



Original Article

Overexpression of sprouty 1 protein in human oral squamous cell carcinogenesis



Yen-Yun Wang^{a,b}, Wen-Chen Wang^{a,c,d†}, Chiang-Wei Su^e,
Ching-Wei Hsu^e, 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

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KEYWORDS

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Abstract Background/purpose: Sprouty (SPRY) has four isoforms, SPRY1–4, and its deficiency produces haphazard ‘sprouting’ of tracheal tubules. This study investigated SPRY1 protein expression in human oral potentially malignant disorders (OPMDs) and oral squamous cell carcinomas (OSCCs).

Materials and methods: 90 OSCCs, 10 OPMDs with malignant transformation (MT), 17 OPMDs without MT, and six normal oral mucosa (NOM) tissue samples were subjected to immunohistochemical staining. Three human oral cancer cell lines (OCCLs), an oral precancer cell line (DOK), and a primary culture of normal oral keratinocytes (HOK) were used for western blotting.

* Corresponding author. School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan. Fax: +886 7 3210637.

** Corresponding author. Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan. Fax: +886 7 3210637.

E-mail addresses: yuanssf@ms33.hinet.net (S.-S. Yuan), k0285@ms22.hinet.net (Y.-K. Chen).

† Wen-Chen Wang: equal contribution to first author Yen-Yun Wang.

Results: Significantly increased expression of SPRY1 protein from NOM and OPMD without MT to OSCC was observed. The protein expressions of SPRY1 in OCCLs were significantly enhanced as compared with DOK and HOK. Increased phosphor/total-ERK expression was observed in OCCLs as compared with HOK. A significantly increased SPRY1 protein level was noted in OPMDs with MT as compared with those without MT, in addition to a significant increase in DOK in comparison with HOK.

Conclusion: Our results indicated that overexpression of SPRY1 protein is potentially associated with human oral squamous cell carcinogenesis.

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Introduction

Oral squamous cell carcinoma (OSCC) is the most common human oral epithelial malignancy, comprising approximately 3% of all newly-confirmed cancer cases.^{1,2} In Taiwan, OSCC represents the fourth most common cancer, and is also the fourth leading cause of death due to cancer in males.^{3,4} Perhaps due to diagnosis at a late stage, the five-year survival rate of OSCC patients is low,⁵ highlighting the necessity to improve our understanding of the pathogenesis of human oral squamous cell carcinogenesis.⁶ Moreover, a proportion of OSCC cases undergo malignant transformation (MT) from a spectrum of oral potentially malignant disorders (OPMDs).^{7,8} Hence, avoidance of MT in patients with OPMDs is imperative.⁹

Sprouty (SPRY) is an intracellular protein that has four isoforms (SPRY1–4) and is related to the modulation of branching in tracheal development, and absence of SPRY could induce haphazard 'sprouting' of tracheal tubules.¹⁰ SPRY has been found to be an inhibitor protein that participates in changing of the components of the Ras/ERK (receptor tyrosine kinase, RTK) pathway.¹¹ Binding of SPRY to the plasma membrane leads to inhibition of mitogen-activated protein kinase (MAPK) signaling.^{12–17} Additionally, the targets for SPRY repression could vary, as it regulates upstream of RAS or downstream at the level of RAF.^{18–21} On the other hand, SPRY has been confirmed as being not only capable of suppressing MAPK activation, but also of upregulating the MAPK pathway.^{22–25}

To the best of our knowledge, SPRY1 expression has not been documented in human oral squamous cell carcinogenesis. So, the present study aimed to investigate the SPRY1 expression in human OPMDs and OSCCs.

Material and methods

SPRY1 expression in human OSCCs and OPMDs

Immunohistochemistry

Tissue specimens from 90 primary OSCC patients with the habits of drinking alcohol, chewing betel quid, and smoking cigarettes under the approval of the Ethics

Committee for Scientific Research on Human Beings of the institution (KMUHIRB-E(II)-20150269) were fixed in 10% neutral buffered formalin solution, dehydrated in graded alcohols, cleaned in xylene, and embedded in paraffin for subsequent immunohistochemical staining. Six normal oral mucosal tissues acquired from healthy persons without the aforementioned oral habits that are risk factors for oral malignancy were employed as controls. Tissue samples were also obtained from 27 human OPMD patients (including patients with hyperkeratosis/epithelial hyperplasia with or without oral epithelial dysplasia, oral submucous fibrosis, and verrucous hyperplasia) for whom 10 patients exhibited MT and 17 who did not.

Deparaffinization of paraffin-embedded 4- μ m-thick tissue sections was performed using xylene solutions, and rehydration was achieved using graded alcohols. Then, the tissue sections were treated three times with microwave radiation in a 10 mM citrate buffer (pH 6.0) for 5 min each time. The sections were subsequently immersed in methanol with 0.3% H₂O₂ for 45 min to block the endogenous peroxidase activity, and incubated in normal goat serum to reduce non-specific binding. Sections were finally incubated for 60 min at room temperature with primary anti-SPRY1 antibody (ABGENT, San Diego, CA, USA; Cat. No. ALS14077; 1:100). The sections were then processed using the standard avidin–biotin peroxidase complex method in accordance with the manufacturer's protocol (Vector Laboratories).²⁶ Diaminobenzidine (Roche; Cat. No. 1718096) was used as a chromogen, and hematoxylin was used for counterstaining. Each set of experiments included a human colon squamous cell carcinoma specimen known to express SPRY1 protein, which served as a positive control and ensured the reproducibility of the staining process. Negative controls were employed using the same procedure, with omission of the primary antibody.

The scores of the percentage of positive staining (P) were categorized as follows: 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 designated 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 $P \times I$ for each section.²⁷ All stained slides were observed and quantified using the semi-automated image analysis software Image J, Version 1.51e, to determine the patterns of positive staining microscopically.

Cell cultures

Three human oral cancer cell lines (OECM1, SAS, and Ca922) were used in the current study. OECM1²⁸ and SAS²⁹ were respectively derived from primary gingiva squamous cell carcinoma with OECM1 being obtained from a Taiwanese betel-quid chewer, and SAS²⁹ shown extensive epidermal growth receptor expression. Ca922³⁰ was obtained from a Japanese patient with high-grade primary squamous cell carcinoma. Ca922, OECM1, and SAS cells were cultured in high-glucose DMEM (HyClone, Logan, UT, USA) together with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (HyClone) at 37°C in a 5% CO₂ incubator. In addition, for primary culture, normal human oral keratinocytes (HOK) (ScienCell, USA) were cultured in Oral Keratinocyte Medium (OKM, ScienCell). The culture medium was replaced every three days.

A human oral precancer cell line DOK³¹ derived from mild to moderate dysplastic oral keratinocytes from the tongue of a Caucasian patient with a keratin profile similar to the original dysplasia, which has been shown to be non-tumorigenic in athymic nude mice, was cultured in high-glucose DMEM (HyClone) 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 5% CO₂ incubator.

Western blot analysis of human oral cancer cell lines and oral precancer cell line

After rinsing Ca922, OECM1, SAS, DOK, and HOK cells with PBS (Sigma-Aldrich, St Louis, MO, USA) followed by treatment with Radio-Immunoprecipitation Assay (RIPA) lysis buffer, the lysates were centrifuged at 4°C, 14,000 rpm, for 15 min. The protein concentration was then quantified using a Thermo Pierce Protein Assay Kit. Equal amounts of protein were denatured with sodium dodecyl sulfate (SDS) running buffer (Sigma-Aldrich) and β-mercaptoethanol (Sigma-Aldrich). The samples were then analyzed using 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Sigma-Aldrich) gels, and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich) using Bio-Rad's transblot with SPRY1 primary antibody (ABGENT; 1:1000), with an observed molecular weight of 35 kDa; and GAPDH (Sigma-Aldrich; 1:1000), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich; 1:5000). The protein level was measured using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan). The ratio was normalized to the GAPDH signal.

Further, samples were analyzed using 10% SDS-PAGE (Sigma-Aldrich) gels, and the proteins were transmitted onto a PVDF membrane (Sigma-Aldrich) using Bio-Rad's transblot with primary antibodies against phosphor-ERK (Boster Biological Technology, CA, USA; Cat. No. P00104; 1:1000) and total-ERK (Boster Biological Technology; Cat. No. P00104; 1:1000), with species specificity for human tissues and an observed molecular weight of 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 measured using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co.). The ratio was normalized to the β-actin signal.

Statistical analyses

All statistical analyses were performed using the SAS Statistical Package (Version 9.1.3, SAS Institute Inc.). Nonparametric Kruskal–Wallis tests were employed to compare the immunohistochemical expressions of SPRY1 protein and to analyze the results of western blots. Statistical significance was considered when the *P* value was less than 0.05.

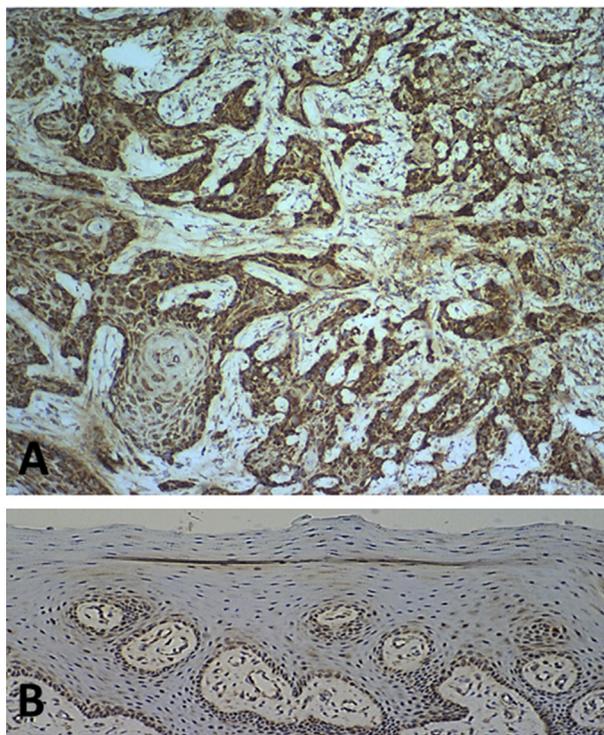


Figure 1 Immunohistochemical staining of SPRY1 protein in human oral squamous cell carcinoma and normal oral mucosa. Representative strong immunohistochemical staining of SPRY1 protein in human oral squamous cell carcinoma (A, ×100) and weak staining of SPRY1 protein in normal oral mucosa (B, ×100).

Results

SPRY1 expression in human OSCCs

Immunohistochemistry

Positive staining of SPRY1 protein was present in 90 cases of OSCC (Fig. 1A) and in six normal oral mucosa samples (Fig. 1B). A significant increase was noted in the mean IS of the OSCC cases (mean IS = 4.18) in comparison with that of the normal oral mucosa samples (mean IS = 1.50) ($P = 0.001$).

The statistical comparison of the mean IS of SPRY1 protein in OSCC patients with histopathological features and oral risk factors is summarized in the [Supplementary Table](#). A significant increase in the mean IS of SPRY1 protein in the male OSCC patients was observed as compared with the female patients ($P = 0.044$). The mean IS of SPRY1 protein in the T2, T3, and T4 patients was increased in comparison with the T1 patients, and an increased mean IS was also observed in stage II, III, and IV patients as compared with stage I patients; however, neither of these increases were of statistical significance. The mean IS of SPRY1 protein for patients with lymph node metastasis was nearly the same as for patients without lymph node metastasis. An increase in the mean IS of SPRY1 protein was noted in OSCC patients with the habits of drinking alcohol, chewing betel quid, and smoking cigarettes as compared

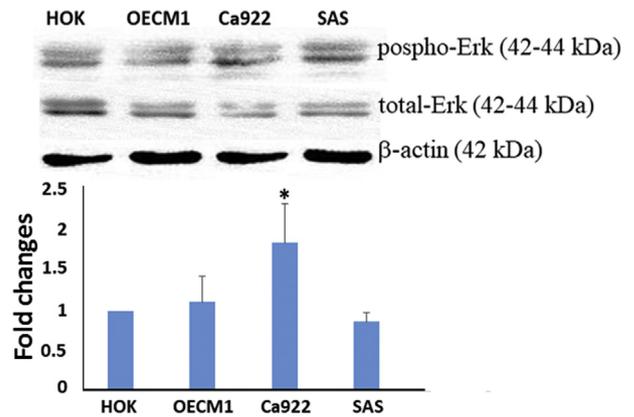


Figure 3 Western blot analyses: phosphor-ERK and total-ERK in human oral cancer cell lines as compared with a primary culture of normal oral mucosa (HOK). Upregulation of phosphor-ERK (normalized to total-ERK) in OECM1 and Ca922 cell lines as compared with HOK, and a slightly decreased expression in SAS as compared with HOK. Results were quantified using densitometric analysis, normalized to the level of β -actin, and expressed as a fold change relative to the normal oral mucosa. Bars represent means \pm standard deviation of the mean ($*P < 0.05$). A representative result of three independent experiments is shown.

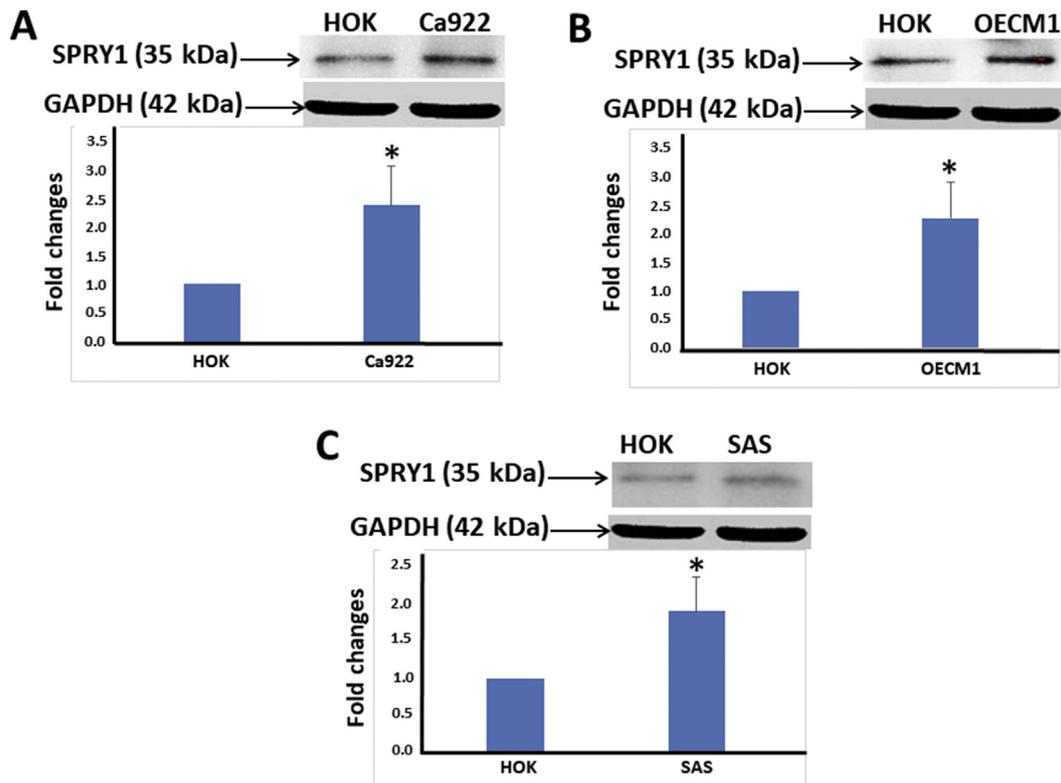


Figure 2 Western blot analyses: SPRY1 protein expression in human oral cancer cell lines as compared with a primary culture of normal oral mucosa (HOK). Upregulation of SPRY1 protein expression in human oral cancer cell lines (A, Ca922; B, OECM1; C, SAS) as compared with a HOK. Results were quantified using densitometric analysis, normalized to the level of GAPDH, and expressed as a fold change relative to the normal oral mucosa. Bars represent means \pm standard deviation of the mean ($*P < 0.05$). A representative result of three independent experiments is shown.

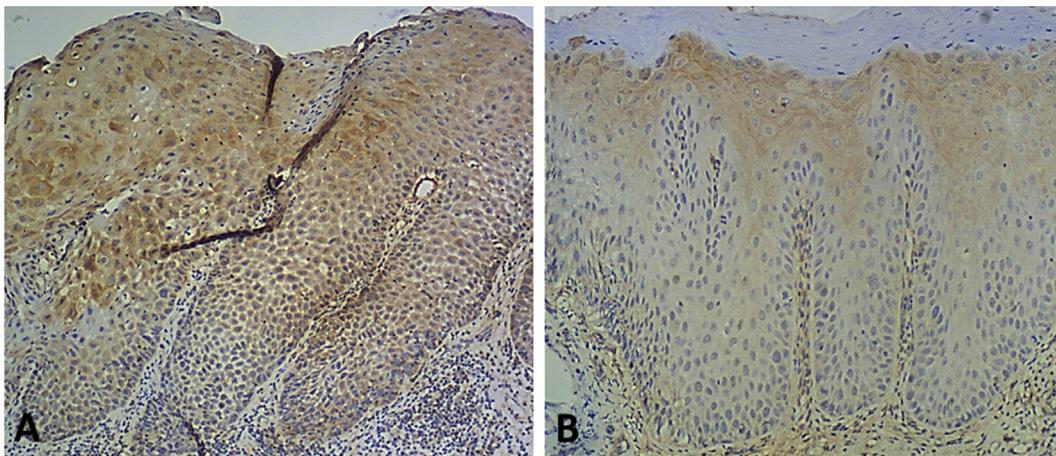


Figure 4 Immunohistochemical staining of SPRY1 protein in human oral potentially malignant disorders (OPMDs) with and without malignant transformation (MT). Representative stronger immunohistochemical staining of SPRY1 protein in a human OPMD with MT (A, $\times 100$) and weaker staining of SPRY1 protein in a human OPMD without MT (B, $\times 100$).

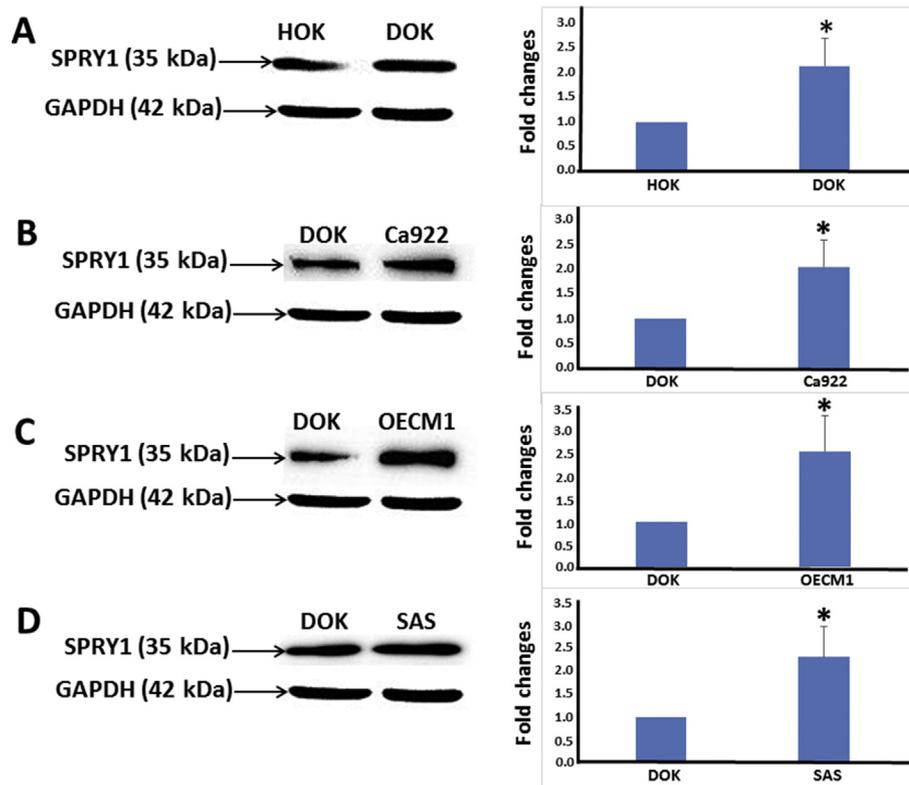


Figure 5 Western blot analyses: SPRY1 protein expression in human oral premalignant cell line DOK as compared with a primary culture of human normal oral mucosa (HOK) and with human oral cancer cell lines. Upregulation of SPRY1 protein expression was observed for DOK as compared with HOK (A). Upregulation of SPRY1 protein expression in human oral cancer cell lines Ca922 (B), OECM1 (C), and SAS (D) as compared with DOK. Results were quantified using densitometric analysis, normalized to the level of GAPDH, and expressed as a fold change relative to the normal oral mucosa (A) and to human oral cancer cell lines (B–D). Bars represent means \pm standard deviation of the mean ($*P < 0.05$). A representative result of three independent experiments is shown.

with patients without these oral habits, but statistical significance was not identified.

Western blot

Western blot analysis demonstrated statistically significant overexpression of SPRY1 protein in the three human oral cancer cell lines (Ca922, OECM1, and SAS) as compared with primary culture of HOK ($P < 0.05$) (Fig. 2A–C). On the other hand, overexpression of phospho-ERK (normalized to total-ERK) was noted in OECM1 and Ca922 cells as compared with primary culture of HOK, with only Ca922 showing statistical significance ($P < 0.05$); however, there was a slight decrease in expression in SAS in comparison with primary culture of HOK ($P > 0.05$) (Fig. 3).

SPRY1 expression in human OPMDs

Immunohistochemistry

Positive staining of SPRY1 protein was observed in all cases of human OPMD with and without MT (Fig. 4A & B). The mean IS of SPRY1 protein in the human OPMDs with and without MT was 3.30 and 1.53, respectively, a significant increase being noted when comparing the two mean IS values ($P < 0.001$). On the other hand, SPRY1 protein expression in human OSCC tissue specimens was significantly increased in comparison with human OPMD tissue specimens without MT ($P < 0.001$). Moreover, SPRY1 protein expression in human OPMD tissue specimens with MT was significantly increased as compared with normal mucosa tissue specimens ($P < 0.05$).

Western blot

Western blot analysis of human oral premalignant cell line DOK indicated overexpression of SPRY1 protein (Fig. 5A) as compared with the primary culture of HOK, this difference being of statistical significance ($P < 0.05$). On the other hand, statistically significant upregulation of SPRY1 protein (Fig. 5B–D) expression was also noted in human oral cancer cell lines Ca922, OECM1, and SAS as compared with DOK ($P < 0.05$).

Discussion

Significant upregulation of SPRY1 protein expression in human OSCC tissue specimens in comparison with human normal oral mucosa was confirmed by immunohistochemical staining in the current study. Additionally, the *in vivo* data demonstrating overexpression of SPRY1 protein in human OSCC as compared with normal oral mucosa were consistent with the *in vitro* findings in human oral cancer cell lines when compared with a human normal oral mucosa primary culture (HOK). Thus, to our knowledge, this was the first study to demonstrate SPRY1 overexpression in human oral squamous cell carcinoma.

Upregulation of SPRY1 protein expression was noted in human oral cancer cell lines as compared with human oral premalignant cell line (DOK). Significant overexpression of SPRY1 protein was also observed in DOK as compared with HOK in the present study. Furthermore, SPRY1 protein expression in human OSCC tissue specimens was significantly increased in comparison with human OPMD tissue specimens without MT in the current study. Moreover, SPRY1 protein expression in human OPMD tissue specimens with MT was significantly increased as compared with normal mucosa tissue specimens. So, collectively, to the best of our knowledge, we have reported for the first time the upregulation of SPRY1 protein in human oral squamous cell carcinogenesis.

A statistically higher SPRY1 protein expression was noted in human OPMDs with MT in comparison with cases without MT in our investigation. Thus, the aforementioned experimental evidence derived in the current study indicated that SPRY1 is possibly involved in human OSCC formation, and could have a potential association with MT of human OPMD.

In contrast to the findings of the present study, SPRY1 protein has been demonstrated to be downregulated in breast cancer^{32,33} and prostate cancer^{34,35} as compared with normal tissues; however, compatible with the results of this study, a significant proportion of cancerous tissues exhibited overexpression of SPRY1 as compared with the corresponding normal epithelium in the same study on prostate cancer.³⁵ Additionally, consistent with the results of the current study, enhanced SPRY1 protein expression has also been reported in ovarian cancer cell lines³⁶ and also with our previous study on SPRY2 on human oral squamous cell carcinogenesis.³⁷

An elevated SPRY1 protein expression in parallel with an enhanced phospho-ERK expression was noted in only two oral cancer cell lines in the current investigation, indicating that other potential mechanisms could also be present. Further studies exploring other possible mechanisms involved in the overexpression of SPRY1 protein are warranted.

In conclusion, our results indicated that SPRY1 overexpression is positively associated with OSCC formation. Nevertheless, the potential genetic and epigenetic alterations of the *SPRY1* gene associated with human oral squamous cell carcinogenesis remain uncertain.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgment

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Appendix

Supplementary Table Statistical comparison of immunohistochemical expression of SPRY1 protein in human oral squamous cell carcinomas, including gender, oral risk factors and histopathological features.

	Number	Mean	Standard deviation	P value
Gender				
Male	84	4.32143	2.49897	0.0435
Female	6	2.16667	2.31667	
Alcohol drinking				
No	21	3.33333	2.05757	0.1137
Yes	67	4.31343	2.55965	
Betel-quid chewing				
No	14	3.28571	2.16364	0.1921
Yes	74	4.22973	2.51324	
Cigarette smoking				
No	11	2.81818	1.77866	0.0704
Yes	77	4.25974	2.51523	
T (tumor size)				
T1	37	3.75676	2.47662	0.1893
T2+T3+T4	53	4.4717	2.55406	
N (lymph node metastasis)				
Yes	23	4.17391	1.99208	0.9933
No	67	4.1791	2.7076	
TNM stage				
I	32	3.875	2.62433	0.5905
II	17	4.76471	2.63461	
III	10	4.7	1.94651	
IV	31	4	2.58199	

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