Abstract. Sprouty2 is known for its tumor-suppressing effect in various human malignant diseases. In head and neck squamous cell carcinoma (HNSCC), the role of sprouty2 in tumorigenesis and clinical implication remains elusive. The aim of the present study was to investigate the expression of sprouty2 in patients with HNSCC and its function in vitro. Quantitative analysis of mRNA expression of sprouty2 was performed on frozen tumor samples from 42 patients with HNSCC and 19 with oral verrucous hyperplasia (OVH) with paired counterparts of normal mucosa. Downregulation of sprouty2 expression was demonstrated in 79% of HNSCC samples and in 58% of OVH samples compared with paired samples of normal mucosa. Enhanced expression of sprouty2 protein suppressed the growth of HNSCC cells and signaling of the phosphorylated AKT pathway. Following transfection of the sprouty2 plasmid, HNSCC cells were more sensitive to sorafenib, a tyrosine kinase inhibitor of Raf and vascular endothelial growth factor receptor. The present study suggested that sprouty2 expression was downregulated and behaved as a tumor suppressor in HNSCC. Sprouty2 expression in tumor cells enhanced sensitivity to sorafenib. Further studies are required to define the clinical impact of sprouty2 in patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents one of the six most common types of cancer in the world, with up to 500,000 novel cases annually; this includes malignancies arising in the oral cavity, larynx and pharynx. The highest rates of HNSCC are found in South-Central Asia, Central and Eastern Europe; rates are lowest in Africa and Central America, with no variation in prevalence between genders (1,2). Significant risk factors for HNSCC carcinogenesis include tobacco use, alcohol consumption and infection with human papilloma virus. In Taiwan, a positive correlation has been reported between the consumption of betel nuts and incidence of HNSCC (3). Furthermore, ~30% of HNSCC revealed mutations in genes that regulate squamous differentiation, including Notch1, interferon regulatory factor 6 and p63, therefore implicating their dysregulation as a major driver of HNSCC carcinogenesis (4). However, the mechanisms underlying the malignant progression of HNSCC remains to be fully elucidated.

Epidermal growth factor (EGF) receptor (EGFR) is overexpressed in 40-90% of HNSCC and is associated with a poorer outcome for HNSCC patients (5,6). Ligand binding triggers the EGFR receptor to either homodimerize or heterodimerize with other members of the Erb family, both leading to EGFR autophosphorylation. The activated receptor recruits signaling complexes and activates Ras-mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3K)-Akt, signal transducers and activators of transcription and the phospholipase C gamma pathway (7). These unique cascades converge with the upregulation of cyclin D1, a key mediator of mitosis and G1 to S-phase progression (8). Angiogenesis has recently been recognized
as a novel therapeutic strategy due to its important role in the pathogenesis of HNSCC. Vascular endothelial growth factors (VEGFs) and their receptors are expressed in the majority of HNSCC tumors. Several preclinical studies indicated that these markers are associated with tumor progression, changes in microvessel density and the development of lymph node metastasis (9,10).

Sprouty was first reported as an antagonistic regulator of fibroblast growth factor (FGF) signaling during tracheal branching in Drosophila (11). Four mammalian sprouty genes have been defined, based on their sequence similarity with Drosophila sprouty (12). In analogy with Drosophila sprouty, mammalian sprouty proteins were proven to antagonize FGF-stimulated organogenesis (13). It was reported that the overexpression of mammalian sprouty1 and sprouty2 inhibited FGF-, VEGF- and EGF-induced proliferation and migration in human umbilical vein endothelial cells and a mouse embryonic fibroblast cell line, NIH3T3 (14,15). It has become evident that expression of sprouty protein is dysregulated in numerous types of human cancer. A decrease in Sprouty2 expression has been demonstrated in hepatocellular carcinoma, lung cancer and other cancer types, this is consistent with its inhibitory on ERK1/2 signaling and proliferation (16,17). A previous study by our group indicated that sprouty2 was downregulated in colon cancer and had a tumor suppressive role (18). However, the tumor-suppressing effect of sprouty2 in HNSCC has not yet been investigated.

Cetuximab is a well-known targeted therapy in HNSCC, which has FDA approval. In addition to EGFR targeted therapy, angiogenesis-based cancer treatment is under extensive investigation. Therapeutic strategies that have been developed in treating cancer patients include monoclonal antibodies and kinase inhibitors. VEGF activation has been demonstrated in HNSCC (19), and high VEGF expression in HNSCC tumor samples has been associated with progression of lymph node spread and poor prognosis (20). Sorafenib, also known as NSC 724772, has been associated with progression of lymph node spread and poor prognosis (20). Sorafenib, also known as NSC 724772, is a multi-targeted agent that inhibits VEGF, and other cancer types, this is consistent with its inhibitory on ERK1/2 signaling and proliferation (16,17). A previous study by our group, identified that sprouty2 was downregulated in colon cancer and had a tumor suppressive role (18). However, the tumor-suppressing effect of sprouty2 in HNSCC has not yet been investigated.

RNA extraction and reverse transcription. Total RNA was extracted from primary tumor tissue and normal mucosa of patients using RNA extraction kits (RNeasy Midi kit; Qiagen, Hilden, Germany). RNA was treated according to the manufacturer's instructions of the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (PCR). For each PCR reaction, 2 ng of cDNA was used for PCR amplification on each sample with 12.5 µl of TagMan PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1.25 µl of TagMan Assay-on-Demand kit (Applied Biosystems) that included forward primer, reverse primer and probe. Samples were run on an ABI HT7900 Sequence Detector (Applied Biosystems) according to the manufacturer's instructions. The following primers were used: human Sprouty2, 5'-GCCATCAGGAGTCCAG-3' (forward) and 5'-GTTGAGTCTTCTGTTG-3' (reverse); and human GAPDH, 5'-GGTGTAACATGAAG-3' (forward) and 5'-CCACATTCGAGG-3' (reverse). The crossing points that were calculated with SDS software version 2.2 (Applied Biosystems) into account the difference in amplification efficiency of the target (Sprouty2) and the reference genes. The delta-delta Ct assay was used for the calculation of quantitative gene expression. Fold changes in gene expression of sprouty2 between tumor and normal tissue were calculated.

Immunoblot analysis. Cells were washed twice with phosphate-buffered saline (PBS) and harvested for immunoblot analysis. Cell lysates were prepared in the SDS sample buffer and proteins were separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and then blocked with 5% BSA in PBS-T (0.1% Tween-20 in PBS). The primary antibodies were diluted according to the manufacturer's recommendation in 5% BSA in PBS-T and incubated with the membrane at 4°C overnight. The antibodies used were: Rabbit anti-human monoclonal antibodies to Sprouty2 (1:2,000; Upstate Biotechnology, Temecula, CA, USA), rabbit polyclonal anti-human-actin (1:3,000; A 2668; Sigma Aldrich, St. Louis, MO, USA), rabbit anti-human monoclonal antibodies to phosphatase and tensin homolog (PTEN, 1:1,000; Epitomics, Burlingame, CA, USA), mouse anti-human monoclonal antibodies to phosphorylated Akt.
Plasmids and transfection. The pSprouty2-EGFP-N3 plasmid encoding Sprouty2-EGFP fusion protein was derived from pEGFP-N3 (Clontech, Mountain View, CA, USA) and rabbit anti-human monoclonal antibodies to Akt (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA) and rabbit anti-human monoclonal antibodies to Akt (1:1,000; Cell Signaling Technology, Inc.) as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse IgG (Santa Cruz Biotechnology) were used as secondary antibodies where appropriate and protein-antibody complexes were visualized using the enhanced chemoluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, USA).

In vitro proliferation assay. Cell proliferation was measured by MTT assay to assess the effect of sprouty2 and sorafenib on the growth of human HNSCC cell lines. Cells transfected with pSprouty2-EGFP-N3 or control cells were cultured in 96-well plates (Corning Costar, Corning, NY, USA) at a density of 4,000 cells per well. Cell proliferation was determined with the MTT assay 72 hours after cell plating. The absorbance was measured at 570 nm using a microtiter plate reader (MRX Revelation, DYNeX, Chantilly, VA, USA). To evaluate the cytotoxic effect of sorafenib (Bayer HealthCare, Whippny, NJ, USA) influenced by sprouty2 and sorafenib regulation, SCC-4 and HONE-1 cells were transiently transfected with pSprouty2-EGFP-N3 with Lipofectamine 2000 (Invitrogen Life Technologies). The control cells were transfected with empty vector.

Statistical analysis. The Kaplan-Meier method was used to analyze time-to-event variables, and the 95% confidence interval (CI) for the median time-to-event was computed. The Student's t test and paired t-test were used for the difference between groups. Any P-value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed using SigmaPlot 9.0 statistical software (Jandel Scientific).

Results

Sprouty2 expression is downregulated in human HNSCC and oral verrucous hyperplasia (OVH) - the downregulation was correlated with the severity of the disease. mRNA expression of sprouty2 was determined in tumor tissues at varying disease stages and tissues from adjacent normal mucosa from 19 OVH patients and 42 HNSCC patients by real-time quantitative PCR. Expression of sprouty2 mRNA in human tumors relative to that in their normal tissue counterparts from the same patients was compared. Overall, the mRNA expression of sprouty2 was decreased in 44 of 61 (72%) paired tumor samples compared with normal mucosal tissues. Specifically, a decrease in sprouty2 mRNA expression was found in 58% (11/19) of OVH and 28% (11/42) of stage III-IV HNSCC samples (Fig. 1A and B). The mean sprouty2 mRNA expression levels relative to their adjacent normal mucosa from three determinations were significantly lower in HNSCC (mean=0.634; 95% CI=0.425-0.844) than in OVH (mean=1.164; CI=0.599-1.730; P=0.029) (Fig. 1C). These findings showed that sprouty2 was downregulated in OVH and HNSCC.

Sprouty2 expression is not associated with features of HNSCC, including tumor size, differentiation grading, nodal metastasis and recurrence - downregulation of sprouty2 has no role in the recurrence-free survival of HNSCC. The downregulation of sprouty2 was correlated with invasiveness of HNSCC. In prostate cancer, the mean expression of sprouty2 was significantly decreased in patients experiencing recurrences during the follow-up period. The expression of sprouty2...
tended to be decreased in advanced-stage cancers and in cases with lymph node metastasis in comparison with early stage cancer (T2 vs >T2) (22). Among 42 cases of invasive HNSCC samples in the present study, the expression of sprouty2 did not correlate with recurrence, nodal metastasis, tumor size or histological grading (Fig. 2A-D). Song et al (23) showed that hepatoma patients negative for sprouty2 had poorer survival. In the present study, the correlation of sprouty2 expression with recurrence-free survival was estimated by the Kaplan-Meier method in HNSCC tumors. The expression of sprouty2 was not associated with any significant changes in recurrence-free survival (Fig. 2E).

Sprouty2 enhances the expression of PTEN and unexpectedly suppresses Akt phosphorylation, which indicates the tumor suppressive role of sprouty2 in HNSCC. Western blot analysis showed that HSC-3, HONE-1, and SCC-4, cells expressed lower levels of sprouty2 among the five HNSCC cell lines examined (Fig. 3). To verify the effect of sprouty2 in signaling pathways in HNSCC, HONE-1 and SCC-4, cells were transfected with pSprouty2-EGFP-N3 plasmid to enhance the expression of sprouty2. HSC-3 cells did not demonstrate ectopic expression of sprouty2 due to inadequate transfection efficacy by lipofectamine. As shown in Fig. 3, the overexpression of sprouty2 reduced the phosphorylation of Akt and increased the expression PTEN. Of note, PTEN is known to be a tumor suppressor gene, implicating that sprouty2 may synergize with PTEN to suppress HNSCC cell growth (18,24). Furthermore, the effect of sprouty2 on the proliferation of HONE-1 and SCC-4 cells was investigated. Fig. 4A shows that cell proliferation, examined by the MTT assay, was decreased in both HONE-1 and SCC-4 cells overexpressing sprouty2 compared with their control counterparts, which were transfected with empty vector (P<0.01).

Sprouty2 enhances the sensitivity of sorafenib in HNSCC cells, SCC-4 and HONE-1. The influence of sprouty2 expression on the effect of sorafenib was determined. SCC-4 and HONE-1 cells were transfected with pSprouty2-EGFP-N3
plasmid to express sprouty2 protein. Overexpression of sprouty2 enhanced the sensitivity of both SCC-4 and HONE-1 cells to sorafenib compared with control cells. The half maximal inhibitory concentration (IC$_{50}$) values of sorafenib were 1.78±0.18 µM in SCC-4 control cells and 3.54±0.24 µM in SCC-4/sprouty2 cells. In HONE-1 cells, the IC$_{50}$ values of sorafenib were 1.86±0.08 µM and 3.30±0.15 µM, respectively. Enhanced sprouty2 expression sensitized SCC-4 and HONE-1 cells to sorafenib. HNSCC, head and neck squamous cell carcinoma; SCC, squamous cell carcinoma; IC$_{50}$, half maximum inhibitory concentration.

![Figure 4](https://example.com/figure4.png)

Discussion

Sprouty proteins have been identified as modulators of receptor tyrosine kinase signaling in normal development and disease states (25). Evidence suggested that the expression of sprouty proteins is dysregulated in a number of cancers; in most types of cancer it is downregulated, with the exception of colon cancer and melanoma, where it is upregulated (Table I) (16-18,22,23,26-37). In prostate cancer, downregulated sprouty2 expression in invasive prostate cancer cell lines and high-grade clinical prostate cancer is similar to that of benign prostatic hyperplasia and well-differentiated tumors (32). A previous study by our group disclosed that sprouty2 was deregulated in human colon cancer and associated with inverse regulation by microRNA 21 (18). It implied that deregulation of sprouty2 expression may promote tumorigenesis. To date, it has remained elusive whether sprouty2 acts as a negative inhibitor of growth factors in HNSCC. The present study confirmed that sprouty2 was downregulated in preneoplasia and HNSCC. Importantly, it was identified that the decrease in sprouty2 expression in HNSCC was significant compared with OVH, a precursor of verrucous carcinoma. It is noteworthy that sprouty2 expression decreased in 58% of OVH samples, but was significantly decreased in invasive carcinoma. This result suggested that the decrease of sprouty2 expression may be an early event in the carcinogenesis of HNSCC. Previous studies on human cancers suggested the prognostic impact of sprouty2. In a hepatoma study, Lee et al (30) found that significant downregulation of sprouty2 protein and sprouty2 mRNA expression characterized most hepatomas with poorer outcomes. Differentiation grading is a strong predictor for prognosis in most types of cancers. Sprouty2 protein has been found to be associated with high-grade cancers. In human glioma, sprouty2 protein levels were significantly decreased in 79% of invasive World Health Organization (WHO) grade II-IV tumors, but not in non-invasive WHO grade I tumors and normal tissues (28). Furthermore, by assessing the expression of sprouty2 mRNA in resected prostate cancer, McKie et al (34) reported a trend between downregulated sprouty2 mRNA expression and increasing Gleason score (less differentiated) tumors. The present study focused on invasive HNSCC and did not suggest sprouty2 was a prognostic predictor, in part due to the limited number of stage I and stage II samples. Only fourteen tumor samples of stage I and stage II disease were analyzed in the present study. To verify the conclusion, the inclusion of a larger number of stage I and stage II disease samples would be required.
Sprouty proteins have been demonstrated to inhibit signaling by a diverse range of stimuli, including VEGF, PDGFR, hepatocyte growth factor, glial-derived growth factor and nerve growth factor (38,39). The inhibitory function of sprouty is mainly associated with the control of the Ras/Raf/MAPK/ERK pathway at multiple levels (25). Sprouty2 interacts with different components of the EGF signaling pathway. Sprouty2 interacts with E3 ubiquitin ligase/c-Casitas B-lineage lymphoma (c-Cbl), which leads to the reduction of EGFR endocytosis and degradation (40). Thus, sprouty2 serves as a negative regulator of FGF and other growth factors but as a positive enhancer of EGF signaling. Enhanced sprouty2 protein expression has been shown to suppress Akt phosphorylation and promote PTEN expression in cancer cells (18,24). In HNSCC cell lines, HONE-1 and SCC-4, ectopic expression of sprouty2 exerted a similar regulation pattern of signaling pathways. The present study showed that the expression of sprouty2 protein suppressed Akt phosphorylation and enhanced PTEN expression. This correlated with the tumor-suppressive effect of sprouty2 in HNSCC.

Several mechanisms have been proposed to define the decrease of sprouty2 in cancer cells, including the methylation of the sprouty2 promoter, increasing degradation of sprouty2 by neural precursor cell expressed developmentally down-regulated 4 (NEDD4) and upregulation of microRNA-21. McKie et al (34) found the suppression of sprouty2 expression correlated with methylation of the CpG region in clinical samples of prostate cancer. Sprouty2 inactivation by promoter methylation in human B-cell lymphoma was suggested by Frank et al (31). Sprouty2 can be proteolytically degraded by c-Cbl, seven in absentia homolog-2 (SIAH-2), or NEDD4 (30,40). In a hepatoma study, all 13 hepatoma samples exhibiting NEDD4 upregulation displayed low sprouty2 protein expression (30). Furthermore, upregulated microRNA-21 was associated with a decrease in sprouty2 protein levels in glioma cells (28) and reduced sprouty2 mRNA expression in colon cancer (18), implying that the deregulation of sprouty2 is triggered differently depending on cancer biology. Further studies are warranted to address the mechanisms of deregulated sprouty2 in HNSCC.

Sorafenib is an oral inhibitor of serine/threonine kinases C-Raf and B-Raf and the receptor tyrosine kinases VEGFR-2, VEGFR-3, PDGFR, FLT-3 and KIT. It inhibits cancer cell activity via two major pathways: The EGFR/Ras/Raf/MAPK/ERK signaling pathway, which is a key regulator in cancer biology, and the VEGF/VEGFR pathway, which is essential for angiogenesis (41,42). Sorafenib has been applied clinically for the treatment of patients with advanced hepatocellular carcinoma. Combining antiangiogenics to overcome resistance of anticancer therapy poses a promising strategy in the future of cancer medicine. In HNSCC, antiangiogenic agents, including bevacizumab and sorafenib, have been integrated into the treatment strategies but none of these agents have reached approval in clinical practice, despite extensive clinical studies. The proposed mechanisms of sorafenib resistance include EGFR activation (43), multidrug resistance protein 2 expression (44) and the activation of the PI3K/Akt signaling pathway in hepatocellular carcinoma (45). Accurate predictors of patient response to anticancer therapy would minimize the use of ineffective and expensive treatment with possible

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Sample origin</th>
<th>First author, year (reference)</th>
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<tbody>
<tr>
<td>Sprouty2 downregulated cancer</td>
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<tr>
<td>Breast cancer</td>
<td>Clinical human tissue</td>
<td>Lo et al, 2004 (24)</td>
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<td>Colon cancer</td>
<td>Clinical human tissue</td>
<td>Feng et al, 2011 (18)</td>
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<tr>
<td>Endometrial cancer</td>
<td>Clinical human tissue</td>
<td>Velasco et al, 2011 (25)</td>
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<tr>
<td>Glioma</td>
<td>Clinical human tissue and cell lines</td>
<td>Kwak et al, 2011 (26)</td>
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<tr>
<td>Squamous cell carcinoma of the head and neck</td>
<td>Clinical human tissue</td>
<td>Present study</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Clinical human tissue and cell lines</td>
<td>Fong et al, 2006 (17)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Cell lines</td>
<td>Frank et al, 2009 (29)</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>Clinical human tissue and cell lines</td>
<td>Sutterlüty et al, 2007 (16)</td>
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<tr>
<td>Sprouty2 upregulated cancer</td>
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<tr>
<td>Melanoma</td>
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<td>Bloethner et al, 2005 (35)</td>
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Table I. Sprouty2 expression in human cancer disease.
toxic side effects. Previous research efforts have focused on ‘forward-signaling’ mechanisms of pathway activation and have implied that the loss of negative feedback control can also lead to aberrant pathway activation. PTEN, a well-known tumor suppressor gene, has been extensively studied in EGFR inhibition of colon cancer therapy as well as trastuzumab treatment in breast cancer. Sprouty2 protein has been shown to be involved in trastuzumab resistance in breast cancer (46) and gefitinib resistance in colon cancer cells (47). The ability of sprouty2 to suppress Akt phosphorylation and modulate EGFR activation has encouraged researchers to explore the role of sprouty2 in altering the effectiveness of sorafenib in HNSCC. The present study indicated that the overexpression of sprouty2 protein suppressed HNSCC proliferation and enhanced sensitivity to sorafenib. Therefore, sprouty2 provides a potential target to increase sorafenib effectiveness in clinical practice. However, further investigations and validation through clinical trials are required prior to application of sprouty2 as a novel treatment for cancer patients in the clinic.

In conclusion, the present study suggested that sprouty2 was downregulated in a high proportion of OVH and HNSCC. Sprouty2 had a negative feedback role in the suppression of Akt phosphorylation and upregulation of PTEN, which had an inhibitory effect on cell proliferation. Sprouty2 may serve as a potential target in HNSCC treatment and a predictor of response to sorafenib therapy.

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