5×10^5 cells per well in 6-well-plates and transfected with 2 μg of the plasmid DNA by Arrest-in reagent (Thermo Scientific, Huntsville, AL, USA).

Results

SIAH2 is overexpressed in clinical specimens of oral cancer. Using aCGH assays, we have previously found gain of chromosomal regions 1p, 3q, 7p, 8q, 9, 11q, 16, 17, 19, 20 and 22, as well as loss of 3p, 4q, 5q, 8p, 10, 18q and Y, in clinical specimens of oral cancer (22). Because the *SIAH2* gene is located at 3q25, we examined if SIAH2 undergoes genomic amplification in oral cancer. Ten paired clinical specimens of oral cancer and their non-neoplastic counterparts were analyzed by aCGH assays for changes of *SIAH2* gene copy number. As shown in Figure 1, aCGH results indicated that eight out of 10 oral cancer samples exhibited *SIAH2* gene amplification.

Next, real-time PCR assays were performed to verify the aCGH results. A total of 25 additional clinical specimens of oral cancer were collected. The majority of the study patients were male and averaged 50 years old, with clinical stages distributed from I to III, and the differentiation status from poor to well differentiation (Table I). Genomic DNA was successfully extracted from 20 of these samples. Results from real-time PCR assays, as shown in Figure 2A, revealed that 14 out of 20 specimens (70%) exhibited *SIAH2* DNA amplification of greater than 1.2-fold, only one tumor specimen (5%) exhibited a reduced *SIAH2* DNA level. When compared with the non-neoplastic corresponding tissues, an average increase of 1.46±0.2-fold was found. This result confirmed the finding from aCGH, indicating a positive correlation of *SIAH2* gene amplification in OSCC tumor.

Quantitative RT-PCR was next performed to address whether the amplification of SIAH2 gene resulted in elevated mRNA expression. As shown in Figure 2B, *SIAH2* mRNA expression in most tumors (23 out of 25, 92%) displayed over 1.5-fold increase relative to their corresponding non-tumor specimens. Only one sample (1/25, 4%) exhibited reduction of *SIAH2* mRNA expression.

The expression of SIAH2 protein in tissues was examined by immunohistochemistry. As shown in Figure 2C, OSCC stained positively for SIAH2. Together, our results show a strong correlation of *SIAH2* DNA amplification and mRNA overexpression with OSCC.

Increased DNA copy number and mRNA expression of SIAH2 in cultured oral cancer cells. We further evaluated the DNA copy number and mRNA expression of *SIAH2* by real-time PCR and RT-PCR assays, respectively, in six OSCC-derived cell lines. Human foreskin keratinocytes (HFKs) (23) were simultaneously analyzed as a control normal cell. As shown in Figure 3A, four out of the six OSCC cell lines analyzed exhibited significantly increased levels of *SIAH2* DNA. Amplification of *SIAH2* gene was highest in the Ca922 cell line, whereas no apparent changes were detected in SAS and OECM1. Interestingly, RT-PCR assays revealed that all six OSCC cell lines displayed at least 10-fold increases in *SIAH2* mRNA levels when compared with control HFK cells (Figure 3B). These results suggest that amplification of the *SIAH2* gene does not fully explain the increased mRNA levels. It is clear that *SIAH2* mRNA expression in OSCC may be regulated at the transcriptional post-transcriptional levels. Consistent with elevated mRNA expression, immunocytochemical analyses demonstrated that the SIAH2 protein was present in all six OSCC cell lines, but not in the normal control HFK (Figure 3C).

Knockdown of SIAH2 suppresses cell growth and induces apoptosis of oral cancer cells. Given that *SIAH2* is overexpressed in oral cancer, next we addressed its role in the growth of oral cancer cells using the cultured Ca922 OSCC cells which expressed abundant *SIAH2*. Expression of *SIAH2* in Ca922 was knocked down by two *SIAH2*-specific siRNAs (Siah2-sh1 and Siah2-sh2). As indicated by RT-PCR (Figure 4A) and immunocytochemical assays (Figure 4B), respectively, expression of SIAH2 mRNA and protein was effectively suppressed by both siRNAs. Examination by MTT assays showed that depletion of *SIAH2* significantly inhibited the proliferation rates of Ca922 cells (Figure 4C). Furthermore, *SIAH2* knockdown-induced growth suppression was observed in all OSCC cell models (Figure 4D). Next, we addressed whether apoptosis contributes to the depletion of SIAH2-induced growth suppression. Expression of SIAH2 in Ca922 cells was silenced by siRNAs, then cells were stained with annexin V and PI and analyzed by flow cytometry. As shown in figure 4E, depletion of *SIAH2* resulted in an proportion of annexin V-positive cells. Consistently, increased cleavage of Poly ADP ribose polymerase (PARP) (Figure 4F), an apoptotic marker, was also released upon *SIAHh2* knockdown. These results together show that apoptosis contributes, at least in part, to the growth suppression induced by *SIAH2* knockdown.

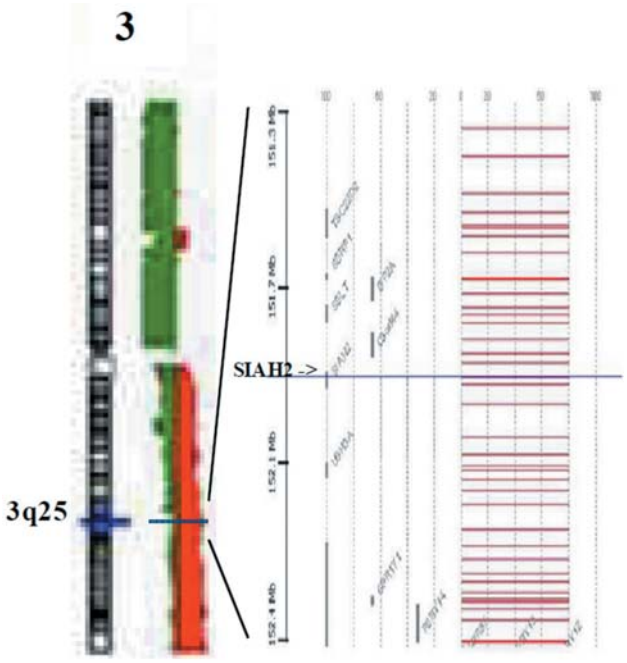

Figure 1. *Analyses by Array-based comparative genomic hybridization (aCGH) on chromosome 3 of clinical specimens of oral squamous cell carcinoma (OSCC). aCGH assays were performed on 10 specimens of oral cancer and their corresponding non-neoplastic tissues. The relative amounts of DNA in tumors to those in normal tissues are shown. Red indicates regions that displayed gain of chromosome, while green designates areas with loss of chromosome. Increase of seven in absentia homologues (SIAH2) gene at 3q25 locus was found in 8 out of 10 OSCC specimens. Statistical significance of the difference as analyzed by Student's t test indicated p<0.05.*

Next, we examined whether p53 is involved in the *SIAH*-depletion-induced apoptosis. As a central player of cell fate determination, the tumor suppressor p53 arrests cell growth and triggers apoptosis of a wide variety of cells under stress. The abundance and the activation of p53 were examined by immunoblotting. Our results indicate that levels of p53 and its Ser46-phosphorylated form (active p53) decreased in the *SIAH2*-knockdown cells (Figure 4G), suggesting that p53 is unlikely to participate in the apoptosis induced by SIAH2 depletion.

Discussion

In this study, we have provided evidence demonstrating for the first time that SIAH2 ubiquitin ligase is an oncogenic factor overexpressed in oral cancer. *SIAH2* gene is located in a chromosomal region usually presenting gain of genetic material, commonly found in oral cancer. Using aCGH assays, we show tha the *SIAH2* gene is frequently amplified in the clinical specimens of OSCC, which also present elevated levels of *SIAH2* mRNA and protein. The tight correlation between *SIAH2* overexpression and oral cancer is shown by the finding that 70% (14/20) of patients harbor *SIAH2* gene amplification, and over 90% (23/25) displayed elevated levels of *SIAH2* mRNA compared with the corresponding non-neoplastic tissues. Overexpression of *SIAH2* in oral cancer is further supported by its abundant mRNA levels in all six analyzed OSCC cell lines. The functional significance of *SIAH2* overexpression in the tumorigenesis of oral cancer is shown by the suppression of growth and induction of apoptosis in *SIAH2*-deficient OSCC cells. Together, these results suggest a tumor promoting role of *SIAH2* in oral cancer.

There are discrepant observations on the role of *SIAH2* in tumor cells. A number of studies suggest that *SIAH2* functions as a tumor suppressor in cancer. While *SIAH2*-knockout mice are largely physiologically normal, an expansion of myeloid precursors, particularly those sensitive to macrophage colony-stimulating factor, was noticed in the bone marrow (24). Analyses of six large cohorts of breast and non-small cell lung carcinoma have revealed that low levels of *SIAH2* expression correlate with reduced disease-free survival (25). Moreover, loss of *SIAH2* expression is associated with more advanced and tamoxifen-resistant estrogen receptor-positive tumors (26). In contrast, a number of studies implicate a tumor-promoting role of *SIAH2* in tumors. Inhibition of *SIAH2* function in a syngeneic breast cancer cell model resulted in suppressed tumor cell growth (27). Analyses of breast cancer specimens revealed a significant correlation between the expression of *SIAH2* and the incidence of tumor with basal-like phenotype (28). The oncogenic roles of *SIAH2* have also been suggested in lung and prostate cancer cells (29-31). Consistent with the present finding, Ahmed *et al*. reported that silencing of *SIAH2* triggered apoptosis in cultured lung cancer cell (30). It appears that the effects of *SIAH2* on tumorigenesis are complicated and may be cellular context-dependent.

In clear distinction to Western countries, a significant population of patients with OSCC in Taiwan consume betel nuts. Our data indicate that whereas 64% of patients with OSCC were betel nut eaters, overexpression of *SIAH2* mRNA was found in 92% of tumor specimens. Likewise, all of the six OSCC cell lines exhibited high levels of *SIAH2* mRNA expression, whereas only two cell lines, OECM1 and TW206, were from patients who were habitual betel nut eaters. It is interesting to note that OECM1 cells do not harbor *SIAH2* gene amplification, which further excludes the association between betel nut mutagens and SIAH2 genomic alterations. Based on these findings, we suggest that increased *SIAH2* DNA and mRNA levels do not seem to be a direct consequence of betel nut consumption. Instead, our results support the notion that *SIAH2* overexpression is a common event of OSCC. The molecular basis of *SIAH2* gene

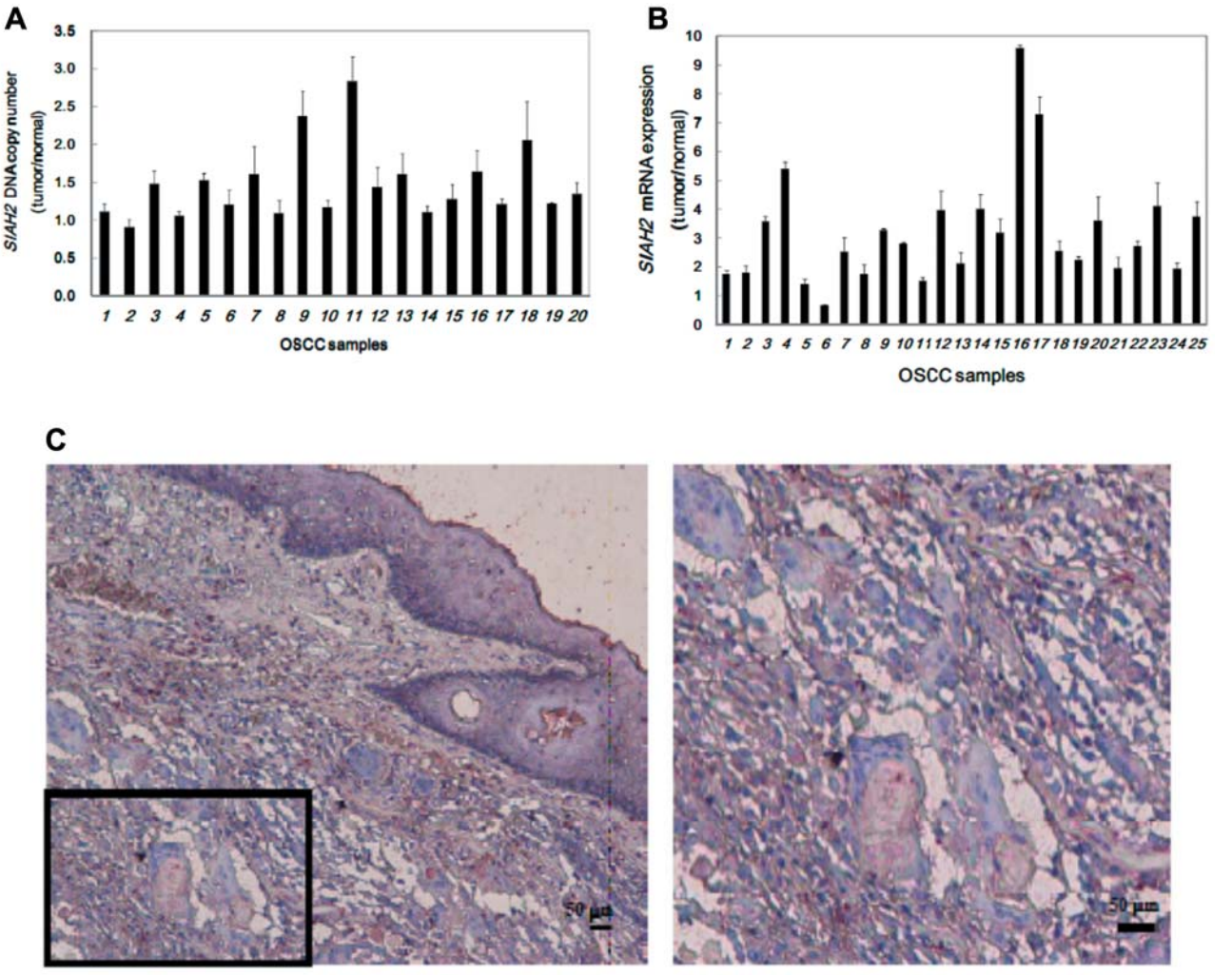

Figure 2. *Increased levels of seven in absentia homologues-2 (SIAH2) DNA and mRNA in clinical specimens of oral cancer. A: Genomic DNA was extracted from 20 paired oral cancer specimens and their adjacent non-neoplastic tissues, and amplified by real-time PCR for SIAH2 and ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4) which served as an internal control. The amounts of DNA in cancer relative to those in normal tissues are shown. B: RNA was extracted from 25 paired oral cancer tissues, and subjected to real-time RT-PCR amplifications for SIAH2 mRNA, expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined as an internal control. Relative mRNA values of tumor to those of normal tissues as them. Bars in A and B are the mean±SD from at least three independent experiments. C: Expression of SIAH2 in oral cancer tissues. Consecutive tissue sections were subjected to immunohistochemistry using an antibody to SIAH2 (left panel). Inset: the area shown in the left panel as viewed at a higher magnification (right). SIAH2-positive cells were revealed by dark red staining.*

amplification and mRNA overexpression in oral cancer warrants further investigation.

Mechanisms underlying the *SIAH2* silence-induced apoptosis/growth suppression remain unclear. Attempts to link p53 to the SIAH2-mediated cellular activities in OSCC failed to ascribe a role of the tumor suppressor. On the contrary, we have shown that the abundance of p53 and its Ser-46 phosphorylated form decreased in the *SIAH2*-deficient cells. Our finding of a positive correlation between p53 and SIAH2 levels is consistent with a report by Chan *et al*. indicating that activation of p53 is associated with increased SIAH2 expression in the basal-like breast cancer (28). We have also examined the possibility whether hypoxia-inducible factor-1α (HIF-1α) may be involved in the SIAH2-mediated action in OSCC cells. HIF-1α which is hydroxylated and stabilized by the SIAH2 substrate prolyl hydroxylase-3 (PHD3) (32), is reported to be overexpressed in OSCC under both normal and hypoxic conditions (33). Moreover, overexpression of HIF-1α is shown to be correlated with poor prognosis of patients with OSCC (34). However, we were unable to detect HIF-1α protein in the OSCC cells under unstimulated conditions.

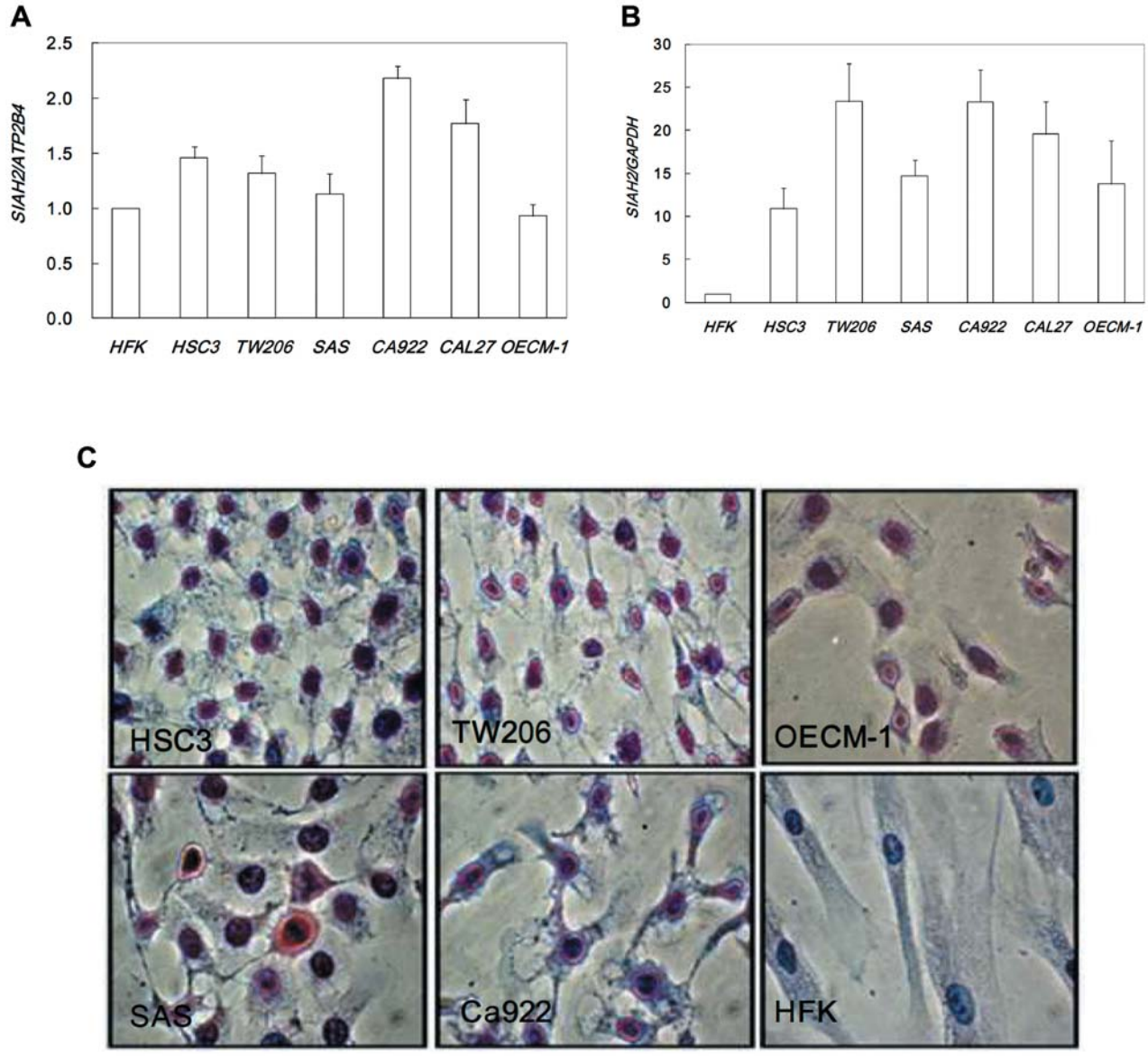

Figure 3. *Increased DNA copy number and mRNA expression of seven in absentia homologues-2 (SIAH2) in cultured oral cancer cell lines. A: Genomic DNA was extracted from the indicated oral squamous cell carcinoma (OSCC) cell lines, and assayed by real-time PCR using specific primers for SIAH2, expression of ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4) gene served as an internal control. B: RNA was extracted and assayed by real time RT-PCR for SIAH2 mRNA expression, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed as an internal control. Expression of SIAH2 gene in the Human foreskin keratinocytes (HFKs) cells was determinded as the normal cell control. The ratios of SIAH2 to internal control obtained from each OSCC cell line were calculated and normalized to that of HFK which was set as 1. Bars are the mean±SD from three independent experiments. C: Immunocytochemical analysis indicating abundant SIAH2 protein expression in OSCC cells compared with that in HFK cells.*

The ubiquitin proteasome pathway-mediated degradation of proteins is implicated in a multitude of cellular processes, including cellular growth and apoptosis. It is not surprising that aberrations of components in the specific pathway have been linked to carcinogenesis. We have previously reported that the WW domain containing E3 ubiquitin protein ligase-1 (WWP1) is frequently amplified in oral cancer (22). We now show that gain of DNA and mRNA expression of yet another E3 ligase, *SIAH2*, is closely-correlated with oral cancer. The ability of *SIAH2* silencing in promoting apoptosis/growth suppression plus its frequent overexpression in OSCC tissues suggests an

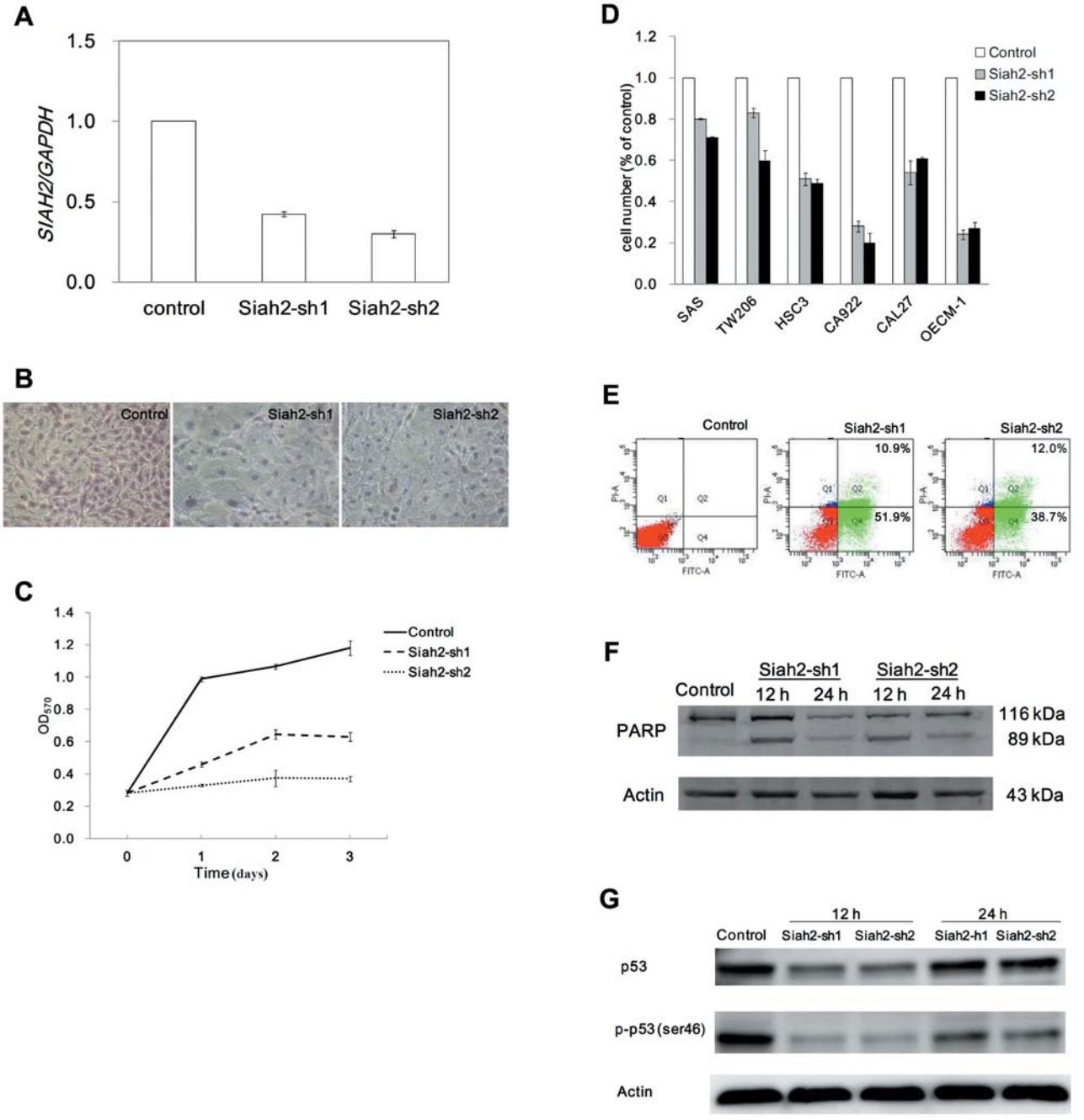

Figure 4. *Knockdown of seven in absentia homologues-2 (SIAH2) expression suppresses growth and induces apoptosis of oral squamous cell carcinoma (OSCC) cells. A, B: Expression of SIAH2 mRNA and protein is effectively suppressed by SIAH2-specific siRNAs. The Ca922 cells were separately transfected with plasmids expressing SIAH2 siRNA (Siah2-sh1 and Siah2-sh2). Expression of SIAH2 mRNA was determined by quantitative RT-PCR 24 h after transfection (A). B: Suppression of SIAH2 protein was analyzed by immunocytochemistry assays using a SIAH2-specific antibody. C: Expression of SIAH2 was knocked-down in Ca922 cells by Siah2-sh1 and Siah2-sh2, and the viability of cells was determined by MTT assays, as measured at the absorbance of 570 nm. D: Expression of SIAH2 in the indicated OSCC cell lines was knocked down in the presence of specific siRNAs as shown. The viability of cells was analyzed by MTT assays 24 h after transfection, numbers were normalized to those of the knockdown control of the individual cell line. E: Expression of SIAH2 in Ca922 cells was inhibited by specific siRNAs (Siah2-sh1, Siah2-sh2) or scrambled siRNA (control). After 24 h, cells were fixed and stained with annexin V and PI, and the percentage of apoptotic cells as determined by flow cytometry is indicated. F: Cells were treated as in (E), lysates were prepared and analyzed by immonoblotting for Poly-ADP ribose polymerase (PARP) cleavage. Expression of actin is shown as a protein loading control. G: Similarly as in F, abundance of total and Ser46-phosphorylated p53 was analyzed by immunoblotting using specific antibodies.*

oncogenic role of SIAH2 in the development of OSCC. These results provide a potential strategy for therapeutic intervention of oral cancer.

Conflicts of Interest

We declare that there was no any financial and personal relationship with other people or organization that could inappropriately influence our work.

Acknowledgements

We thank the tissue-bank of China Medical University Hospital for technical assistance and Dr. Guan-Chin Tseng (China Medical University Hospital) for the pathological measurement of tissue sections in this study. This study was supported by research grants from the China Medical University (CMU95-299, CMU97-096 and CMU100-TC-07).

References

1 Moore RJ, Doherty DA, Do KA, Chamberlain RM and Khuri FR: Racial disparity in survival of patients with squamous cell carcinoma of the oral cavity and pharynx. Ethnicity Health *6(3-4)*: 165-177, 2001.

2 Petersen PE: The World Oral Health Report 2003: Continuous improvement of oral health in the 21st century-the approach of the WHO Global Oral Health Programme. Commun Denti Oral Epidemiol *31(Suppl 1)*: 3-23, (2003)

3 Jeng JH, Chang MC and Hahn LJ: Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. Oral Oncol *37(6)*: 477-492, 2001.

4 O'Regan EM, Toner ME, Smyth PC, Finn SP, Timon C, Cahill S, Flavin R, O'Leary JJ and Sheils O: Distinct array comparative genomic hybridization profiles in oral squamous cell carcinoma occurring in young patients. Head Neck *28(4)*: 330-338, 2006.

5 Uchida K, Oga A, Okafuji M, Mihara M, Kawauchi S, Furuya T, Chochi Y, Ueyama Y and Sasaki K: Molecular cytogenetic analysis of oral squamous cell carcinomas by comparative genomic hybridization, spectral karyotyping, and fluorescence *in situ* hybridization. Cancer Genet Cytogenet *167(2)*: 109-116, 2006.

6 Hoeller D and Dikic I: Targeting the ubiquitin system in cancer therapy. Nature *458(7237)*: 438-444, 2009.

7 Bedford L, Lowe J, Dick LR, Mayer RJ and Brownell JE: Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. Nat Revi Drug Discov *10(1)*: 29-46, 2011.

8 Carthew RW and Rubin GM: seven in absentia, a gene required for specification of R7 cell fate in the Drosophila eye. Cell *63(3)*: 561-577, 1990.

9 Habelhah H, Frew IJ, Laine A, Janes PW, Relaix F, Sassoon D, Bowtell DD and Ronai Z: Stress-induced decrease in TRAF2 stability is mediated by SIAH2. EMBO J *21(21)*: 5756-5765, 2002.

10 Firth JD: Hypoxia and Mitochondrial Inhibitors Regulate Expression of Glucose Transporter-1 *via* Distinct Cis-acting Sequence. J of Biol Chem *270(49)*: 29083-29089, 1995.

11 Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P and Semenza GL: Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res *63(5)*: 1138-1143, 2003.

12 Bruick RK: Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Acad Scie USA *97(16)*: 9082-9087, 2000.

13 Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH and Harris AL: HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res *61(18)*: 6669-66673, 2001.

14 Feldser D, Agani F, Iyer NV, Pak B, Ferreira G and Semenza GL: Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res *59(16)*: 3915-3918, 1999.

15 Kramer OH, Stauber RH, Bug G, Hartkamp J and Knauer SK: SIAH proteins: Critical roles in leukemogenesis. Leukemia *27(4)*: 792-802, 2013.

16 Sun S, Schiller JH and Gazdar AF: Lung cancer in never smokers – a different disease. Nat Rev Cancer *7(10)*: 778-790, 2007.

17 Sharma SV, Bell DW, Settleman J and Haber DA: Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer *7(3)*: 169-181, 2007.

18 Brambilla E, Travis WD, Colby TV, Corrin B and Shimosato Y: The new World Health Organization Classification of Lung Tumours. Eur Resp J *18(6)*: 1059-1068, 2001.

19 Schubbert S, Shannon K and Bollag G: Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer *7(4)*: 295-308, 2007.

20 Ko SY, Lin SC, Chang KW, Wong YK, Liu CJ, Chi CW and Liu TY: Increased expression of amyloid precursor protein in oral squamous cell carcinoma. Int J Cancer *111(5)*: 727-732, 2004.

21 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods *25(4)*: 402-408, 2001.

22 Lin JH, Hsieh SC, Chen JN, Tsai MH and Chang CC: WWP1 gene is a potential molecular target of human oral cancer. Oral Surg, Oral Medi, Oral Pathol Oral Radiol *116(2)*: 221-231, 2013.

23 Gyongyosi E, Szalmas A, Ferenczi A, Konya J, Gergely L and Veress G: Effects of human papillomavirus (HPV) type 16 oncoproteins on the expression of involucrin in human keratinocytes. Virol J *9*: 36, 2012.

24 Frew IJ, Hammond VE, Dickins RA, Quinn JMW, Walkley CR, Sims NA, Schnall R, Della NG, Holloway AJ, Digby MR, Janes PW, Tarlinton DM, Purton LE, Gillespie MT and Bowtell DDL: Generation and analysis of *Siah2*-mutant mice. Mol Cell Bio *23(24)*: 9150-9161, 2003.

25 Confalonieri S, Quarto M, Goisis G, Nuciforo P, Donzelli M, Jodice G, Pelosi G, Viale G, Pece S and Di Fiore PP: Alterations of ubiquitin ligases in human cancer and their association with the natural history of the tumor. Oncogene *28(33)*: 2959-2968, 2009.

26 Jansen MP, Ruigrok-Ritstier K, Dorssers LC, van Staveren IL, Look MP, Meijer-van Gelder ME, Sieuwerts AM, Helleman J, Sleijfer S, Klijn JG, Foekens JA and Berns EM: Downregulation of SIAH2, an ubiquitin E3 ligase, is associated with resistance to endocrine therapy in breast cancer. Breast Cancer Research and Treat *116(2)*: 263-271, 2009.

27 Moller A, House CM, Wong CS, Scanlon DB, Liu MC, Ronai Z and Bowtell DD: Inhibition of Siah ubiquitin ligase function. Oncogene *28(2)*: 289-296, 2009.

28 Chan P, Moller A, Liu MC, Sceneay JE, Wong CS, Waddell N, Huang KT, Dobrovic A, Millar EK, O'Toole SA, McNeil CM, Sutherland RL, Bowtell DD and Fox SB: The expression of the ubiquitin ligase SIAH2 (seven in absentia homolog 2) is mediated through gene copy number in breast cancer and is associated with a basal-like phenotype and p53 expression. Breast Cancer Res *13(1)*: R19, 2011.

29 House CM, Moller A and Bowtell DD: Siah proteins: Novel drug targets in the Ras and hypoxia pathways. Cancer Res *69(23)*: 8835-8838, 2009.

30 Ahmed AU, Schmidt RL, Park CH, Reed NR, Hesse SE, Thomas CF, Molina JR, Deschamps C, Yang P, Aubry MC and Tang AH: Effect of disrupting seven-in-absentia homolog 2 function on lung cancer cell growth. J Natl Cancer Inst *100(22)*: 1606-1629, 2008.

31 Qi J, Pellecchia M and Ronai ZA: The Siah2-HIF-FoxA2 axis in prostate cancer – new markers and therapeutic opportunities. Oncotarget *1(5)*: 379-385, 2010.

32 Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD and Ronai Z: Siah2 regulates stability of prolyl-hydroxylases, controls HIF1α abundance, and modulates physiological responses to hypoxia. Cell *117(7)*: 941-952, 2004.

33 Mohamed KM, Le A, Duong H, Wu Y, Zhang Q and Messadi DV: Correlation between VEGF and HIF-1 α expression in human oral squamous cell carcinoma. Exp Mol Pathol *76(2)*: 143-152, 2004.

34 Uehara M, Sano K, Ikeda H, Nonaka M and Asahina I: Hypoxia-inducible factor 1 alpha in oral squamous cell carcinoma and its relation to prognosis. Oral Oncol *45(3)*: 241-246, 2009.

Received August 5, 2013
Revised September 26, 2013
Accepted September 30, 2013