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Original Article

Sprouty 4 expression in human oral squamous cell carcinogenesis

Pei-Hsien Liao [a,1], Fu-Hsiung Chuang [a,b,1], Yen-Yun Wang [a,c,1], Wen-Chen Wang [a,d,e], Chiang-Wei Su [f], Ching-Wei Hsu [f], Shyng-Shiou Yuan [c,g,h,i**], Yuk-Kwan Chen [a,d,e*]

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

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KEYWORDS
SPRY4;
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Abstract *Background/purpose:* Reviewing literature, sprouty 4 (SPRY4) has not been studied in human oral squamous cell carcinomas (OSCCs). The study aimed to examine SPRY4 expression in human oral squamous cell carcinogenesis.
Materials and methods: A total of 95 OSCCs, 10 OPMDs with malignant transformation (MT), 17 OPMDs without MT, and six normal oral mucosa (NOM) samples were recruited for immunohistochemical staining; three OSCC tissues with normal tissue counterpart NOM were employed for Western blotting. Three human oral cancer cell lines (OCCLs), an oral precancer cell line

* Corresponding author. School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan.
** Corresponding author. Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan.
E-mail addresses: yuanssf@ms33.hinet.net (S.-S. Yuan), k0285@ms22.hinet.net (Y.-K. Chen).
[1] These three authors contributed equally to this work.

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(dysplastic oral keratinocyte, DOK), and a primary culture of normal oral keratinocytes (HOK) were used for Western blotting; OCCLs and HOK were employed for real-time quantitative reverse transcription-polymerase chain reaction. OCCLs were evaluated in terms of proliferation, migration, and invasion assays.

Results: SPRY4 protein expression was significantly increased in OSCCs compared with NOM. Protein and mRNA SPRY4 expression in OCCLs were significantly elevated compared with HOK. Significant increases in the degrees of proliferation, migration, and invasion were noted in OCCLs with SPRY4 siRNA transfection compared with those without transfection. SPRY4 protein level was increased in OPMD with MT compared to OPMD without MT. SPRY4 protein was significant increase in DOK in comparison with HOK. SPRY4 protein expression was significantly increased from NOM and OPMD without MT to OSCC. SPRY4 protein expression in OCCLs was significantly enhanced compared with DOK and HOK respectively.

Conclusion: Our results indicate that SPRY4 expression is possibly involved in human oral squamous cell carcinogenesis.

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Introduction

Human squamous cell carcinoma (SCC) is the most frequent epithelial malignancy in oral cavity.[1,2] According to the data of World Health Organization, 377,713 and 177,757 individuals have been diagnosed with and expired for oral squamous cell carcinoma (OSCC) respectively; additionally, the frequency of OSCC in Asia has been the highest globally.[3] In Taiwan, OSCC is the fourth most common cancer, and also the fourth most male cancer deaths.[4] The five-year survival rate of late-stage OSCC patients is low.[5,6] Additionally, some OSCC lesions have been reported suffering malignant transformation (MT) from OPMDs in Taiwan.[7–9] So, MT for oral potentially malignant disorders (OPMDs) should be alerted.[10]

Sprouty (SPRY) gene family, consisting of four isoforms (SPRY1—4), controls branching in tracheal formation, and lack of SPRY could cause disorganized sprouting of tracheal tubules.[11] SPRY has been found to be an inhibitor protein regulated via the Ras/ERK (receptor tyrosine kinase, RTK) pathway.[12,13] Increased phosphor/total-ERK expression was observed in human oral squamous cell carcinogenesis.[14,15] Furthermore, SPRY gene encodes RTK inhibitor that has been involved in various living processes such as embryogenesis and stem cell conservation. SPRY4 knock-down enhanced cellular differentiation of the three germ layers in human embryonic stem cells.[16] Additionally, SPRY4 mutation induced malfunction of periderm function, causing aberrant oral epithelial adhesions in an animal study.[17]

Reviewing literature, to our knowledge, SPRY4 expression in human SCC and OPMD has not been studied in oral cavity. Herein, we purported to explore SPRY4 expression in human oral squamous cell carcinogenesis.

Material and methods

Immunohistochemical staining

Upon consent of Ethics Committee for Scientific Research on Human Beings of the institution (KMUHIRB-E(II)-20150269), tissue samples from 95 primary OSCC patients were fixed in 10% neutral buffered formalin solution, dehydrated in graded alcohols, cleaned in xylene, and embedded in paraffin for immunohistochemical staining. All OSCC patients had risk factors for oral malignancy of alcohol drinking, betel-quid chewing, and cigarette smoking. Six normal oral mucosal tissues acquired from healthy persons were employed as controls. Tissue specimens from 27 human

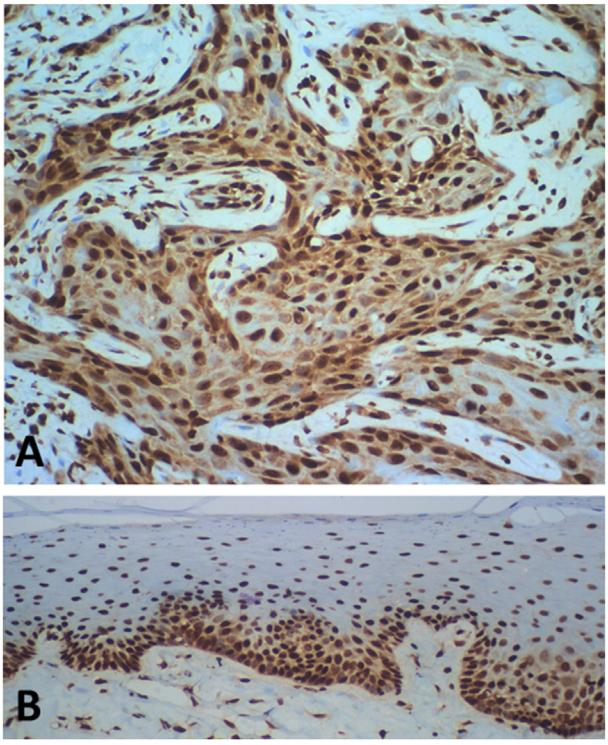

Figure 1 Immunohistochemical staining of SPRY4 protein in human oral squamous cell carcinoma and normal oral mucosa. Representative strong immunohistochemical staining of SPRY4 protein in human oral squamous cell carcinoma (A, × 100) and weak staining of SPRY4 protein in normal oral mucosa (B, × 100).

Table 1 Statistical comparison of immunohistochemical expression of SPRY4 protein in human oral squamous cell carcinomas with gender, oral risk factors and histopathological features.

	Number	Mean ± Standard deviation	*P* value
Gender			
Male	90	5.27 ± 5.15	$P > 0.05$
Female	4	3.00 ± 6.00	
Alcohol drinking			
No	21	5.19 ± 5.65	$P > 0.05$
Yes	73	5.16 ± 5.08	
Betel-quid chewing			
No	19	6.16 ± 6.13	$P > 0.05$
Yes	75	4.92 ± 4.92	
Cigarette smoking			
No	16	3.94 ± 4.81	$P > 0.05$
Yes	78	5.42 ± 5.24	
T (tumor size)			
T1	39	7.31 ± 5.54	[a]$P < 0.05$
T2+T3+T4	56	3.86 ± 4.57	
N (lymph node metastasis)			
Yes	22	4.68 ± 5.16	$P > 0.05$
No	73	5.45 ± 5.30	
TNM stage			
I	32	7.86 ± 5.59	[a]$P < 0.05$
II	22	4.27 ± 4.52	
III	9	3.78 ± 4.92	
IV	32	3.78 ± 4.62	

[a] Statistically significance.

OPMD patients (including patients with hyperkeratosis/epithelial hyperplasia with or without oral epithelial dysplasia, oral submucous fibrosis, and verrucous hyperplasia) for whom 10 patients demonstrated MT and 17 who did not were also obtained.

Deparaffinization of paraffin-embedded 4-μm-thick tissue sections were implemented sequentially through treating with xylene solution and rehydration with graded alcohols. The tissue sections were subsequently treated with microwave under citrate buffer (10 mM, pH 6.0) for three times (5 min each time). The sections were then immersed in methanol with H_2O_2 (0.3%) for 45 min to inhibit endogenous peroxidase activity, and incubated in normal goat serum to limit non-specific binding. Sections were finally incubated with primary anti-SPRY4 antibody (ABGENT, San Diego, CA, USA, Cat. no. ALS11241; 1:100) for 1 h at room temperature. The sections were then processed using standard avidin-biotin peroxidase complex method with reference to the manufacturer's protocol (Vector Laboratories, San Diego, CA, USA).[18] Diaminobenzidine (Roche, San Diego, CA, USA; Cat. no. 1718096) was used as a chromogen, and hematoxylin used for counterstaining. Human colon SCC specimen with SPRY4 protein expression was used as positive control and confirmed reproducibility of the staining processes for each batch of staining. Negative controls, without using the primary antibody, were performed with the same staining procedures.

The scores of percentages of positive staining (P) were quantified as: 0 (<1%); 1 (1–24%); 2 (25–49%); 3 (50–74%); and 4 (75–100%); the scores for staining intensity (I) were regarded 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 determined as P × I for each section.[19] The stained slides were quantified to determine the results of positive staining with semi-automated image analysis software Image J, Version 1.51e.

Cell culture

Three human oral cancer cell lines (OCCLs: OECM1, SAS, and Ca922) were cultured in the study. Both OECM1[20] and SAS[21] were obtained from primary gingival SCC; OECM1 acquired

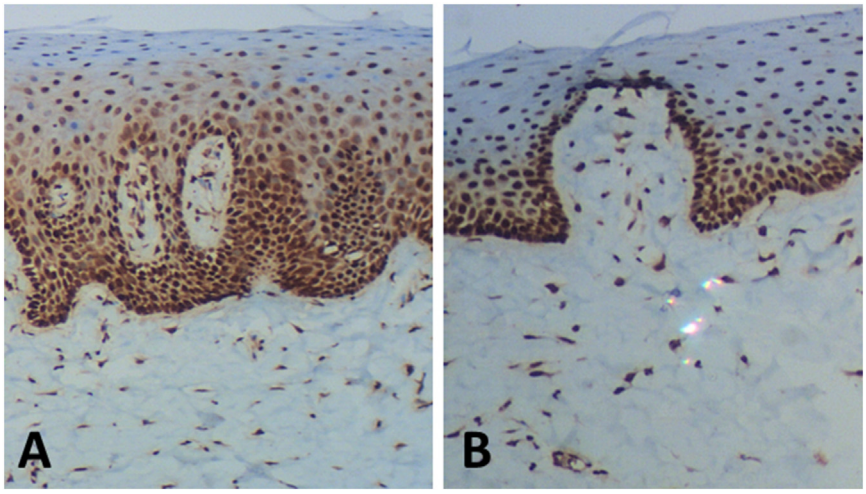

Figure 2 Immunohistochemical staining of SPRY4 protein in human oral premalignant lesions with and without malignant transformation. A representative higher immunoscore for immunohistochemical staining of SPRY4 protein for a human oral premalignant lesion with malignant transformation (A, × 200) and lower immunoscore for immunohistochemical staining of SPRY4 protein for a human oral premalignant lesion without malignant transformation (B, × 200).

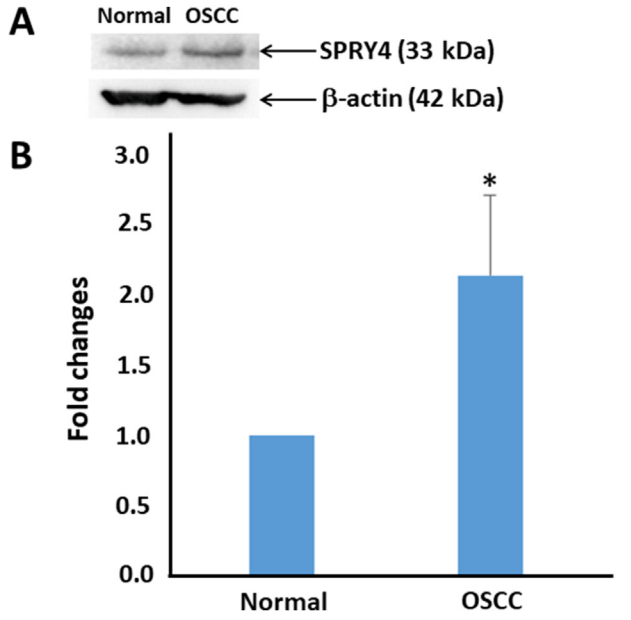

Figure 3 Western blot analyses of SPRY4 protein expression in human oral squamous cell carcinoma as compared with human normal oral mucosa counterparts. Western blot analyses showed upregulation of SPRY4 protein expression in human oral squamous cell carcinoma tissue as compared with normal oral mucosa. 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.

from a betel-quid chewer in Taiwan; SAS[21] had epidermal growth receptor expression; Ca922[22] was obtained from a high-grade primary SCC patient in Japan. The cells of OCCLs were cultured in high-glucose DMEM (HyClone, Logan, UT, USA) with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (HyClone) at 37 °C in 5% CO_2 incubator. Additionally, normal human oral keratinocytes primary culture (HOK) (ScienCell, Carlsbad, CA, USA) was immersed in Oral Keratinocyte Medium (ScienCell). The culture medium was changed every three days. Human oral precancer cell line (DOK)[23] obtained from mild to moderate dysplastic oral keratinocytes in the tongue of a Caucasian patient with keratin profile similar to the original dysplastic lesion that has been proved 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% CO_2 incubator.

Western blot

Being treated the cells of OCCLs, DOK, HOK, and OSCC tissue specimens with phosphate buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA), and radioimmunoprecipitation assay (RIPA) lysis buffer, the lysates were centrifuged with 14,000 rpm for 15 min at 4 °C. The protein level was subsequently quantified using Thermo Pierce Protein Assay Kit (ThemoFisher, Rockford, Illinoi, USA). Equal quantities of protein were denaturized with sodium dodecyl sulfate (SDS) running buffer (Sigma-Aldrich) and β-mercaptoethanol (Sigma-Aldrich). The samples were examined using 10% SDS-plyacrylamide gel electrophoresis (PAGE) (Sigma-Aldrich) gels, and the proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich) using Bio-Rad's transblot with SPRY4 primary antibody (ABGENT; 1: 1000), with molecular weight of 35 kDa; and GAPDH (Sigma-Aldrich; 1: 1000), followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich; 1: 5000). The protein level was quantified with the image analyzer (Fuji Photo Film Co., Tokyo, Japan) with the ratio normalized to the level of GAPDH. Three independent experiments were performed.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA samples were prepared from OCCLs and HOK using TRIzol Reagent (Invitrogen)[24] with RNA quality and concentration evaluated according to the optical density at 260 nm wavelength. The RNA was resuspended in 100 μL of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1 μg/mL Subsequently, the procedures of real-time quantitative reverse transcription-polymerase chain reaction for cells of human OCCLs and HOK were implemented according to our previous study[25] with oligoprimers for SPRY4 mRNA (forward: 5′ CTGGTCCTTCAYGGGTGCTC 3′; backward: 5′ CAGATGACGCTGTTCGTGTG 3′), and β-actin (forward: 5′ CCATGCCATCCTGCGTCTG 3; backward: 5′ CCATCTCTTGCTCGAAGTCCAG 3′), which were designed with reference to the published cDNA sequences in GenBank. Three independent experiments were performed.

SPRY4 gene knock-down in human oral squamous cell carcinoma cell lines

SPRY4 gene knock-down were done with procedures with reference to our previous study[12] with RNA interference performed using commercially-synthesized SPRY4 siRNA (forward: 5′-GCACGUGCAUGUGUUUGGUTT-3′; backward: 3′-TTCGUGCACGUACACAAACCA-5′) as described in the protocols provided by the manufacturer (Dharmacon RNAi, Gene Expression, Buckinghamshire, UK). The cells of OCCLs were incubated with the prepared transfection medium at 37 °C in 5% CO_2 for 72 h.

Proliferation assay, migration assay, and invasion assay

The procedures for the experiments of proliferation, migration, and invasion assays were done according to our previous study.[12] Quantification and analysis were performed using the semi-automated image analysis software Image J Version 1.51e. Three independent experiments were performed.

Statistical analyses

Statistical evaluation was performed using SAS Statistical Package (Version 9.1.3, SAS Institute Inc.) with significance being confirmed when *P* value less than 0.05. Mann-Whitney U tests were used to compare the immunohistochemical expressions of SPRY4 protein among OPMDs with/without MT, OSCCs, and normal oral mucosa, and to compare the differences in degrees of proliferation, migration, and invasion between OCCLs with/without SPRY4 siRNA transfection. SPRY4 protein expression along with the differentiation, tumor size, histological lymph-node involvement, and pathologic stage of OSCC patients were compared with Student's *t*-test. Moreover, results of Western blot and real-time quantitative reverse transcription-polymerase chain reaction were analyzed with nonparametric Kruskal-Wallis test.

Results

Immunohistochemical staining

Positive staining of SPRY4 protein was observed in 95 cases of OSCC (Fig. 1A) and in six normal oral mucosa samples (Fig. 1B). The mean IS of the OSCC cases was 5.27, whilst that of the normal oral mucosa samples was 2.17. A significant increase was noted in the mean IS of the OSCC cases in comparison with that of the normal oral mucosa samples ($P < 0.05$).

The statistical comparison of mean IS of SPRY4 protein in OSCC patients with oral risk factors and histopathological features is summarized in Table 1. A significant decrease in the mean IS of SPRY4 protein was noted as compared T2, T3, and T4 patients with T1 patients ($P < 0.05$); and as also for stage II, III, and IV patients compared with stage I patients ($P < 0.05$). There was a decrease of mean IS of SPRY4 protein for patients with lymph node metastasis as compared with those patients without lymph node metastasis but no statistical significance was found. An increase of mean IS of SPRY4 protein without statistical significance was noted for OSCC patients without alcohol drinking, and betel-quid chewing as compared with those with these two oral risk factors respectively; however, there was a decrease of mean IS of SPRY4 protein without statistical significance for patients without cigarette smoking in comparison with those patients with cigarette smoking. The mean IS of SPRY4 protein for male patients were higher but without statistical significance as compared with female patients.

Positive staining of SPRY4 proteins was observed in all cases of human OPMD with and without MT (Fig. 2A and B). The mean IS of the human OPMDs with and without MT for SPRY4 protein was 4.09 and 3.31 respectively; however, no

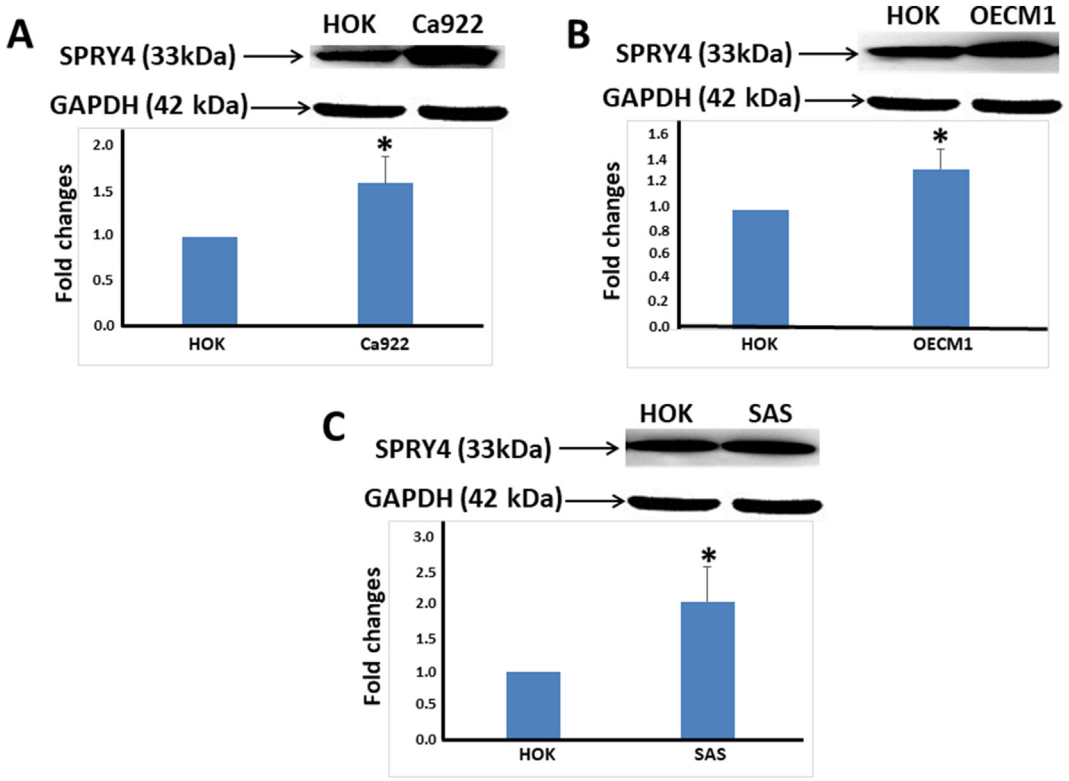

Figure 4 Western blot analyses: SPRY4 protein expression in human oral cancer cell lines as compared with the primary culture of normal oral mucosa (HOK). Upregulation of SPRY4 protein expression in human oral cancer cell lines (A, Ca922; B, OCEM1; C, SAS) as compared with HOK. Results were quantified using densitometric analysis, normalized to the level of GAPDH, and expressed as a fold change relative to the normal oral mucosa. Bars represent means ± standard deviation of the mean (*$P < 0.05$). A representative result of three independent experiments is shown.

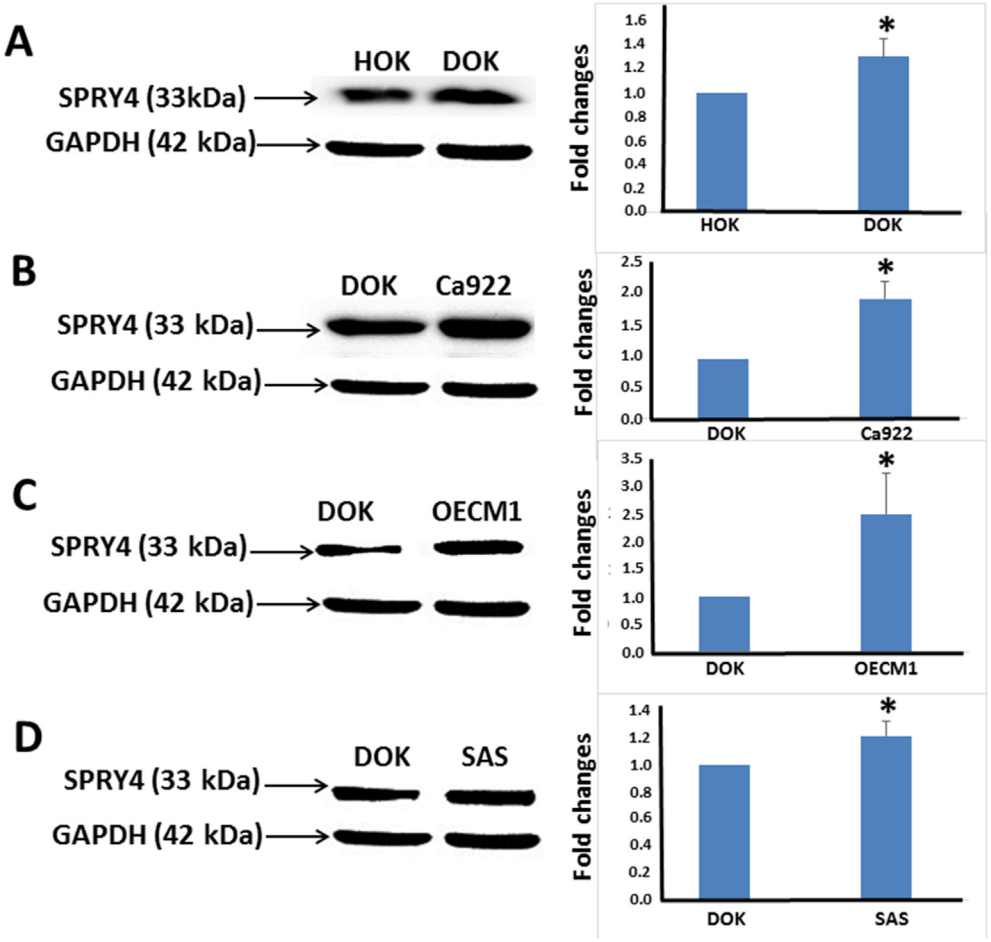

Figure 5 Western blot analyses: SPRY4 protein expression in human oral premalignant cell line DOK as compared with the primary culture of human normal oral mucosa (HOK) and with human oral cancer cell lines. Upregulation of SPRY4 protein expression was observed for DOK as compared with HOK (A). Upregulation of SPRY4 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 the fold change relative to the normal oral mucosa (A) and to human oral cancer cell lines (B—D). Bars represent means ± standard deviation of the mean (*$P < 0.05$). A representative result of three independent experiments is shown.

significant increase being noted when comparing the mean IS of human OPMDs with MT with those without MT for SPRY4 protein.

Western blot

Western blot of human OSCC tissues revealed overexpression of SPRY4 protein as compared with HOK, the difference being of statistical significance ($P < 0.05$) (Fig. 3). On the other hand, Western blot also demonstrated statistically significant overexpression of SPRY4 protein in the three OCCLs as compared with HOK ($P < 0.05$) (Fig. 4A—C).

Western blot of human OSCC tissues revealed overexpression of SPRY4 protein as compared with normal oral mucosa, the difference being of statistical significance ($P < 0.05$) (Fig. 3). On the other hand, Western blot demonstrated statistically significant overexpression of SPRY4 protein in the three OCCLs as compared with HOK ($P < 0.05$) (Fig. 4A-C). Western blot also confirmed significant SPRY4 protein overexpression for DOK as compared with HOK (Fig. 5A); and significant SPRY4 protein overexpression for the three OCCLs as compared with DOK (Fig. 5B-D).

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time quantitative reverse transcription-polymerase chain reaction analyses of the three OCCLs indicated upregulation of SPRY4 mRNA expression respectively, as compared with HOK (Fig. 6).

Proliferation assay, migration assay, invasion assay

The degrees of proliferation (Fig. 7), migration (Figs. 8A, 9A and 10A), and invasion (Figs. 8B, 9B and 10B) for the three OCCLs with SPRY4 siRNA transfections were significantly increased as compared with those without SPRY4 siRNA transfections respectively ($P < 0.05$).

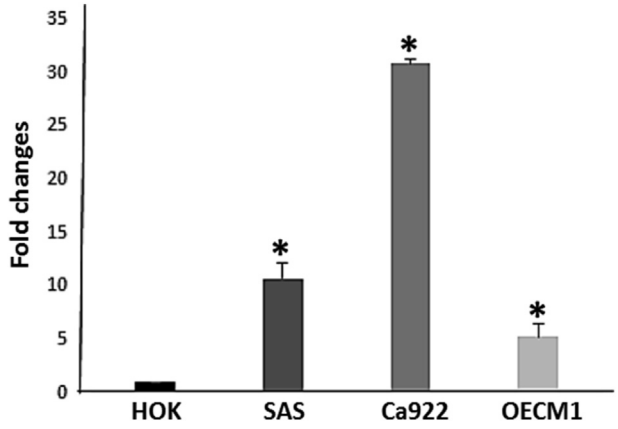

Figure 6 Real-time quantitative reverse transcription-polymerase chain reaction of SPRY4 in human oral cancer cell lines as compared with the primary culture of normal oral mucosa (HOK). Upregulation of SPRY4 mRNA expression was observed in human oral cancer cell lines (OECM1, SAS, and Ca922) as compared with HOK. Results were normalized by the level of GAPDH and expressed as fold change relative to HOK. Bars represent means ± standard deviation of the mean (*$P < 0.05$).

Discussion

To our knowledge, the potential prognostic and clinical relevance of SPRY4 expression in human oral squamous cell carcinogenesis has not been explored. In the present study, there was a significant SPRY4 protein overexpression in human OSCC tissues as compared with normal oral mucosa upon immunohistochemical and Western blot investigations. Overexpression of SPRY4 protein and mRNA was also demonstrated in human OCCLs as compared with HOK, which were consistent to the aforementioned *in vivo* results. Moreover, significant SPRY4 protein overexpression was observed for OCCLs and DOK as compared with HOK in the current study; also noted for human OCCLs as compared with DOK. A higher SPRY4 protein expression was found in human OPMDs with MT in comparison with those without MT although statistical significance could not be confirmed. Hence, the experimental data suggested that SPRY4 would perhaps involve in human OSCC formation, and could putatively relate to MT of human OPMDs. Our results are also consistent to the most recent finding that SPRY4 has been overexpressed in human colorectal carcinoma attributed to the epigenetically upregulation of SPRY4 gene.[26]

On the other hand, significant decreases in the proliferation, migration, and invasion of OCCLs without SPRY4 siRNA transfection were noted as compared with the OCCLs with SPRY4 siRNA transfection. The *in vitro* data are parallel with the *in vivo* immunohistochemical results in which SPRY4 expressions were decreased in OSCC patients with larger tumor size, higher disease stage, and with lymph node metastasis. Thus, considering the results for human OPMDs and OSCCs together, the present study, to the best of our knowledge, could be the first to confer the potential involvement of SPRY4 protein expression in human oral squamous cell carcinogenesis. Furthermore, the results of our study indicates that SPRY4 is a probable candidate for a tumor suppressor in human OSCC, which is compatible to different kinds of cancers including ovarian cancer,[27] endometrial adenocarcinoma,[28] breast carcinoma,[29] lung carcinoma,[30] and prostate carcinoma.[31]

In conclusion, SPRY4 has not only documented being associated with human oral squamous cell carcinogenesis,

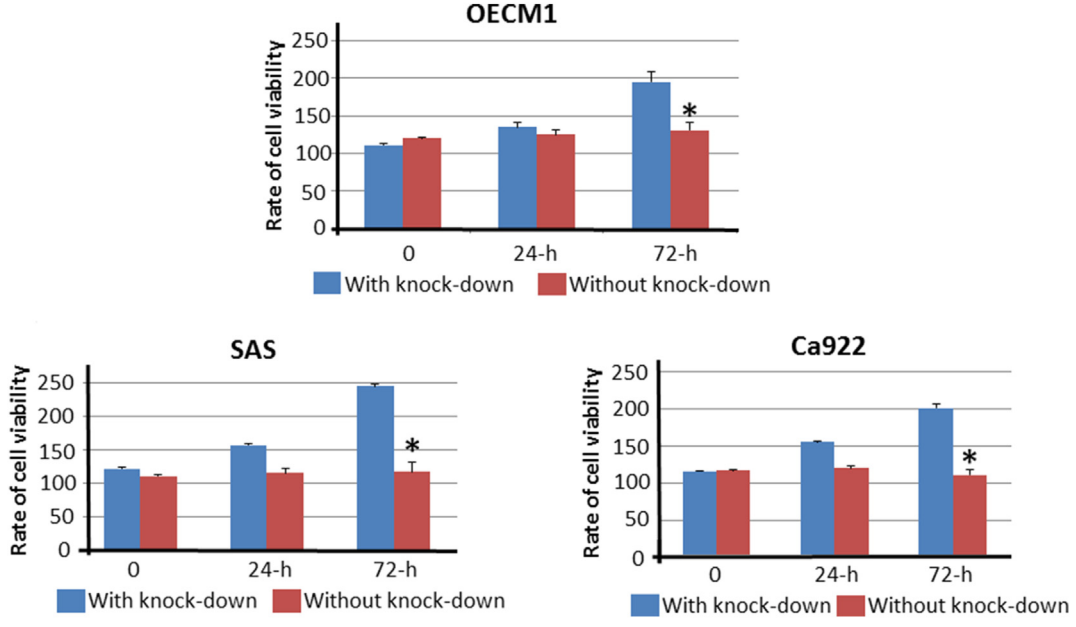

Figure 7 Proliferation assays of oral cancer cell lines (SAS, Ca922, and OECM1) with 24 h, and 72 h after SPRY4 siRNA transfection as compared with the three oral cancer cell lines without SPRY4 siRNA transfection. The proliferation rates of the oral cancer cell lines (A) OECM1, (B) SAS, and (C) Ca922 without SPRY4 siRNA transfection were significantly lower than those of the three oral cancer cell lines with SPRY4 siRNA transfection (*$P < 0.05$).

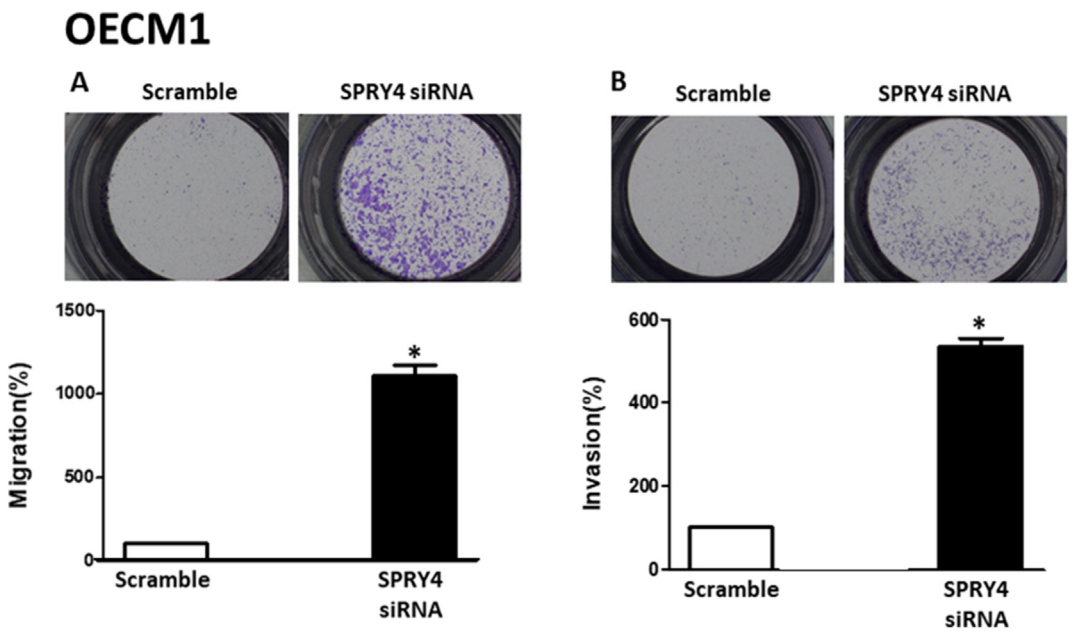

Figure 8 Migration assay (A) and invasion assay (B) of the oral cancer cell line (OECM1) with 48 h after SPRY4 siRNA transfections as compared with the oral cancer cell line without SPRY4 siRNA transfection (scramble). The degree of migration and degree of invasion of the oral cancer cell lines (OECM1) without SPRY4 siRNA transfections was significantly lower as compared with the oral cancer cell line with SPRY4 siRNA transfections respectively (A) (*$P < 0.05$).

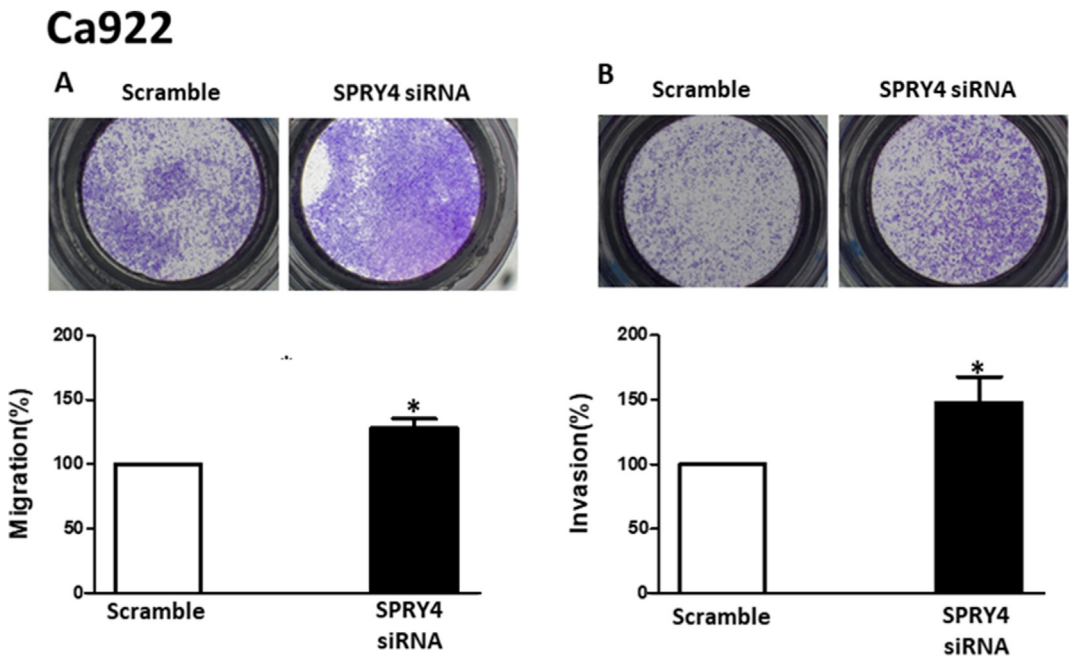

Figure 9 Migration assay (A) and invasion assay (B) of the oral cancer cell line (Ca922) with 48 h after SPRY4 siRNA transfections as compared with the oral cancer cell line without SPRY4 siRNA transfection (scramble). The degree of migration and degree of invasion of the oral cancer cell lines (Ca922) without SPRY4 siRNA transfections was significantly lower as compared with the oral cancer cell line with SPRY4 siRNA transfections respectively (A) (*$P < 0.05$).

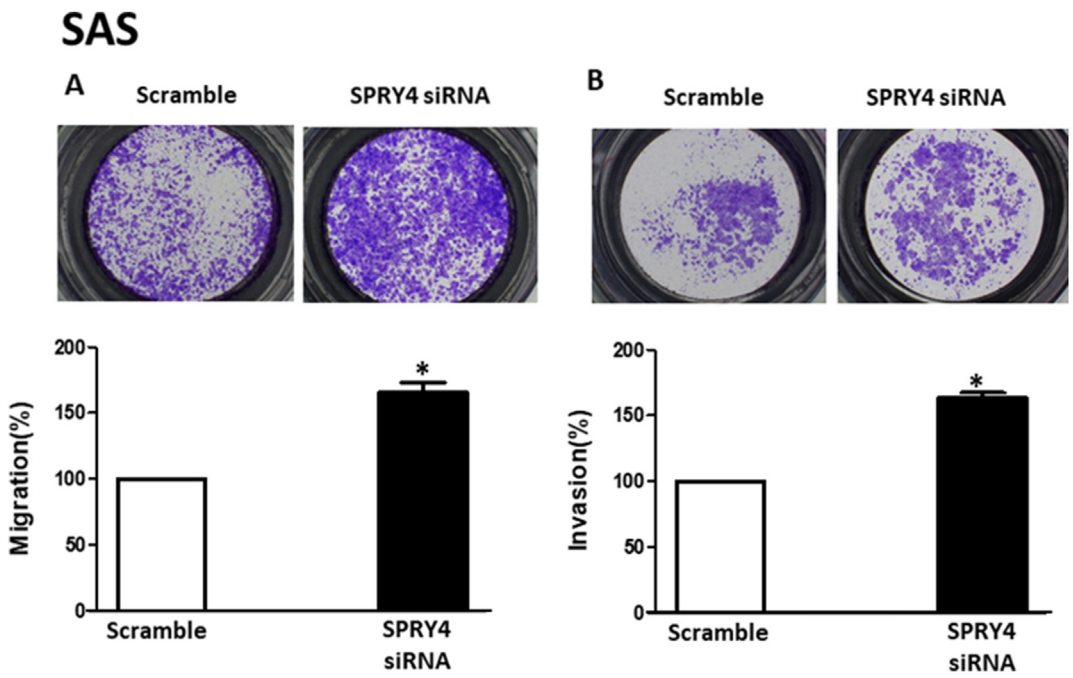

Figure 10 Migration assay (A) and invasion assay (B) of the oral cancer cell line (SAS) with 48 h after SPRY4 siRNA transfections as compared with the oral cancer cell line without SPRY4 siRNA transfection (scramble). The degree of migration and degree of invasion of the oral cancer cell lines (SAS) without SPRY4 siRNA transfections was significantly lower as compared with the oral cancer cell line with SPRY4 siRNA transfections respectively (A) ($*P < 0.05$).

but also a possible candidate for a tumor suppressor in OSCCs due to the lower proliferation, migration, and invasion without SRPY4 siRNA knock-down experiments in the current study. This warrants future investigation to validate the atypical roles of this putative tumor suppressor of SPRY4 in human oral squamous cell carcinogenesis whether SPRY4 could be compensatory overexpressed via epigenetic regulation, as confirmed in colorectal carcinoma.[25]

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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