Archives of Oral Biology 87 (2018) 131–142

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Overexpression of sprouty2 in human oral squamous cell carcinogenesis

Pei-Hsien Liao[a,1], Yen-Yun Wang[a,b,1], Wen-Chen Wang[a,c,d,1], Chung-Ho Chen[e], Yu-Hsun Kao[e], Jing-Wei Hsu[e], Ching-Yi Chen[c,d], Ping-Ho Chen[a], Shyng-Shiou Yuan[b,f,g,h,**], Yuk-Kwan Chen[a,c,d,*]

[a] School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Division of Oral Pathology & Maxillofacial Radiology, Department of Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[d] Oral & Maxillofacial Imaging Center, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Division of Oral & Maxillofacial Surgery, Department of Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[f] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[g] Translational Research Center, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[h] Department of Obstetrics and Gynecology and Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

ARTICLE INFO

Keywords:
Sprouty2
Oral carcinogenesis
Oral squamous cell carcinoma
Oral potentially malignant disorder
Human

ABSTRACT

Objective: This study investigated SPRY2 expression in human oral potentially malignant disorders (OPMDs) and oral squamous cell carcinomas (OSCCs).

Methods: 75 OSCCs, 23 OPMDs with malignant transformation (MT), 17 OPMDs without MT, and eight normal oral mucosa (NOM) tissues were used for immunohistochemical staining; three OSCC tissues with normal tissue counterparts were used for western blotting. Three human oral cancer cell lines (OCCLs), an oral precancer cell line (DOK), and a NOM primary culture (NOMPC) were used for western blotting; OCCLs and NOMPC were employed for real-time quantitative reverse transcription-polymerase chain reaction. OCCLs were evaluated in terms of proliferation, migration, invasion and BRAF V600E point mutation assays.

Results: Significantly increased SPRY2 protein expression was observed in OSCCs as compared with NOM, and SPRY2 expression also differed between OSCC patients with and without lymph-node metastasis. SPRY2 protein and mRNA expressions were significantly enhanced as compared with NOMPC. Increased phospho-ERK expression was observed in OCCLs as compared with NOMPC. Significant decreases in the proliferation rate, degrees of migration and invasion were noted in OCCLs with *SPRY2* siRNA transfection as compared with those without *SPRY2* siRNA transfection. No BRAF V600E point mutation was observed for OCCLs as compared with NOMPC. A significantly increased SPRY2 protein level was noted in OPMDs with MT as compared to those without MT, and was also found in OPMDs with MT in comparison with NOM, as well as in DOK in comparison with NOMPC.

Conclusions: Our results indicated that SPRY2 overexpression is associated with human oral squamous-cell carcinogenesis.

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of human oral malignancies and is the 11th most common cancer worldwide; it makes up 3% of all newly-diagnosed cancer cases (Cheng, Rees, & Wright, 2014; Tang, Wu, Zhang, & Su, 2013). Because of the high prevalence (16.5% in men) of betel quid-chewing in Taiwan (Tovosia et al., 2007), OSCC is one of the leading types of cancer; it is the fourth most frequently occurring cancer and the fifth leading cause of cancer death in men in Taiwan (Health Promotion Administration, 2014). The claim made in recent research that the five-year survival rate of oral cancer patients is still low might be attributable to most cases of OSCC being diagnosed at an advanced stage. The five-year survival rate of patients with early-stage (I and II) OSCC is approximately 80%, but that of patients with advanced-stage (III and IV) OSCC is only approximately 20% (Scott, Grunfeld, & McGurk, 2005). This highlights the need for continued efforts to gain an understanding of the potential pathogenesis of oral carcinogenesis as an essential step

* Corresponding author at: School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.
** Corresponding author at: Translational Research Center, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan.
E-mail addresses: yuanssf@ms33.hinet.net (S.-S. Yuan), k0285@ms22.hinet.net (Y.-K. Chen).
[1] These authors contributed equally to this paper.

https://doi.org/10.1016/j.archoralbio.2017.12.021
Received 13 May 2017; Received in revised form 23 November 2017; Accepted 21 December 2017

towards improving treatment (Chen, Huang, Lin, & Lin, 1999).

On the other hand, quite a number of cases of OSCC are preceded by clinically-evident oral potentially malignant disorders (OPMDs) (Hsue et al., 2007; Wang et al., 2014). Most recently, it has been found that the malignant transformation rates of a cohort of 5071 patients with OPMDs were 4.84% for epithelial dysplasia with oral submucous fibrosis, 6.58% for epithelial dysplasia with hyperkeratosis/epithelial hyperplasia, 2.91% for hyperkeratosis/epithelial hyperplasia, 6.79% for verrucous hyperplasia, 0.52% for lichen planus, and 3.72% for oral submucous fibrosis; the overall malignant transformation rate was 4.32% (Wang et al., 2014). Thus, it is important to prevent malignant change in those patients diagnosed with OPMDs (Van der Waal, 2009).

Sprouty (SPRY) is an intracellular protein, and is called SPRY because it has been implicated in the regulation of branching in tracheal development, and its deficiency produces random 'sprouting' of tracheal tubules (Hacohen, Kramer, Sutherland, Hiromi, & Krasnow, 1998). SPRY has been confirmed to be one of the inhibitor proteins involved in modifying the components of the Ras/ERK (receptor tyrosine kinase, RTK) pathway (Lo et al., 2006). To date, four mammalian SPRY proteins (SPRY1–4) have been identified. These proteins possess highly-conserved cysteine-rich C termini (c-cbl), which are regarded as pivotal to the binding of SPRY to plasma membrane so as to suppress MAPK signals, and generally variable N termini that contain several small conserved sequences (de Maximy et al., 1999; Minowada et al., 1999; Tefft, Lee, Smith, Leinwand, & Zhao, 1999).

Mitogen-activated protein kinase (MAPK) signals from different RTKs [such as epidermal growth factor (EGF)] (Cabrita & Christofori, 2008; Guy et al., 2003; Kim & Bar-Sagi, 2004) are repressed by SPRY. The targets for SPRY suppression are variable, because SPRY may modulate upstream of RAS or downstream at the level of RAF (Gross, Bassit, Benezra, & Licht, 2001; Hanafusa, Torii, Yasunaga, & Nishida, 2002; Lee et al., 2004; Sasaki et al., 2003). On the other hand, it has also been demonstrated that SPRY does not suppress EGF-induced MAPK activation, but instead produces elevated activation of the MAPK pathway in a number of different cells (Lim et al., 2002; Rubin et al., 2003; Wong, Lim, Low, Chen, & Guy, 2001). Thus, SPRY seems able to either upregulate or downregulate MAPK signaling according to the ligand RTK, and also appears to be cell-specific.

Of the four SPRY proteins, SPRY2 has been the target of investigation in a number of cancers (Grose & Dickson, 2005). Experimental results have suggested that SPRY2 protein is an essential regulator of different pathways for tumorigenesis, for instance, angiogenesis, cell growth, invasion, and migration, which are crucial to the development of different types of cancer, such as breast cancer (Li et al., 2013; Lo et al., 2004; Takai & Jones, 2002), prostate cancer (Ahmad, Gao, Patel, & Leung, 2013; Jennifer & Gail, 2012; McKie et al., 2005; Patel et al., 2013) liver cancer (Sirivatanauksorn, Sirivatanauksorn, Srisawat, Khongmanee, & Tongkham, 2012; Song et al., 2012; Wang et al., 2012), and melanoma (Bloethner et al., 2005; Davies et al., 2002; Dong et al., 2003; Tsavachidou et al., 2004; Tuveson, Weber, & Herlyn, 2003; Wellbrock et al., 2004). However, the potential prognostic and clinical relevance of SPRY2 protein expression in human oral squamous cell carcinogenesis, to our knowledge, has not been fully elucidated. The aim of the current study was to explore the SPRY2 protein expression in human OPMDs and OSCCs.

2. Material and methods

2.1. SPRY2 expression in human OSCCs

2.1.1. Tissue microarray and immunohistochemistry

All tissues for tissue microarray were obtained from formalin-fixed, paraffin-embedded tissue blocks. Slides from hematoxylin-eosin stained sections were reviewed by an oral and maxillofacial pathologist to select representative areas of tumor to be cored. Construction of the tissue microarray was performed using Booster Arrayer & TMA designer software (Alphelys, France), the detailed procedure of which was described in a previous report (Yuan et al., 2012).

Table 1
Characteristics of the oral squamous cell carcinoma patients.

Patient characteristics		Number (%)
Mean age (years)	Male	54.4
	Female	59.8
Sex	Male	71 (94.7)
	Female	4 (5.3)
Differentiation	Good	66 (88.0)
	Moderate to poor	9 (12.0)
Tumor size	≤ 2 cm	62 (82.7)
	> 2 cm	13 (17.3)
Lymph-node metastasis	Yes	61 (81.3)
	No	14 (18.7)
Pathologic stage	I + II	54 (72.0)
	III + IV	21 (18.0)

Tissue specimens of primary OSCCs from 75 patients (71 males, mean age 54.4 years; 4 females, mean age 59.8 years) were retrieved from the Oral Pathology Department at Kaohsiung Medical University Hospital, with the approval of the Ethics Committee for Scientific Research on Human Beings of the institution (KMUH-IRB-20140272). All patients had the habits of drinking alcohol, chewing betel quid, and smoking cigarettes. The characteristics of the patients, including age, gender, differentiation, tumor size, histopathological lymph-node involvement, and stage, are summarized in Table 1. Normal oral mucosal tissue, used as a control, was taken from eight healthy individuals without the aforementioned habits that are risk factors of oral malignancy. The tissue specimens were fixed in 10% neutral buffered formalin solution for approximately 24 h, dehydrated in graded alcohols, cleaned in xylene, and embedded in paraffin for subsequent immunohistochemical staining.

Paraffin-embedded 4-μm-thick tissue sections were stained for SPRY2 protein using a primary rabbit polyclonal anti-SPRY2 antibody (Proteintech, Rosemont, IL, USA; Cat. No. 11383-1-AP). Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed using graded alcohols. To retrieve the antigenicity, tissue sections were treated three times with microwave radiation in a 10 mM citrate buffer (pH 6.0) for 5 min each. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 45 min to block the endogenous peroxidase activity, and were subsequently incubated in normal goat serum to reduce non-specific binding. Sections were finally incubated for 60 min at room temperature with primary anti-SPRY2 antibody (Proteintech; 1:100).

The sections were then processed using the standard avidin-biotin peroxidase complex method in accordance with the manufacturer's recommendations (Vector Laboratories) (Hsu, Raine, & Fanger, 1981) Diaminobenzidine (DAB, Roche, Cat. No. 1718096) was used as a chromogen, and commercial hematoxylin was used for counterstaining. Each set of experiments included a human colon squamous cell carcinoma specimen known to express SPRY2, which served as a positive control and ensured the reproducibility of the staining process. Negative controls were included following the same procedure, but with omission of the primary antibody. The scores of the percentage of positive staining (P) were classified as: 0 (< 1%); 1 (1–24%); 2 (25–49%); 3 (50–74%); and 4 (75–100%), whereas the scores for the intensity of staining (I) were classified as 0, no staining; 1, light yellow color (weak staining); 2, brown color (moderately strong staining); and 3, dark brown color (strong staining). The total score (S) was then calculated as P × I for each section (Sarbia et al., 1999).

Quantification and analysis of the immunohistochemical stained slides were performed by two experienced board-certified oral and

maxillofacial pathologists independently using the semi-automated image analysis software Image J Version 1.51e. The whole stained slides were observed under different magnifications (40-, 100-, 200-fold) to determine cell types and patterns of positive staining microscopically. Then, for quantification of positive staining, ten views of every immunostained section were randomly selected under 200-fold magnification. When disagreement existed between the two observers, a consensus was reached by mutual discussion.

2.1.2. Western blot for fresh tissue specimens of OSCCs

Fresh tissue specimens of OSCCs from three male patients aged between 60 and 71 years (mean age 65 years) along with their normal tissues counterparts were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer. The lysates were subsequently centrifuged at 4 °C, 14,000 rpm, for 15 min. The protein concentrations were measured using a Thermo Pierce Protein Assay Kit. Equal amounts of protein were denatured by adding SDS running buffer (Sigma-Aldrich, St Louis, MO, USA) and β-mercaptoethanol (Sigma-Aldrich). The samples were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Sigma-Aldrich) on 10% gels, and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich) using Bio-Rad's transblot with the primary antibody against SPRY2 (Proteintech; Cat. No. 11383-1-AP, 1: 1000), which has species specificity for human tissues with observed molecular weight 60–65 kDa, and β-actin (Sigma-Aldrich; 1: 1000), which has species specificity for human tissues with observed molecular weight 42 kDa, followed by HRP-conjugated secondary antibodies (Sigma-Aldrich; 1: 5000). The amount of protein was quantified using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan). The ratio was normalized by the β-actin signal.

2.1.3. Cell cultures

The characteristics of the human oral cancer cell lines (OECM1, SAS and Ca922) have been summarized in (Table 2). Briefly, OECM1 (Yang & Meng, 1994) was derived from the primary gingival SCC of a Taiwanese with betel-quid chewing history whilst both SAS (Takahashi et al., 1989) and Ca922 (Kimura, 1978) were obtained from Japanese with primary of tongue (high-grade) and OSCC of gingiva (with extensive epidermal growth receptor production) respectively. The three human oral cancer cell lines (OECM1, SAS and Ca922), kindly provided by Professor Shyng-Shiou Yuan (Kaohsiung Medical University), and normal oral mucosa (gingival tissue from a healthy male without the habits of alcohol drinking, betel-quid chewing, and cigarette smoking) primary cultures, kindly provided by Dr. Ping-Ho Chen, were cultured in high-glucose DMEM (Hyclone, Logan, UT, USA) with the addition of 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Hyclone) at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was changed on every third day.

2.1.4. Western blot for cells of human oral cancer cell lines

Cells of human oral cancer cell lines (OECM1, SAS and Ca922) and human normal oral mucosa primary culture were rinsed with PBS (Sigma-Aldrich, St Louis, MO, USA) and lysed with RIPA lysis buffer. The lysates were subsequently centrifuged at 4 °C, 14,000 rpm, for 15 min. The protein concentrations were measured using a Thermo Pierce Protein Assay Kit. Equal amounts of protein were denatured by adding SDS running buffer (Sigma-Aldrich) and β-mercaptoethanol (Sigma-Aldrich). The samples were then analyzed by SDS-PAGE (Sigma-Aldrich) on 10% gels, and the proteins were transferred onto a PVDF membrane (Sigma-Aldrich) using Bio-Rad's transblot with the primary antibody against SPRY2 (Proteintech; 1: 1000), phosphor-ERK (Cell Signaling, Beverly, MA, USA, Cat. No. 4370; 1: 1000), with species specificity for human tissues and observed molecular weight 42–44 kDa, total-ERK (Cell Signaling, Cat. No. 4695; 1: 1000), with species specificity for human tissues and observed molecular weight 42–44 kDa, and β-actin (Sigma-Aldrich; 1: 1000), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich; 1: 5000). The amount of protein was then quantified using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan). The ratio was normalized by the β-actin signal.

2.1.5. Real-time quantitative reverse transcription-polymerase chain reaction for cells of human oral cancer cell lines

Total RNA samples were prepared from human oral cancer cell lines (OECM1, SAS and Ca922) and normal oral mucosa primary culture using TRIzol Reagent (Invitrogen) (Chomczynski & Sacchi, 1987). The quality and concentration of RNA were assessed according to the optical density at a wavelength of 260 nm (using an optical density 260 unit equivalent to 40 μg/mL of RNA). The RNA was resuspended in 100 μL of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1 μg/mL. Reverse transcription was performed from 500 ng of total RNA template using a High Capacity cDNA Reverse Transcription Kit. cDNA was amplified by PCR with oligoprimers for *SPRY2* and β-actin, which were designed with reference to the published cDNA sequences in GenBank (Supplementary Table 1).

The reaction was performed on a thermal cycler with Maxima™ SYBR Green qPCR Master Mix (2′) added to the PCR reaction mixture at an annealing temperature of 60 °C for 50 cycles. After normalization to the expression level of β-actin mRNA, the relative expression levels of each of human oral cancer cell line and β-actin mRNA were determined as the percentage change as compared with the normal oral mucosa primary culture.

2.1.6. Establishment of human OSCC cell cultures with SPRY2 gene knockdown

RNA interference was performed using commercially-synthesized *SPRY2* siRNA (ON-TARGET plus Human *SPRY2* (10253) siRNA-SMART pool; Supplementary Table 2) as described in the protocols provided by the manufacturer (Dharmacon RNAi, Gene Expression, Buckinghamshire, UK), as follows: *SPRY2* siRNA stock solution at a concentration of 5 μM in RNase-free solution was first prepared. In separate tubes, *SPRY2* siRNA (Tube 1) and the appropriate DharmaFECT (Tube 2) transfection reagent were diluted with serum-free medium, respectively. The contents of Tube 1 and Tube 2 were gently mixed by pipetting carefully up and down and incubated for 5 min at room temperature. Then, the contents of Tube 1 were added to Tube 2 and mixed by pipetting carefully up and down and incubated for 20 min at room temperature. Subsequently, an antibiotic-free complete medium

Table 2
Summary of the human oral cancer and precancer cell lines used in the current study.

Cell line	Tumor source/characteristic	Profile	Ethnicity
OECM1 (Yang & Meng, 1994)	Primary OSCC from gingiva	A history of betel-quid chewing	Taiwanese
SAS (Takahashi et al., 1989)	Primary OSCC from tongue	A high-grade, poorly-differentiated OSCC	Japanese
Ca922 (Kimura, 1978)	Primary OSCC from gingiva	EGF receptor produced extensively	Japanese
DOK (Chang et al., 1992)	Dysplastic oral keratinocyte from tongue	Mild to moderate ED, with a keratin profile similar to the original dysplasia Non-tumourigenic in athymic nude mice	Caucasian

OSCC: oral squamous cell carcinoma; EGF: epidermal growth receptor; ED: epithelial dysplasia.

was added to the mixed solution to attain a final *SPRY2* siRNA concentration of 50 nM. Finally, the culture medium from the dishes was removed and the transfection medium was added to each dish. The cells of oral cancer cell lines (OECM1, SAS and Ca922) were incubated with the prepared transfection medium at 37 °C in 5% CO_2 for 48 h.

2.1.7. Proliferation assay

The cell proliferation assay experiments were measured for 24 h and 48 h post-transfection respectively using a Cell Proliferation Kit (XTT based, Biological Industries Israel Beit Haemek Ltd. Kibbutz Beit Haemek, Israel; Cat. No. 20-300-1000) according to the following procedure: oral cancer cells (OECM1, SAS and Ca922) at a concentration of 100 µL were plated respectively into flat 96-well plates (5×10^3–1×10^4 cells/well) and transfected with *SPRY2* siRNA for 48 h. Then, a reaction solution sufficient for one plate (96 wells) was prepared, and 0.1 mL of activation solution was subsequently added to 5 mL XTT reagent. 50 µL of the reaction solution was added to each well (with and without *SPRY2* siRNA transfection) and the plate was incubated for 24 h, and 48 h after transfection respectively. with gentle shaking to evenly distribute the dye in the wells. Finally, the absorbance of the samples against a background control as a blank was measured at a wavelength of 450–500 nm using a spectrophotometer (Benchmark Plus Microplate Spectrophotometer System #1706930, BioRad, Hercules, California, USA). Three independent experiments were performed.

2.1.8. Migration assay

The migration assay experiments were measured 24 h and 48 h post-transfection respectively as per the procedures described below. After seeding cells of oral cancer cell lines (OECM1, SAS and Ca922) without *SPRY2* siRNA transfection in a 6-cm dish overnight, cells were transfected with *SPRY2* siRNA for 48 h, then trypsinized with 0.25% Trypsin-EDTA (1×) and Phenol Red (Cat. No. 25200072; Gibco, Glen Burnie, MD, USA), following which cells were counted. Cells at a concentration of 500 µL (with and without *SPRY2* siRNA transfection) were subsequently plated into each Matrigel chamber [5×10^4 cells/chamber; Corning Matrigel Invasion Chamber 24-well Plate 8.0 µm (BioCoat)] and incubated at 37 °C in a 5% CO_2 incubator for 24 h, and 48 h after transfection respectively. Finally, the cells were stained with 0.5% crystal violet (Shimaku's pure chemicals, Samchun, Korea) for 30 min and counted by light microscopy based on five-field digital images taken randomly at 200× magnification. Quantification and analysis were performed using the semi-automated image analysis software Image J Version 1.51e. Three independent experiments were performed.

2.1.9. In vitro transwell invasion assay

The invasion assay experiments were measured 24 h and 48 h post-transfection respectively as follows: cells of oral cancer cell lines (OECM1, SAS and Ca922) without *SPRY2* siRNA transfection were seeded in a 6-cm dish overnight. After transfecting the cells with *SPRY2* siRNA for 48 h, the cells were trypsinized with 0.25% Trypsin-EDTA (1×) and Phenol Red (Cat. No. 25200072; Gibco, Glen Burnie, MD, USA), and then counted. *In vitro* cancer cell invasion assay was then performed using a 12-well transwell inserts with 8-µm pore size and coated with matrigel (Corning Life Sciences, Corning, NY). The upper wells of the inserts were coated with 100 µL Matrigel (1 mg/mL) (BD Bioscience, San Jose, CA) and polymerized at 37 °C for 4 h. The coating was then rinsed once with serum-free medium. Subsequently, oral cancer cells at a concentration of 500 µL (with and without *SPRY2* siRNA transfection) were added to the upper well; the bottom chamber contained growth medium with 10% FBS. After incubation for 24 h, and 48 h after transfection respectively, cells in the top well were removed by peeling off the matrigel and swiping the top of the membrane with cotton swabs. Cells on the underside of the membrane were stained with 0.4 g/L crystal violet (Sigma, St Louis, MO) and counted by light microscopy based on five-field digital images taken randomly at 200× magnification. Quantification and analysis were performed using the semi-automated image analysis software Image J Version 1.51e. Three independent experiments were performed.

2.1.10. BRAF V600E point mutation analyses

Genomic DNA was extracted from cells of oral cancer cell lines (OECM1, SAS and Ca922) and a primary culture of normal oral mucosa by proteinase K digestion and phenol-chloroform extraction, as described elsewhere (Sambrook & Russell, 2001), using a QIAamp DNA FFPE Tissue Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA was then quantified by spectrophotometry (NanoDrop instrument; Thermo Fisher Scientific, Wilmington, DE, USA). A quantitative polymerase chain reaction was performed using a specific TaqMan probe (BRAF_rs113488022) (Life Technologies, Carlsbad, CA, USA) to detect the T > A transversion at position c.1799.

Cells of oral cancer cell lines (OECM1, SAS and Ca922) and a primary culture of normal oral mucosa were further analyzed by direct sequencing (Chen, Hsue, & Lin, 2011). Oligoprimers developed with reference to the published cDNA sequences in GenBank (Supplementary Table 3) were used for PCR amplification under the following conditions: 1 cycle at 95 °C for 15 min; 40 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 7 min. Amplified PCR products were purified using an ABI cleanup kit and sequenced in both directions using a BigDye Terminator, version 3.1, Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI3730 system running ABI Prism DNA Sequence Analysis Software version 2.6 (Applied Biosystems).

2.2. SPRY2 expression in human OPMDs

2.2.1. Immunohistochemistry

Samples from two different cohorts of human OPMD patients (including patients with hyperkeratosis/epithelial hyperplasia with or without oral epithelial dysplasia, oral submucous fibrosis, and verrucous hyperplasia) with (Table 3) and without (Table 4) malignant transformation were retrieved from the Oral Pathology Department at Kaohsiung Medical University Hospital, with approval from the Ethics Committee for Scientific Research on Human Beings of the institution.

The histopathological criteria for oral epithelial dysplasia (Van der Waal, 1986) include: (i) basal layer hyperplasia, (ii) nuclear enlargement and hyperchromatism, (iii) loss of intercellular adhesion and normal polarization, (iv) abnormal mitoses above the basal cell layer, (v) individual cell keratinization within the spinous layer, (vi) cellular pleomorphism, (vii) drop-shaped epithelial ridges, (viii) irregular stratification, and (ix) an altered nuclear-cytoplasmic ratio. The degree of dysplasia was graded in accordance with the following criteria (Wright & Shear, 1985): (i) mild epithelial dysplasia: dysplastic alterations limited to the lower third of the oral epithelium; (ii) moderate epithelial dysplasia: dysplastic changes noted for up to two-thirds of the thickness of the oral epithelium; and (iii) severe epithelial dysplasia: dysplastic cells observed within more than two-thirds but less than the whole thickness of the oral epithelium. On the other hand, two criteria to confirm malignant transformation must be fulfilled in order to diagnose progression to oral cancer: the first is that the potential oral cancer must develop at the same site as the initial lesion; the second is that the time taken for this progression must exceed six months. This latency time is designed to exclude the possibility of concomitant progression (Wang et al., 2014).

The tissue processing of all human OPMDs specimens, the subsequent immunohistochemical staining, and the grading of IS were performed as described above for the OSCC tissue specimens.

2.2.2. Cell cultures

Human oral precancer cell line DOK (Table 2) (Chang, Foster, Betts,

Table 3
Characteristics of the oral potentially malignant disorder (OPMD) patients with malignant transformation (MT).

Patient No.	Age (years)	Gender	OPMD	Duration of MT (months)
1	56	Male	Severe oral epithelial dysplasia	20.70
2	53	Male	Moderate oral epithelial dysplasia	24.60
3	41	Male	Epithelial hyperplasia with mild oral epithelial dysplasia	69.90
4	44	Male	Epithelial hyperplasia with mild epithelial dysplasia	19.77
5	42	Male	Epithelial hyperplasia and hyperparakeratosis	46.70
6	55	Male	Hyperorthokeratosis	38.83
7	57	Female	Epithelial hyperplasia with hyperparakeratosis and oral submucous fibrosis	70.77
8	62	Male	Oral submucous fibrosis	31.97
9	61	Male	Oral submucous fibrosis and hyperparakeratosis	45.60
10	57	Male	Verrucous hyperplasia	29.80
11	53	Male	Oral submucous fibrosis	19.23
12	46	Male	Severe oral epithelial dysplasia	47.83
13	54	Male	Epithelial dysplasia and oral submucous fibrosis	33.50
14	46	Male	Verrucous hyperplasia	64.33
15	88	Female	Severe oral epithelial dysplasia	18.87
16	36	Male	Verrucous hyperplasia	16.77
17	57	Male	Oral submucous fibrosis	21.10

Table 4
Characteristics of the oral potentially malignant disorder (OPMD) patients without malignant transformation.

Patient No.	Age (years)	Gender	OPMD
1	51	Male	Severe oral epithelial dysplasia
2	54	Male	Severe oral epithelial dysplasia
3	40	Male	Severe oral epithelial dysplasia
4	69	Male	Verrucous hyperplasia with oral submucous fibrosis
5	49	Male	Severe oral epithelial dysplasia
6	70	Male	Severe oral epithelial dysplasia
7	32	Male	Severe oral epithelial dysplasia
8	51	Male	Severe oral epithelial dysplasia
9	58	Male	Severe oral epithelial dysplasia
10	40	Male	Hyperparakeratosis with mild oral epithelial dysplasia
11	56	Male	Severe oral epithelial dysplasia with oral submucous fibrosis
12	65	Male	Severe oral epithelial dysplasia
13	53	Male	Severe oral epithelial dysplasia
14	27	Male	Hyperparakeratosis with mild epithelial dysplasia
15	47	Male	Severe oral epithelial dysplasia
16	69	Male	Severe oral epithelial dysplasia
17	55	Male	Severe oral epithelial dysplasia
18	50	Male	Severe oral epithelial dysplasia
19	39	Male	Severe oral epithelial dysplasia
20	70	Male	Severe oral epithelial dysplasia
21	52	Male	Moderate to severe oral epithelial dysplasia
22	32	Male	Severe oral epithelial dysplasia
23	49	Male	Severe oral epithelial dysplasia

& Marnock, 1992), kindly provided by Professor Shyng-Shiou Yuan (Kaohsiung Medical University), was cultured in high-glucose DMEM (Hyclone, Logan, UT, USA) with the addition of 10% fetal bovine serum (Hyclone), 2 mM glutamine (Hyclone), 5 μg/ml hydrocortisone (Hyclone), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. On the other hand, human oral cancer cell lines OECM1 and SAS, and a normal oral mucosa (gingiva) primary culture, were cultured as described above in 2.1.3.

2.2.3. Western blot

Cells of the human oral precancer cell line DOK, human oral cancer cell lines OECM1 and SAS, and a human normal oral mucosa primary culture were rinsed with phosphate-buffered saline (PBS; Sigma-Aldrich) and lysed with RIPA lysis buffer (Sigma-Aldrich). The lysates were subsequently centrifuged at 4 °C, 14,000 rpm, for 15 min. The protein concentrations were measured using a Thermo Pierce Protein Assay Kit. Equal amounts of protein were denatured by adding SDS running buffer (Sigma-Aldrich) and β-mercaptoethanol (Sigma-Aldrich). The samples were then analyzed by SDS-PAGE (Sigma-Aldrich) on 10% gels, and the proteins were transferred onto a PVDF membrane (Sigma-Aldrich) using Bio-Rad's transblot with the primary antibody against SPRY2 (Proteintech; 1: 1000) and β-actin (1: 1000), followed by HRP-conjugated secondary antibodies (1: 5000). The amount of protein was then quantified using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan). The ratio was normalized by the β-actin signal.

2.3. Statistical analyses

All statistical analyses were performed using the SAS Statistical Package (Version 9.1.3, SAS Institute Inc.). Mann-Whitney *U* tests were used to compare the immunohistochemical expressions of SPRY2 protein among the OPMDs with/without malignant transformation, OSCCs, and normal oral mucosa, and to assess the differences in proliferation rate and degrees of migration and invasion between oral cancer cells with and without *SPRY2* siRNA transfection. Chi-square analysis was employed to compare the expression of SPRY2 protein along with the differentiation, tumor size, histological lymph-node involvement, and pathologic stage of the OSCC patients. The disease-free survival rate of the OSCC patients with respect to high and low SPRY2 expression was examined using Kaplan-Meier analysis, and the differences in survival rate were analyzed using the log-rank test. Additionally, nonparametric Kruskal-Wallis tests were used to analyze the results of western blots and real-time quantitative reverse transcription-polymerase chain reactions. Statistical significance was considered when the P value < 0.05.

3. Results

3.1. SPRY2 expression in human OSCCs

3.1.1. Tissue microarray and immunohistochemistry

Positive cytoplasmic staining of SPRY2 protein was observed in 71 cases (94.6%) of OSCC (Fig. 1A), and negative staining was noted in 4 cases (5.4%). On the other hand, positive staining of SPRY2 protein was observed in six normal oral mucosa samples (75.0%), while two samples demonstrated negative staining (25.0%) (Fig. 1B). The mean IS of the OSCC cases was 6.67, whilst that of the normal oral mucosa samples was 1.88 (Table 5). A significant increase was noted in the mean IS of the OSCC cases in comparison with that of the normal oral mucosa samples (Table 5).

A significant difference in the mean IS of SPRY2 protein was confirmed in a comparison of the OSCC patients with and without

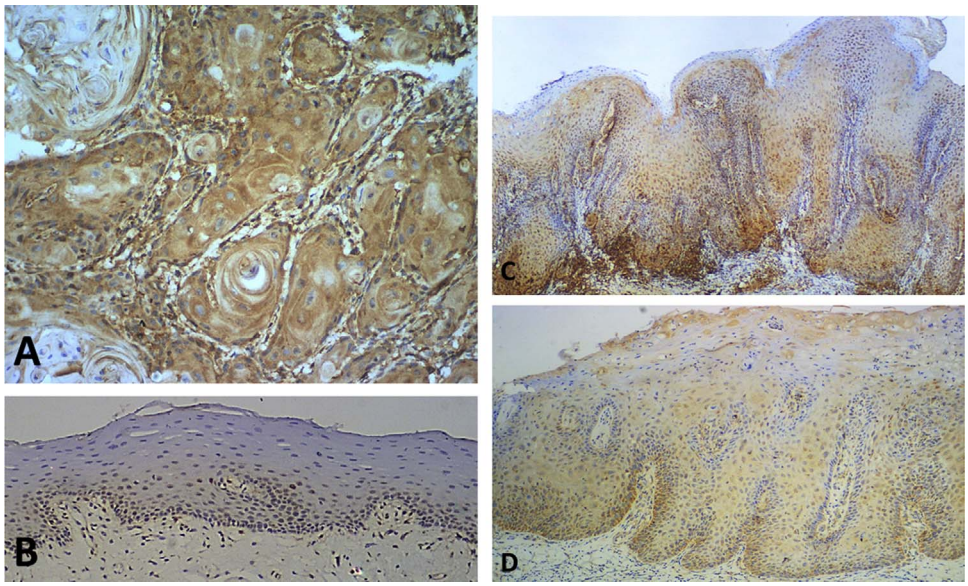

Fig. 1. Immunohistochemical staining of SPRY2 protein in human oral squamous cell carcinoma and normal oral mucosa as well as in human oral premalignant lesions with and without malignant transformation.
A representative strong immunohistochemical staining of SPRY2 protein in human oral squamous cell carcinoma (A, ×100), and weak staining of SPRY2 protein in normal oral mucosa (B, ×100). A representative stronger immunohistochemical staining of SPRY2 protein for a human oral premalignant lesion with malignant transformation (C, ×100) and weaker staining of SPRY2 protein for a human oral premalignant lesion without malignant transformation (D, ×100).

histological cervical lymph-node metastasis ($P = 0.03$; Table 6) with the cut-off value determined to be 7 and specificity, and sensitivity to be 0.6183 and 0.8571 respectively upon ROC (receiver operating characteristic) curve analysis; however, there were no significant correlations of the mean IS of SPRY2 protein with histopathological differentiation, stage, or tumor size ($P > 0.05$; Table 6). Additionally, the disease-free survival rate of the OSCC patients with a high IS (IS > 7) of SPRY2 protein was poorer than that of the OSCC patients with a low IS (IS < 7), but the difference was not significant ($P < 0.05$; Supplementary Fig. 1).

3.1.2. Western blot for fresh tissue specimens of OSCC

By western blot analysis of fresh tissues of human OSCC, overexpression of SPRY2 protein of approximately five-fold was observed as compared with primary cultures of samples of normal oral mucosa (Fig. 2), this difference being of statistical significance ($P < 0.05$, non-parametric Kruskal-Wallis test).

3.1.3. Western blot for cells of human oral cancer cell lines

Western blot analysis of the three human oral cancer cell lines (OECM1, SAS and Ca922) indicated overexpression of SPRY2 protein by two-, six- and 14-fold, respectively, as compared with primary cultures of normal oral mucosa samples (Fig. 3A), these results being of statistical significance ($P < 0.05$, non-parametric Kruskal-Wallis test). On the other hand, overexpression of phospho-ERK by 2.7- and 1.7-fold (normalized to total-ERK) was noted in OECM1 and Ca922, respectively, as compared with primary cultures of normal oral mucosa, which was of statistical significance ($P < 0.05$); however, there was a slight decrease in expression in SAS in comparison with primary cultures of normal oral mucosa ($P > 0.05$) (Fig. 3B).

3.1.4. Real-time quantitative reverse transcription-polymerase chain reaction for cells of human oral cancer cell lines

Real-time quantitative reverse transcription-polymerase chain reaction analyses of the three human oral cancer cell lines (OECM1, SAS and Ca922) indicated upregulation of *SPRY2* mRNA expression by 3.2-fold ($P < 0.05$), 5.5-fold ($P < 0.05$), and 15.3 fold ($P < 0.05$), respectively, as compared with primary cultures of normal oral mucosa samples (Fig. 4).

3.1.5. Proliferation assay of human OSCC cell cultures with SPRY2 gene knock-down

The proliferation rates of the oral cancer cell lines (OECM1, SAS and Ca922) with *SPRY2* siRNA transfection were significantly decreased as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection ($P < 0.05$) for 24 h (Fig. 5) and 48 h (Supplementary Fig. 2) after transfection respectively.

3.1.6. Migration assay of human OSCC cell cultures with SPRY2 gene knock-down

The degrees of migration of the oral cancer cell lines (OECM1, SAS and Ca922) with *SPRY2* siRNA transfection were significantly decreased as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection ($P < 0.05$) 24 h (Fig. 6) and 48 h (Supplementary

Table 5
Statistical analyses of immunohistochemical expression of SPRY2 protein among human oral potentially malignant disorders (OPMDs) with/without malignant transformation (MT), oral squamous cell carcinomas (OSCCs), and normal oral mucosa (NOM).

	P values			
	OSCC	OPMD with MT	OPMD without MT	NOM
OSCC (9.34 ± 3.61)#		*<0.005	*<0.005	*<0.005
OPMD with MT (3.53 ± 1.95)	*<0.005		*0.049	*0.015
OPMD without MT (2.46 ± 1.72)	*<0.005	*0.049		0.565
NOM (1.88 ± 1.27)	*<0.005	*0.015	0.565	

#Values within brackets: immunoscore (mean ± standard deviation).
*$P < 0.05$ (Mann-Whitney *U* test).

Table 6
Statistical comparison of immunohistochemical expression of SPRY2 protein in human oral squamous cell carcinomas with histopathological features.

Histopathological feature		Immunoscore (mean ± standard deviation)	*P* value
Differentiation	Well	6.80 ± 2.16	$P = 0.21$
	Moderate to poor	5.78 ± 3.11	
Tumor size	≤2 cm	6.64 ± 2.33	$P = 0.78$
	> 2 cm	6.84 ± 2.15	
Lymph-node metastasis	Yes	7.86 ± 2.38	$P = 0.03$*
	No	6.41 ± 1.36	
Pathologic stage	I + II	6.52 ± 2.45	$P = 0.33$
	III + IV	7.10 ± 1.81	

*$P < 0.05$ (Chi-square analysis).

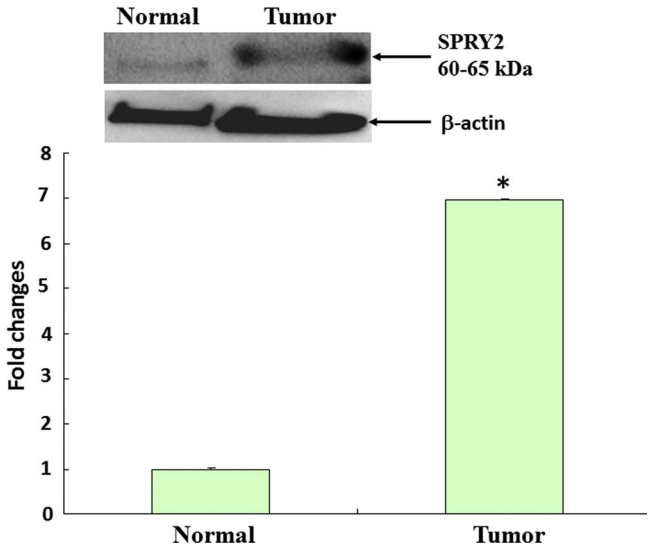

Fig. 2. Western blot analyses of SPRY2 protein expression in human oral squamous cell carcinoma as compared with human normal oral mucosa counterparts.
Western blot analyses showed upregulation of SPRY2 protein expression in human oral squamous cell carcinoma tissue as compared with normal oral mucosa. Results were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to the normal oral mucosa. Bars represent means ± standard deviation of the mean (* $P < 0.05$ compared with normal oral mucosa). A representative result of three independent experiments is shown.

Fig. 2) after transfection respectively.

3.1.7. In vitro transwell invasion assay of human OSCC cell cultures with SPRY2 gene knock-down

The degrees of invasion of the oral cancer cell lines (OECM1, SAS and Ca922) with *SPRY2* siRNA transfection were significantly decreased as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection ($P < 0.05$) 24 h (Fig. 7) and 48 h (Supplementary Fig. 2) after transfection respectively.

3.1.8. BRAF V600E point mutation analyses

No BRAF V600E point mutation was observed for the three human oral cancer cell lines (OECM1, SAS and Ca922) as compared with the primary culture of normal oral mucosa (Supplementary Fig. 3).

3.2. SPRY2 expression in human OPMDs

3.2.1. Immunohistochemistry

Positive cytoplasmic staining of SPRY2 protein was observed in all cases of human OPMD with and without malignant transformation (Fig. 1C & D). The mean IS of the human OPMDs with and without malignant transformation was 3.53 and 2.46, respectively (Table 5), a significant increase being noted when comparing the mean IS of human OPMDs with malignant transformation with those without malignant transformation (Table 5). On the other hand, a significant increase in the mean IS was noted when comparing cases of human OPMDs with malignant transformation with normal mucosa samples; no significant increase of IS was found when comparing human OPMDs without malignant transformation with normal mucosa samples (Table 5).

3.2.2. Western blot

Western blot analysis of human oral premalignant cell line DOK and human oral cancer cell lines OECM1 and SAS indicated overexpression of SPRY2 protein by two-, three-, and six-fold, respectively, as compared with the primary culture of normal oral mucosa (Fig. 3C), these differences being of statistical significance ($P < 0.05$). On the other hand, upregulation of SPRY2 protein expression was also noted for human oral cancer cell lines OECM1 and SAS) as compared with DOK, statistical significance having been established for the difference between SAS and DOK ($P < 0.05$), but not between OECM1 and DOK ($P > 0.05$) (Fig. 3C).

4. Discussion

To our knowledge, the potential prognostic and clinical relevance of SPRY2 expression in human oral squamous cell carcinogenesis has not been fully explored.

In the current study, significant upregulation of SPRY2 protein expression in human OSCC tissue specimens in comparison with human normal oral mucosa was confirmed by immunohistochemical and western blot analyses. Compatible with the *in vivo* data, overexpressions of SPRY2 protein and mRNA were also demonstrated in human oral cancer cell lines as compared with a human normal oral mucosa primary culture. Additionally, a significantly higher SPRY2 protein expression was noted in OSCC patients with lymph-node metastasis in comparison with those without. Moreover, significant decreases in the proliferation rate, migration and invasion of the oral cancer cells with *SPRY2* siRNA transfection were noted as compared with the oral cancer cells without *SPRY2* siRNA transfection. In the present study, a lower disease-free survival was noted for OSCC patients with high SPRY2 protein expression as compared with OSCC patients with low SPRY2 protein expression, although this difference was not statistically significant. Taken together, the aforementioned experimental evidence produced in the current study indicated that SPRY2 is involved in human OSCC formation, and could have a potential impact on the disease-free survival rate of OSCC patients.

On the other hand, significant overexpressions of SPRY2 protein were observed in human oral premalignant cell line DOK and oral cancer cell lines as compared with the primary culture of normal oral mucosa in the current study. Upregulation of SPRY2 protein expression was also noted in human oral cancer cell lines as compared with DOK. A significantly greater SPRY2 protein expression was noted in human OPMDs with malignant transformation in comparison with cases without malignant transformation, which indicated that SPRY2 protein

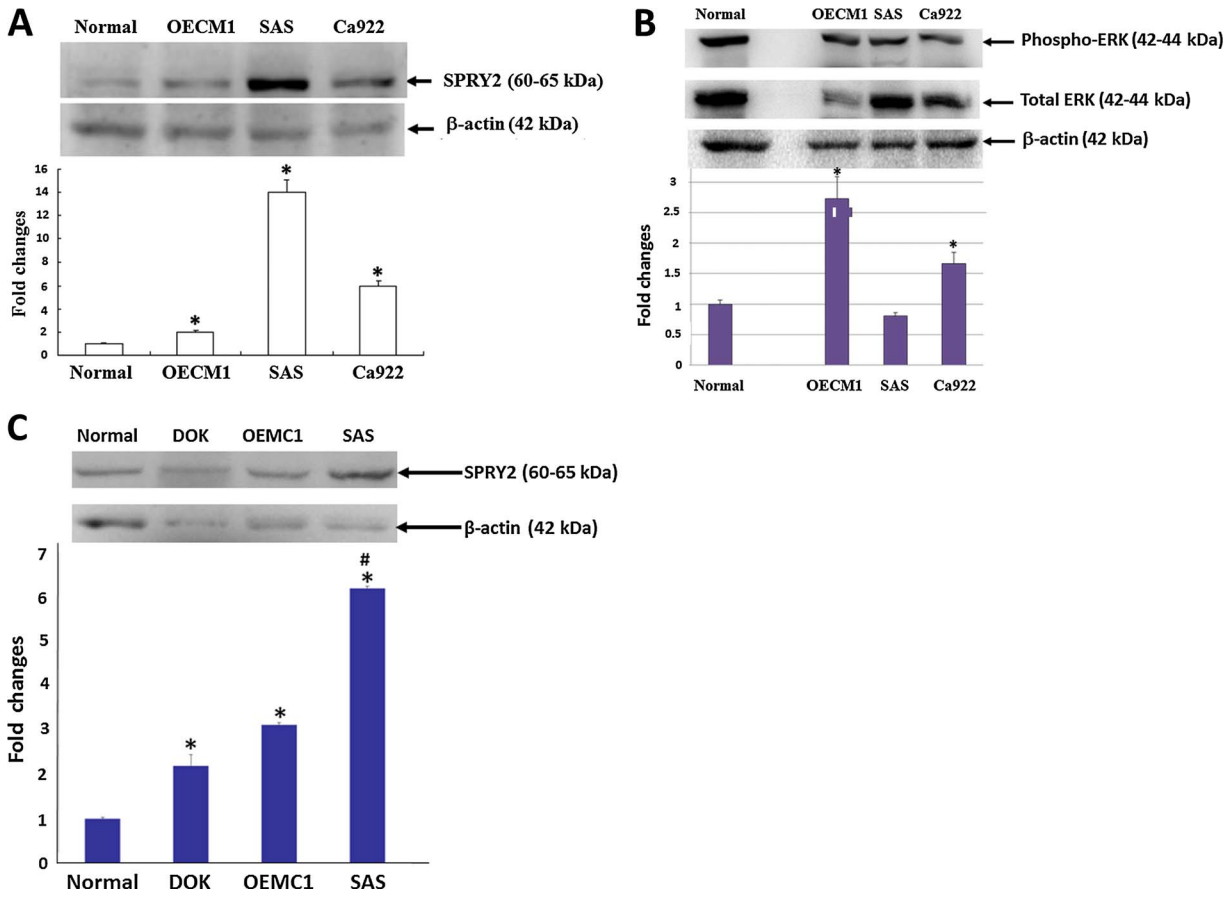

Fig. 3. Western blot analyses: SPRY2 protein expression in human oral cancer cell lines as compared with a primary culture of normal oral mucosa; phosphor-ERK and total-ERK in human oral cancer cell lines as compared with a primary culture of normal oral mucosa; SPRY2 protein expression in human oral premalignant cell line DOK, and human oral cancer cell lines OECM1 and SAS as compared with a primary culture of human normal oral mucosa.
(A) Upregulation of SPRY2 protein expression in human oral cancer cell lines (OCEM1, SAS, and Ca922) as compared with a primary culture of human normal oral mucosa. (B) Upregulation of phosphor-ERK (normalized to total-ERK) in OECM1 and Ca922 cell lines as compared with a primary culture of normal oral mucosa, and a slightly decreased expression in SAS as compared with a primary culture of normal oral mucosa. (C) Upregulation of SPRY2 protein expression in DOK and in human oral cancer cell lines OECM1 and SAS as compared with a primary culture of human normal oral mucosa. Upregulation of SPRY2 protein expression was also observed for the human oral cancer cell lines as compared with DOK.
Results were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to the normal oral mucosa. Bars represent means ± standard deviation of the mean (* $P < 0.05$ compared with normal oral mucosa primary culture; # $P < 0.05$ compared with DOK). A representative result of three independent experiments is shown.

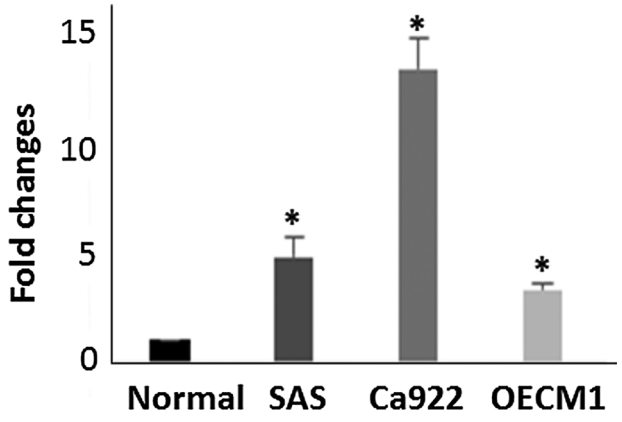

Fig. 4. Real-time quantitative reverse transcription-polymerase chain reaction of SPRY2 in human oral cancer cell lines as compared with a primary culture of normal oral mucosa.
Upregulation of SPRY2 mRNA expression was observed in human oral cancer cell lines (OECM1, SAS, and Ca922) as compared with a primary culture of human normal oral mucosa. Results were normalized by the level of GAPDH and expressed as fold change relative to the normal oral mucosa. Bars represent means ± standard deviation of the mean (* $P < 0.05$ compared with normal oral mucosa primary culture).

could be associated with malignant transformation of human OPMDs. Taking the data for human OPMDs and OSCCs together, the present study, to the best of our knowledge, was the first to demonstrate the potential prognostic and clinical relevance of SPRY2 protein expression in human oral squamous cell carcinogenesis.

In contrast to the findings of the present study, SPRY2 protein has been demonstrated to be downregulated in a number of other cancers, such as breast cancer (Takai & Jones, 2002), prostate cancer (Fritzsche et al., 2006; McKie et al., 2005), non-small-cell lung cancer, (Sutterlüty et al., 2007) colon cancer (Feng et al., 2011), endometrial cancer (Velasco et al., 2011), hepatocellular carcinoma (Sirivatanauksorn et al., 2012), and head and neck cancer (Lin et al., 2015).

On the other hand, consistent with the experimental data obtained in the current study, upregulation of SPRY2 was observed in melanoma containing the BRAF V600E mutation (Bloethner et al., 2005; Wellbrock et al., 2004). Experimental evidence has suggested that BRAF mutations cause BRAF kinase activity and increased ERK signaling, leading to cell proliferation in melanoma cells (Davies et al., 2002; Dong et al., 2003; Tuveson et al., 2003).

Additionally, compatible with the results of this study, upregulation of SPRY2 protein expression has also been reported in colon cancers (Barbáchano et al., 2010; Holgren et al., 2010). It has been shown that, perhaps due to involvement of c-Met upregulation, SPRY2 is

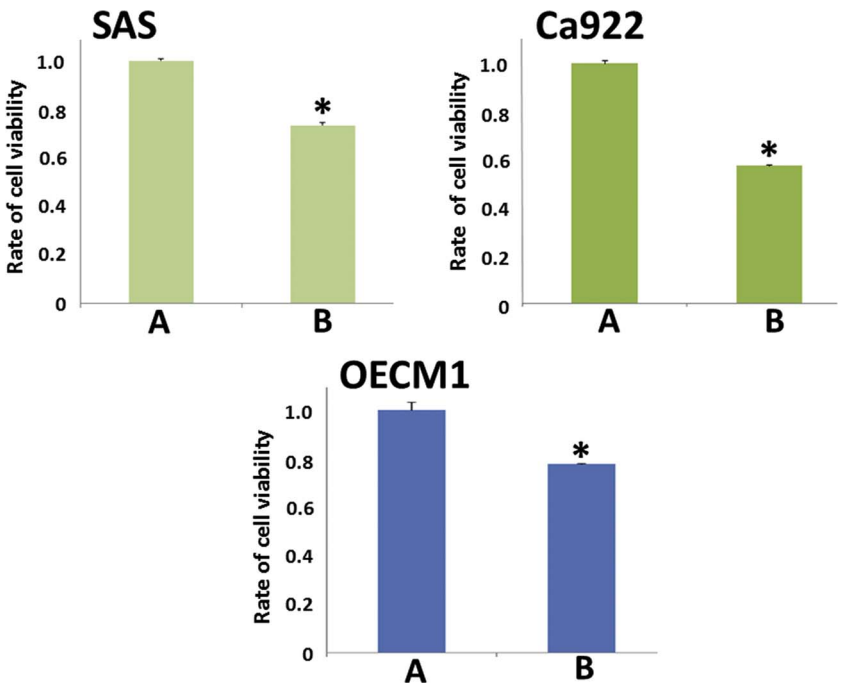

Fig. 5. Proliferation assays of oral cancer cell lines (SAS, Ca922, and OECM1) with 24 h after *SPRY2* siRNA transfection as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection.
The proliferation rates of the oral cancer cell lines (SAS, Ca922, and OECM1) with *SPRY2* siRNA transfection (B) were significantly lower than those of the three oral cancer cell lines without *SPRY2* siRNA transfection (A) (* $P < 0.05$).

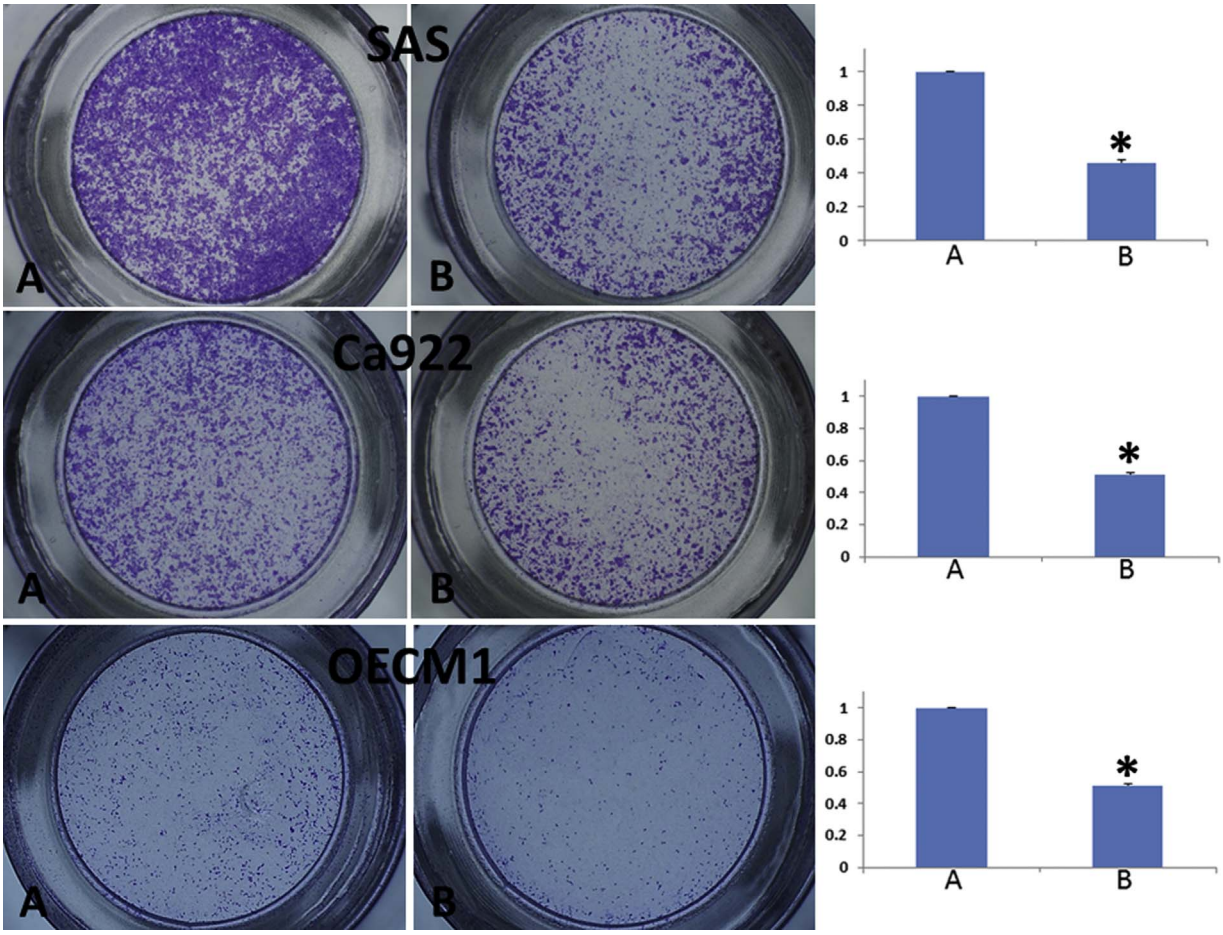

Fig. 6. Migration assays of the oral cancer cell lines (SAS, Ca922, and OECM1) with 24 h after *SPRY2* siRNA transfection as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection.
The degree of migration of the oral cancer cell lines (SAS, Ca922, and OECM1) with *SPRY2* siRNA transfection (B) was significantly lower as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection (A) (* $P < 0.05$).

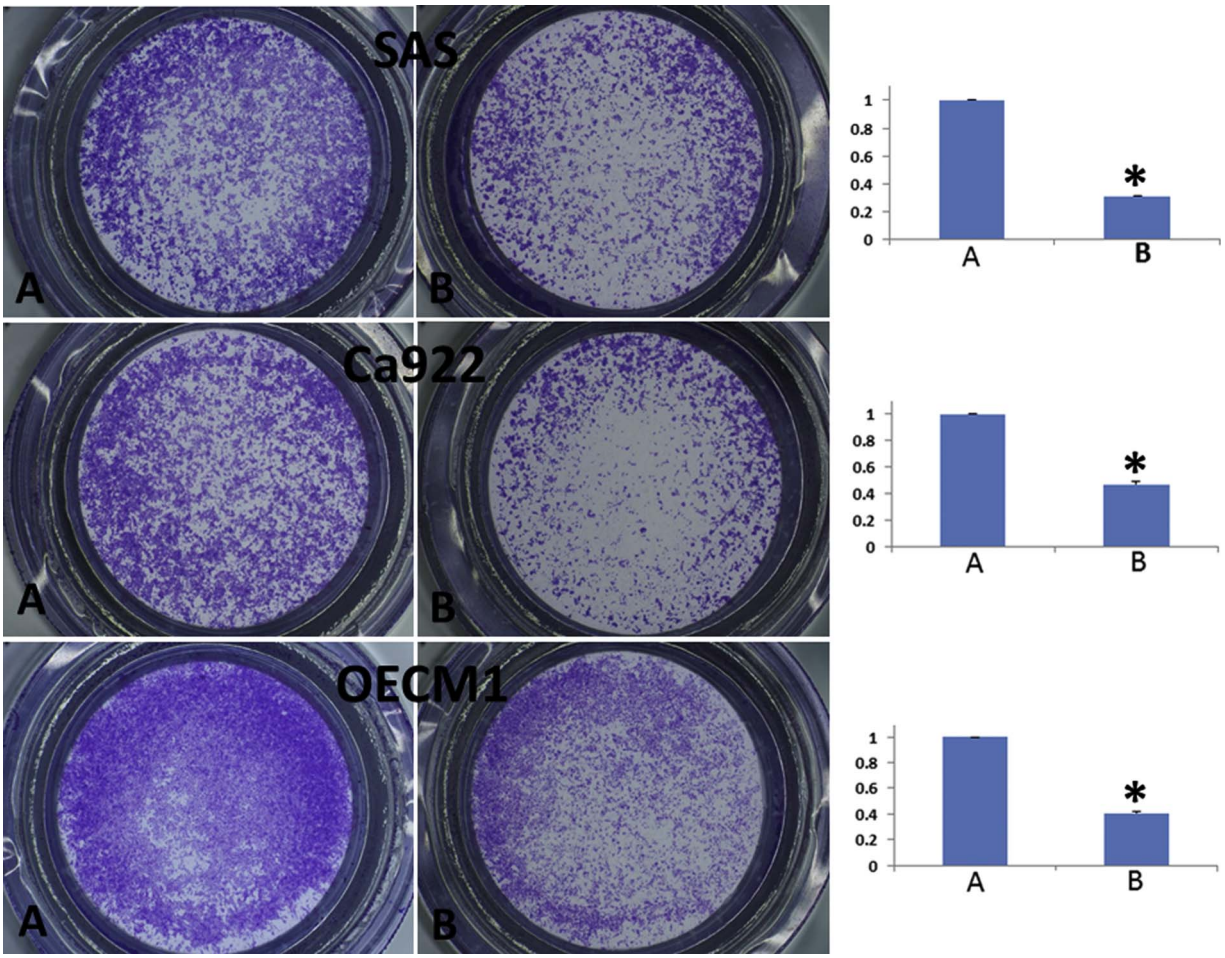

Fig. 7. Invasion assays of the oral cancer cell lines (SAS, Ca922, and OECM1) with 24 h after *SPRY2* siRNA transfection as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection.
The degree of invasion of the oral cancer cell lines (SAS, Ca922, and OECM1) with *SPRY2* siRNA transfection (B) was significantly lower as compared with the three oral cancer cell lines without *SPRY2* siRNA transfection (A) (* $P < 0.05$).

upregulated in colon cancer cells, and SPRY2 upregulation was also found to modulate the metastatic potential of colon cancer cells (Holgren et al., 2010). Thus, in consideration of the previous findings (Davies et al., 2002; Dong et al., 2003; Barbáchano et al., 2010; Bloethner et al., 2005; Holgren et al., 2010; Tuveson et al., 2003; Wellbrock et al., 2004) together with the experimental results of the current study, we propose that SPRY2 can either initiate inhibitory modulation by impeding the RAS pathway or activate functions via different pathways (such as c-Met (Holgren et al., 2010)). However, in the presence of RAS mutations, the inhibitory effects of SPRY2 might be avoided, subsequently permitting substantial stimulatory functions; this could be attributed to the previous finding that the N-terminal tyrosine residue of SPRY2 (Tyr55) can behave as a docking place for the SH2 domain of c-Cbl (Fong et al., 2006; Hall et al., 2003). Then, SPRY2 could contest with EGFR to combine with c-Cbl, thus inhibiting EGFR ubiquination and degradation (Egan, Hall, Yatsula, & Bar-Sagi, 2002 Fang, Yu, Eder, Mao, & Boyd, 1999; Lim et al., 2002). Consequently, EGFR would not be downregulated, and could even enhance continued stimulation of MAPK signaling (Egan et al., 2002; Fang et al., 1999; Lim et al., 2002).

In the present study, an increased SPRY2 protein expression in parallel with an increased phospho-ERK expression in oral cancer cell lines was noted; however, BRAF V600E mutation was not demonstrated in the oral cancer cell lines used in this study in comparison with the normal oral mucosa primary culture. It has also been reported that BRAF and KRAS mutations are uncommon in human OSCCs (Bruckman, Schönleben, Qiu, Woo, & Su, 2010). Thus, based on the experimental data of the current study, and the fact that RAS mutation in OSCC is rare (Bruckman et al., 2010). RAS mutation would not contribute in a dominant manner to the mechanism of SPRY2 upregulation.

On the other hand, in a review of the literature, some potential modulations other than RAS mutation were identified (Acunzo et al., 2013; Brett et al., 2001; Hanafusa et al., 2002;). It has been reported that SPRY2 could separate RTK signaling from RAS activation, probably by confiscating Grb-2, an adaptor protein mandatory for RAS activation (Brett et al., 2001; Hanafusa et al., 2002). Additionally, cross-talk between c-met and EGFR involving miR-27a and SPRY2 has been evidenced in non-small-cell lung cancer (Acunzo et al., 2013). However, an association between SPRY2 and c-met in oral squamous cell carcinogenesis has not been reported, to the best of our knowledge. Therefore, the kind(s) of potential genetic aberration(s) of the *SPRY2* gene implicated in its overexpression in human oral squamous cell carcinogenesis remain uncertain, and need to be explored in the future.

In conclusion, the possible genetic abnormalities of the *SPRY2* gene associated to its upregulation in human oral squamous cell carcinogenesis remain uncertain in the current study. Nevertheless, upregulation of SPRY2 protein expression in human oral squamous cell carcinogenesis was demonstrated both *in vitro* and *in vivo* in the present study. A significant increase in SPRY2 protein expression was noted in OSCC patients with cervical lymph-node metastasis as compared with patients without lymph-node metastasis, as well as in OPMD patients with malignant transformation as compared with those without

malignant transformation. All these findings imply potential prognostic and clinical relevance of SPRY2 overexpression in human oral squamous cell carcinogenesis.

Conflict of interest

None declared.

Acknowledgements

The authors are grateful to the Ministry of Science and Technology, Taiwan (MOST-104-2314-B-037-058) for their financial support. We are also grateful for the receipt of funding from the health and welfare surcharge of tobacco products, Ministry of Health and Welfare, Taiwan, Republic of China(MOHW106-TDU-B-212-144007).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.archoralbio.2017.12.021.

References

Acunzo, M., Romano, G., Palmieri, D., Laganá, A., Garofalo, M., Balatti, V., et al. (2013). Cross-talk between MET and EGFR in non-small cell lung cancer involves miR-27a and Sprouty2. *Proceedings of the National Academy of Sciences of the United States of America, 110*(21), 8573–8578.

Ahmad, I., Gao, M., Patel, R., & Leung, H. Y. (2013). Modelling synergistic interactions between HER2, Sprouty2 and PTEN in driving prostate carcinogenesis. *Asian Journal of Andrology, 15*(3), 323–327.

Barbáchano, A., Ordóñez-Morán, P., García, J. M., Sánchez, A., Pereira, F., Larriba, M. J., et al. (2010). SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumourigenicity. *Oncogene, 29*(34), 4800–4813.

Bloethner, S., Chen, B., Hemminki, K., Muller-Berghaus, J., Ugurel, S., Schadendorf, D., et al. (2005). Effect of common B-RAF and N-RAS mutations on global gene expression in melanoma cell lines. *Carcinogenesis, 26*(7), 1224–1232.

Brett, D., Kemmner, W., Koch, G., Roefzaad, C., Gross, S., & Schlag, P. M. (2001). A rapid bioinformatic method identifies novel genes with direct clinical relevance to colon cancer. *Oncogene, 20*(33), 4581–4585.

Bruckman, K. C., Schönleben, F., Qiu, W., Woo, V. L., & Su, G. H. (2010). Mutational analyses of the BRAF, KRAS, and PIK3CA genes in oral squamous cell carcinoma. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 110*(5), 632–637.

Cabrita, M. A., & Christofori, G. (2008). Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis, 11*(1), 53–62.

Chang, S. E., Foster, S., Betts, D., & Marnock, W. E. (1992). DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. *International Journal of Cancer, 52*(6), 896–902.

Chen, Y. K., Huang, H. C., Lin, L. M., & Lin, C. C. (1999). Primary oral squamous cell carcinomas: An analysis of 703 cases in southern Taiwan. *Oral Oncology, 35*(2), 173–179.

Chen, Y. K., Hsue, S. S., & Lin, L. M. (2011). Expression of inhibitors of apoptosis family proteins in human oral sqaumous-cell carcinogenesis. *Head & Neck, 33*(7), 985–998.

Cheng, Y. S., Rees, T., & Wright, J. (2014). A review of research on salivary biomarkers for oral cancer detection. *Clinical and Translational Medicine, 3*(1), 3.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry, 162*(1), 156–159.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., et al. (2002). Mutations of the BRAF gene in human cancer. *Nature, 417*(6892), 949–954.

Dong, J., Phelps, R. G., Qiao, R., Yao, S., Benard, O., Ronai, Z., et al. (2003). BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. *Cancer Research, 63*(14), 3883–3885.

Egan, J. E., Hall, A. B., Yatsula, B. A., & Bar-Sagi, D. (2002). The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proceedings of the National Academy of Sciences of the United States of America, 99*(9), 6041–6046.

Fang, X., Yu, S., Eder, A., Mao, M., Bast, R. C., Jr, Boyd, D., et al. (1999). Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene, 18*(48), 6635–6640.

Feng, Y. H., Wu, C. L., Tsao, C. J., Chang, J. G., Lu, P. J., Yeh, K. T., et al. (2011). Deregulated expression of sprouty2 and microRNA-21 in human colon cancer: Correlation with the clinical stage of the disease. *Cancer Biology Therapy, 11*(1), 111–121.

Fong, C. W., Chua, M. S., McKie, A. B., Ling, S. H., Mason, V., Li, R., et al. (2006). Sprouty 2, an inhibitor of mitogen-activated protein kinase signaling, is down-regulated in hepatocellular carcinoma. *Cancer Research, 66*(4), 2048–2058.

Fritzsche, S., Kenzelmann, M., Hoffmann, M. J., Müller, M., Engers, R., Gröne, H. J., et al. (2006). Concomitant down-regulation of SPRY1 and SPRY2 in prostate carcinoma. *Endocrine-Related Cancer, 13*(3), 839–849.

Grose, R., & Dickson, C. (2005). Fibroblast growth factor signaling in tumorigenesis. *Cytokine and Growth Factor Reviews, 16*(2), 179–186.

Gross, I., Bassit, B., Benezra, M., & Licht, J. D. (2001). Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *Journal of Biological Chemistry, 276*(49), 46460–46468.

Guy, G. R., Wong, E. S., Yusoff, P., Chandramouli, S., Lo, T. L., Lim, J., et al. (2003). Sprouty: How does the branch manager work. *Journal of Cell Science, 116*(Pt. 15), 3061–3068.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., & Krasnow, M. A. (1998). Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell, 92*(2), 253–263.

Hall, A. B., Jura, N., DaSilva, J., Jang, Y. J., Gong, D., & Bar-Sagi, D. (2003). hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl. *Current Biology, 13*(4), 308–314.

Hanafusa, H., Torii, S., Yasunaga, T., & Nishida, E. (2002). Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nature Cell Biology, 4*(11), 850–858.

Health Promotion Administration (2014). *Ministry of health and welfare. cancer registry annual report 2011*ROC: Ministry of Health and Welfare3–17.

Holgren, C., Dougherty, U., Edwin, F., Cerasi, D., Taylor, I., Fichera, A., et al. (2010). Sprouty-2 controls c-met expression and metastatic potential of colon cancer cells: Sprouty/c-met upregulation in human colonic adenocarcinomas. *Oncogene, 29*(38), 5241–5253.

Hsu, S. M., Raine, L., & Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabelled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry, 29*(4), 577–580.

Hsue, S. S., Wang, W. C., Chen, C. H., Lin, C. C., Chen, Y. K., & Lin, L. M. (2007). Malignant transformation in 1458 patients with potentially malignant oral mucosal disorders: A follow-up study based in a Taiwanese hospital. *Journal of Oral Pathology and Medicine, 36*(1), 25–29.

Jennifer, L. S., & Gail, R. M. (2012). Sprouty genes function in suppression of prostate tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America, 109*(48), 20023–20028.

Kim, H. J., & Bar-Sagi, D. (2004). Modulation of signalling by Sprouty: A developing story. *Nature Reviews Molecular Cell Biology, 5*(6), 441–450.

Kimura, Y. (1978). Studies on lactate dehydrogenase isoenzymes in a cell line (Ca 9–22) derived from carcinoma of the gingiva. *Kokubyo Gakkai Zasshi, 45*(1), 20–35.

Lee, C. C., Putnam, A. J., Miranti, C. K., Gustafson, M., Wang, L. M., Vande Woude, G. F., et al. (2004). Overexpression of sprouty 2 inhibits HGF/SF-mediated cell growth, invasion, migration, and cytokinesis. *Oncogene, 23*(30), 5193–5202.

Li, X., Liu, X., Xu, W., Zhou, P., Gao, P., Jiang, S., et al. (2013). c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting sprouty2. *Journal of Biological Chemistry, 288*(25), 18121–18133.

Lim, J., Yusoff, P., Wong, E. S., Chandramouli, S., Lao, D. H., Fong, C. W., et al. (2002). The cysteine-rich sprouty translocation domain targets mitogen-activated protein kinase inhibitory proteins to phosphatidylinositol 4,5-bisphosphate in plasma membranes. *Molecular and Cellular Biology, 22*(22), 7953–7966.

Lin, C. L., Chiang, W. F., Tung, C. L., Hsieh, J. L., Hsiao, J. R., Huang, W. T., et al. (2015). Spouty2 protein is downregulated in human squamous cell carcinoma of the head and neck and suppresses cell proliferation in vitro. *Molecular Medicine Reports, 11*(1), 547–554.

Lo, T. L., Yusoff, P., Fong, C. W., Guo, K., McCaw, B. J., Phillips, W. A., et al. (2004). The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer. *Cancer Research, 64*(17), 6127–6136.

Lo, T. L., Fong, C. W., Yusoff, P., McKie, A. B., Chua, M. S., Leung, H. Y., et al. (2006). Sprouty and cancer: The first terms report. *Cancer Letters, 242*(2), 141–150.

McKie, A. B., Douglas, D. A., Olijslagers, S., Graham, J., Omar, M. M., Heer, R., et al. (2005). Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. *Oncogene, 24*(13), 2166–2174.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., et al. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development, 126*(20), 4465–4475.

Patel, R., Gao, M., Ahmad, I., Fleming, J., Singh, L. B., Rai, T. S., et al. (2013). Sprouty2, PTEN, and PP2A interact to regulate prostate cancer progression. *Journal of Clinical Investigation, 123*(3), 1157–1175.

Rubin, C., Litvak, V., Medvedovsky, H., Zwang, Y., Lev, S., & Yarden, Y. (2003). Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. *Current Biology, 13*(4), 297–307.

Sambrook, J., & Russell, D. W. (2001). *Preparation and analysis of eukaryotic genomic DNA. Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press6 [4–6.12].

Sarbia, M., Loberg, C., Wolter, M., Arjumand, J., Heep, H., Reifenberger, G., et al. (1999). Expression of bcl-2 and amplification of c-myc are frequent in basaloid squamous cell carcinomas of the esophagus. *The American Journal of Pathology, 155*(4), 1027–1032.

Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., et al. (2003). Mammalian Sprouty4 suppresses ras-independent ERK activation by binding to raf1. *Nature Cell Biology, 5*(5), 427–432.

Scott, S. E., Grunfeld, E. A., & McGurk, M. (2005). The idiosyncratic relationship between diagnostic delay and stage of oral squamous cell carcinoma. *Oral Oncology, 41*(4), 396–403.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Srisawat, C., Khongmanee, A., & Tongkham, C. (2012). Differential expression of Sprouty genes in hepatocellular carcinoma. *Journal of Surgical Oncology, 105*(3), 273–276.

Song, K., Gao, Q., Zhou, J., Qiu, S. J., Huang, X. W., Wang, X. Y., et al. (2012). Prognostic significance and clinical relevance of Sprouty 2 protein expression in human

hepatocellular carcinoma. *Hepatobiliary and Pancreatic Diseases International, 11*(2), 177–184.
Sutterlüty, H., Mayer, C. E., Setinek, U., Attems, J., Ovtcharov, S., Mikula, M., et al. (2007). Down-regulation of Sprouty2 in non-small cell lung cancer contributes to tumor malignancy via extracellular signal-regulated kinase pathway-dependent and −independent mechanisms. *Molecular Cancer Research, 5*(5), 509–520.
Takahashi, K., Kanazawa, H., Akiyama, Y., Tazaki, S., Takahara, M., Muto, T., et al. (1989). Establishment and characterization of a cell line (SAS) from poorly differentiated human squamous cell carcinoma of the tongue. *Journal of The Japanese Stomatological Society, 38*(1), 20–28.
Takai, D., & Jones, P. A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proceedings of the National Academy of Sciences of the United States of America, 99*(6), 3740–3745.
Tang, H., Wu, Z., Zhang, J., & Su, B. (2013). Salivary lncRNA as a potential marker for oral squamous cell carcinoma diagnosis. *Molecular Medicine Reports, 7*(3), 761–766.
Tefft, J. D., Lee, M., Smith, S., Leinwand, M., Zhao, J., Bringas, P., Jr., et al. (1999). Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. *Current Biology, 9*(4), 219–222.
Tovosia, S., Chen, P. H., Ko, A. M., Tu, H. P., Tsai, P. C., & Ko, Y. C. (2007). Prevalence and associated factors of betel quid use in the Solomon Islands: A hyperendemic area for oral and pharyngeal cancer. *American Journal of Tropical Medicine and Hygiene, 77*(3), 586–590.
Tsavachidou, D., Coleman, M. L., Athanasiadis, G., Li, S., Licht, J. D., Olson, M. F., et al. (2004). SPRY2 is an inhibitor of the ras/extracellular signal-regulated kinase pathway in melanocytes and melanoma cells with wild-type BRAF but not with the V599E mutant. *Cancer Research, 64*(16), 5556–5559.
Tuveson, D. A., Weber, B. L., & Herlyn, M. (2003). BRAF as a potential therapeutic target in melanoma and other malignancies. *Cancer Cell, 4*(2), 95–98.
Van der Waal, I. (1986). Diagnostic and therapeutic problems of oral precancerous lesions. *International Journal of Oral and Maxillofacial Surgery, 15*(6), 790–798.
Van der Waal, I. (2009). Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncology, 45*(4-5), 317–323.
Velasco, A., Pallares, J., Santacana, M., Gatius, S., Fernandez, M., Domingo, M., et al. (2011). Promoter hypermeth¬ylation and expression of sprouty 2 in endometrial carcinoma. *Human Pathology, 42*(2), 185–193.
Wang, C., Delogu, S., Ho, C., Lee, S. A., Gui, B., Jiang, L., et al. (2012). Inactivation of Spry2 accelerates AKT-driven hepatocarcinogenesis via activation of MAPK and PKM2 pathways. *Journal of Hepatology, 57*(3), 577–583.
Wang, Y. Y., Tail, Y. H., Wang, W. C., Chen, C. Y., Kao, Y. H., Chen, Y. K., et al. (2014). Malignant transformation in 5071 southern Taiwanese patients with potentially malignant oral mucosal disorders. *BMC Oral Health, 14*, 99.
Wellbrock, C., Ogilvie, L., Hedley, D., Karasarides, M., Martin, J., Niculescu-Duvaz, D., et al. (2004). V599EB-RAF is an oncogene in melanocytes. *Cancer Research, 64*(7), 2338–2342.
Wong, E. S., Lim, J., Low, B. C., Chen, Q., & Guy, G. R. (2001). Evidence for direct interaction between Sprouty and Cbl. *Journal of Biological Chemistry, 276*(8), 5866–5875.
Wright, A., & Shear, M. (1985). Epithelial dysplasia immediately adjacent to oral squamous cell carcinoma. *Journal of Oral Pathology, 14*(7), 559–564.
Yang, C. Y., & Meng, C. L. (1994). Regulation of PG synthase by EGF and PDGF in human oral, breast, stomach, and fibrosarcoma cancer cell lines. *Journal of Dental Research, 73*(8), 1407–1415.
Yuan, S. S., Hou, M. F., Hsieh, Y. C., Huang, C. Y., Lee, Y. C., Chen, Y. J., et al. (2012). Role of MRE11 in cell proliferation, tumor invasion, and DNA repair in breast cancer. *Journal of the National Cancer Institute, 104*(19), 1485–1502.
de Maximy, A. A., Nakatake, Y., Moncada, S., Itoh, N., Thiery, J. P., & Bellusci, S. (1999). Cloning and expression pattern of a mouse homologue of Drosophila sprouty in the mouse embryo. *Mechanisms of Development, 81*(1-2), 213–216.