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Overexpression of sprouty2 in human oral squamous cell carcinogenesis

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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

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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