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SIP1 predicts progression and poor prognosis in pharyngeal squamous cell carcinoma

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Summary. Objectives: The epithelial-mesenchymal transition (EMT) is a crucial process in tumorigenesis that enables tumor cells to invade and metastasize. The transcription factors SIP1, SLUG, ZEB1, SNAI1, and TWIST are fundamental in regulating EMT. We investigated the relationships between several clinicopathological variables, prognosis, and SIP1, SLUG, or ZEB1 in a retrospective pharyngeal squamous cell carcinoma (PSCC) cohort.

Study Design: Immunohistochemistry was used to evaluate the expression of SIP1, SLUG, and ZEB1 in 108 tumor samples from a retrospective cohort of patients with PSCC.

Results: Tumors with positive epithelial SIP1 immunostaining were more advanced (SIII-IV, p=0.02) and had more lymph node metastases (p=0.04) than SIP1-negative tumors. Tumors with positive stromal staining of SIP1 relapsed more often than SIP1-negative tumors (p=0.007). Negative SIP1 immunoreactivity correlated significantly with better disease-specific survival (DSS) and better overall survival (OS) (p=0.012 and p=0.003 for epithelial reactivity, p=0.018 and p=0.003 for stromal reactivity, respectively). Lack of epithelial SIP1 expression remained an independent and favorable prognostic factor in a Cox proportional hazards model (p=0.046), together with high Karnofsky performance status score and low T class (p<0.001 for both). Co-expression of SNAI1, TWIST, and SIP1 in tumor epithelium predicted even shorter DSS than SIP1 expression alone (p<0.001) in the present study cohort.
Conclusions: SIP1 is related to cancer progression and appears to be an independent prognostic factor in PSCC.

Key words: Pharyngeal squamous cell carcinoma, Epithelial-mesenchymal transition, SIP1, SLUG, ZEB1, SNAI1, TWIST, Prognosis

Introduction

Pharyngeal carcinoma is an aggressive tumor often diagnosed at a locally advanced stage. It includes nasopharyngeal, oropharyngeal, and hypopharyngeal subsites. Histologically, oro- and hypopharyngeal tumors are almost exclusively squamous cell carcinomas (SCCs) (Chin et al., 2006). The incidence of pharyngeal carcinoma has been increasing at a rate of approximately 1% per year for the past 10 years (Lundberg et al., 2011), accounting for 130 000 new cases and causing over 80 000 deaths per year worldwide (Parkin et al., 2005). The prognosis of pharyngeal carcinoma has remained poor despite the availability of multimodal therapies. The prognosis and treatment modality are determined by TNM class (Gospodarowicz et al., 2004). Despite

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Abbreviations. SCC, Squamous cell carcinoma; PSCC, Pharyngeal squamous cell carcinoma; EMT, Epithelial-mesenchymal transition; PBS, Phosphate buffered saline; DSS, Disease-specific survival; OS, Overall survival

extensive research, there is currently no established biomarker for patient survival.

Tumors have been described as wounds that do not heal (Kalluri and Zeisberg, 2006). The epithelial-mesenchymal transition (EMT) is a diverse cellular process that becomes active in both tumorigenesis and wound healing; it is also crucial in embryogenesis. During the EMT, epithelial cells lose their cohesion and gain motile and invasive characteristics that enable them to invade and metastasize (Thiery, 2002). The EMT is defined by the downregulation of adhesion molecules (e.g., E-cadherin) and upregulation of mesenchymal genes (e.g., *N-cadherin, vimentin,* and *β-catenin*) (Thiery, 2002). It is regulated by transcription factors such as SNAI1, TWIST, Smad-interacting protein 1 (SIP1, also known as ZEB2), SLUG (also known as SNAI2), and Zinc-finger E-box-binding homeobox 1 (ZEB1), all of which induce EMT and provoke E-cadherin downregulation by binding to its promoter region (Comijn et al., 2001; Christiansen and Rajasekaran, 2006; Aigner et al., 2007).

SIP1 downregulates E-cadherin transcription, and thus appears to promote invasion in malignant epithelial tumors (Comijn et al., 2001). It also regulates genes that encode structural proteins of tight junctions, desmosomes, and gap junctions (Vandewalle et al., 2005). In addition, SIP1 has an anti-apoptotic effect on the DNA damage response (Sayan et al., 2009). SIP1 expression has independent prognostic value for poor disease-specific overall survival in oral SCC (Maeda et al., 2005), and SIP1 overexpression correlated with delayed neck metastases in another oral SCC series (Sakamoto et al., 2011). In lung carcinoma, SIP1 expression has been associated with tumor growth and poor prognosis (Miura et al., 2009).

SLUG is involved in neural crest specification in chicken and *Xenopus* embryos (Nieto, 2002). In addition to E-cadherin downregulation, SLUG may act synergistically with other E-cadherin repressors (Castro Alves et al., 2007; Alves et al., 2009). Thus, SLUG downregulation promotes apoptosis and decreases invasion capability *in vitro* and *in vivo* (Tang et al., 2011). It has been suggested that SLUG might also have a role in pathological angiogenesis (Welch-Reardon et al., 2014). SLUG overexpression has been associated with aggressive tumor behavior and poor survival in esophageal SCC and colorectal carcinomas (Uchikado et al., 2005; Shioiri et al., 2006). However, in a study of patients with oral SCC, SLUG expression did not correlate with clinicopathological parameters or survival (Wushou et al., 2011).

ZEB1 is mainly involved in the embryonic development of the neural crest and musculoskeletal system (Gheldof et al., 2012). It also inhibits the expression of epithelial genes that are central to adhesion and epithelial polarity (Vandewalle et al., 2005; Aigner et al., 2007). ZEB1 promotes metastasis in colorectal cancer and hepatocellular carcinoma (Spaderna et al., 2008; Zhou et al., 2012), and is aberrantly expressed in aggressive uterine cancers (Spoelstra et al., 2006). However, ZEB1 expression in lung tumors does not correlate with survival (Merikallio et al., 2011). Also, in a study with a large collection of bladder tumor array samples, ZEB1 expression was not associated with tumor stage, histological grade, metastasis, or survival (Kenney et al., 2011).

Our previous study indicates that SNAI1 and TWIST expression in tumor stromal cells is associated with poor prognosis in pharyngeal SCC (PSCC) (Jouppila-Matto et al., 2011a,b). In the present work, we aimed to evaluate the expression of SIP1, SLUG, and ZEB1 in PSCC, and their association with clinicopathological variables and survival. To our knowledge, there are no previous studies that focus on SIP1 expression in pharyngeal carcinoma.

Materials and methods

Patients

The original cohort included 138 patients diagnosed with oropharyngeal or hypopharyngeal SCC in Eastern Finland between 1971 and 1997. One hundred and eight of these patients had sufficient material available for immunohistochemical analyses. The representativeness of the groups was confirmed by χ^2 test (Pukkila et al., 2001). All histological samples were gathered before any oncological treatments were administered. Histological differentiation was evaluated according to World Health Organization (WHO) classification, and tumor staging was based on International Association Against Cancer (UICC) classification (Shanmugaratnam and Sobin, 1991; Sobin and Fleming, 1997). The Karnofsky performance status (KPS) was assessed and recorded at the time of diagnosis (Schag et al., 1984). The patients were surveyed until death or April 2009, and none were lost during follow-up.

Tissue microarray and immunohistochemistry

Two viable and representative areas at or close to invasive front of 108 paraffin-embedded tissue blocks were chosen by an experienced histopathologist (YS) and marked for microarrays, which were constructed using a 1.3-mm core Manual tissue arrayer I (Beecher Instruments, Silver Spring, MD, USA). Four-micrometer-thick sections were deparaffinated and rehydrated in a routine manner. Then, the sections were heated in a microwave oven (800W) for 2×5 min in 0.01 M citrate buffer (pH 6.0), incubated in the last buffer for 18 min and washed twice for 5 min in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with hydrogen peroxide (5%, 5 min), followed by washing with water for 2×5 min and with PBS for 2×5 min. Non-specific binding was blocked with 1.5% normal serum in PBS for 25 min at room temperature. Sections were incubated overnight at 4°C with a rabbit polyclonal anti-SIP1 antibody (1:200

dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit polyclonal anti-SLUG antibody (1:100 dilution) (AB Nova, Taipei city, Taiwan) and a mouse monoclonal anti-ZEB1 antibody (1:500 dilution) (GenWay Biotechnology, San Diego, CA, USA), respectively. In negative controls, the primary antibody was omitted.

The slides were then washed with PBS for 2×5 min. SIP1- and ZEB1-stained slides were incubated with biotinylated secondary antibody (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 35 min at room temperature. Next, the slides were washed twice in PBS for 5 min, incubated for 45 min in pre-formed avidin-biotinylated peroxidase complex (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA), and washed with PBS for 2×5 min. SLUG-stained sections were treated with Dako REAL EnVision secondary antibody (K5007) and incubated for 30 min. The color was developed with diaminobenzidine tetrahydrocloride (DAB) (Sigma, St. Louis, MO, USA). Samples were counterstained with Mayer's hematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). Strongly positive pharyngeal tumor tissues samples for each antibody were identified in preliminary test stainings and were then used as positive controls in each definitive staining series. In negative controls the primary antibody was omitted.

Evaluation of expression

Two observers separately evaluated all samples (AJ-M and YS for SIP1 and SLUG, AJ-M and RS for ZEB1) without being aware of the clinical data. Stained nuclei of tumor epithelial cells, tumor stromal cells, and endothelial cells were counted in array spots. In SIP1 and SLUG samples, there was also cytoplasmic staining in tumor epithelia and stromal tissue, which was classified into four groups according to intensity: no staining=0, weak staining=1, moderate staining=2, and intense staining=3. The percentage of tumor epithelial and stromal cell nuclear staining of SIP1, SLUG, and ZEB1 was counted and divided into five groups: 0-5%=1, 6-25%=2, 26-50%=3, 51-75%=4, and 76-100%=5. We also counted the number of array spots that exhibited detectable endothelial immunostaining (no staining=0, staining in one spot=1, and staining in two spots=2). The two observers re-evaluated together all the spots at which the scores diverged by more than one class to reach a consensus; a mean value of A and B spots of each sample from both observers was counted. The median value of every variable was counted; the samples were divided into positive and negative with respect to the median value. All median values are represented in Table 1.

Table 1. Median values of SIP1, SLUG, and ZEB1 calculated from the classification of positive cell expression.

	SIP1	SLUG	ZEB1
Tumor epithelial cell nuclei	1	2.25	0.5
Tumor stromal cell nuclei	2	1.75	3
Tumor epithelial or stromal cytoplasm	1.25	2.13	0

0-5%=1, 6-25%=2, 26-50%=3, 51-75%=4, 76-100%=5

Statistical analysis

The chi-squared test was used to analyze the

Table 2. Clinicopathological features of patients with pharyngeal squamous cell carcinoma (n=108).

Variable	n (%)
Mean age at the time of presentation, years	65 [40-89]*
Median duration of the symptoms, months	3 [0-76]*
Sex	
Male	81 (75)
Female	27 (25)
Site of primary tumor	
Oropharynx	68 (63)
Hypopharynx	40 (37)
T category	
T1	13 (12)
T2	39 (37)
T3	21 (19)
T4	35 (32)
N category	
N0	62 (58)
N1	16 (15)
N2	27 (25)
N3	3 (3)
M category	
M0	104 (96)
M1	4 (4)
Stage	
S I	9 (8)
S II	24 (22)
S III	21 (19)
S IV	54 (50)
Histologic differentiation	
Gr 1	25 (24)
Gr 2	48 (44)
Gr 3	35 (32)
Karnofsky performance status score	
≥70%	71 (66)
<70%	37 (34)
Primary treatment	
Radiotherapy	68 (63)
Surgery and radiotherapy	31 (28)
Surgery	5 (5)
No cancer-specific treatment	4 (4)
Relapse	
No	38 (35)
Yes	41 (38)
No response	31 (29)
Second primary tumor	
No	98 (91)
Yes	10 (9)
Median OS, months	20.9 [1.1-401.3]*

*Values in square brackets indicate range.

association between immunohistochemical markers and clinicopathological variables. Associations between markers were described as a proportion of similarity in expression. Variables affecting mortality in PSCC, the applied end-point event, were analyzed using the Kaplan-Meier method and Cox's proportional hazards model. The statistical differences between the curves were analyzed using the log-rank test. Disease-specific survival (DSS) was defined as the time between the date of primary diagnostic biopsy and the date of death due to pharyngeal cancer in a 5-year follow-up period. P-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

Ethics

The research plan was approved by the ethical committee of Kuopio University and Kuopio University Hospital and permission for accessing data from the Finnish Cancer Registry and from hospital records was obtained from by the Finnish Ministry of Social Affairs and Health.

Results

Cohort

The clinicopathological data are summarized in Table 2. The mean age at the time of diagnosis was 65 years; three-quarters of patients were male. At the time of diagnosis, 69% of the carcinomas were stages III or IV and 76% were moderately or poorly differentiated (histopathological grades 2-3). The main treatment modality used was radiotherapy, either alone (64%) or postoperatively as adjuvant therapy (28%). The median follow-up time was 43 months (range 1-332 months).

Expression of SIP1, SLUG, and ZEB1

SIP1 expression was abundant in tumor epithelial nuclei, especially at the invasive front. It was also

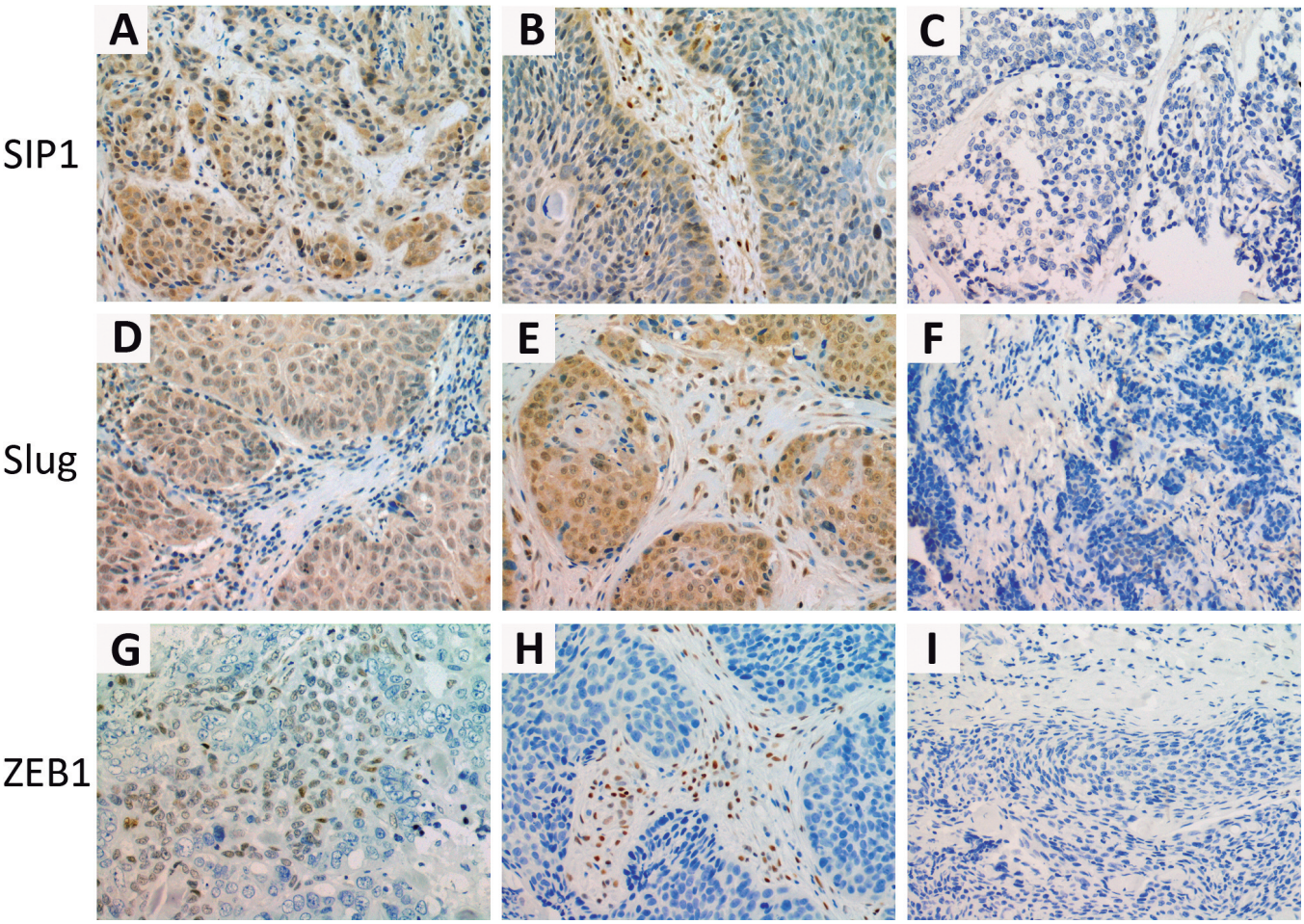

Fig. 1. Immunohistochemical detection of SIP1, SLUG, and ZEB1 in pharyngeal squamous cell carcinoma. Epithelial **(A, D, G),** stromal **(B, E, H)**, and negative immunostainings **(C, F, I)** of SIP1, SLUG, and ZEB1, respectively. The stromal component includes endothelial and fibroblast cells. x 200

frequently observed in tumor stromal cell nuclei, endothelial cell nuclei and the cytoplasm. Forty-four of 108 (41%) samples exhibited epithelial cell nuclear SIP1 positivity and cytoplasmic positivity. When present, the cytoplasm of all cell types exhibited a similar staining pattern. The nuclei of stromal cells were SIP1-positive in 38 of 103 cases (37%), and 63 samples (58%) had SIP1-positive endothelial cell nuclei (Fig. 1). There was an association between cytoplasmic SIP1 and nuclear immunostaining of all the cellular compartments ($p<0001$ for each). In addition, endothelial and stromal cell nuclear staining were associated ($p<0.001$).

Tumor epithelial cell nuclear immunostaining of SLUG was apparent in 57 of 108 (53%) PSCC tissue samples. Stromal cell nuclear staining was detected in 60 of 106 samples (57%), tumor epithelial and stromal cell cytoplasmic stainings were evident in 55 of 108 array spots (51%), and 55% of the array spots (54 of 98 samples) had SLUG-positive endothelial cell nuclei (Fig. 1).

Epithelial cells were rarely positive for ZEB1; only single positive cells were detected in 34 of 108 tumor samples (31%).Thirty-eight samples (35%) featured ZEB1-positive stromal cell nuclei, and positive endothelial cells were observed in 61 spots (56%). The cytoplasm was ZEB1-negative in all samples (Fig. 1).

Immunohistochemistry and the clinicopathological variables

Table 3. Clinicopathological variables and SIP1 expression.

	Positive epithelial cell nuclei, cases (n=44)	Negative epithelial cell nuclei, cases (n=64)	p	Positive stromal cell nuclei, cases (n=38)	Negative stromal cell nuclei, cases (n=65)	p
Age <65	59% (26)	52% (33)		42% (16)	63% (41)	
Age >65	41% (18)	48% (31)	0.44	58% (22)	37% (24)	0.039
Karnofsky perf. status score <70	36% (16)	33% (21)		45% (17)	29% (19)	
Karnofsky perf. status score >70	63% (28)	67% (43)	0.7	55% (21)	71% (46)	0.11
Grade 1-2	82% (36)	56% (36)		79% (30)	60% (39)	
Grade 3	18% (8)	44% (28)	0.006	21% (8)	40% (26)	0.048
T1-2	41% (18)	53% (34)		39% (15)	52% (34)	
T 3-4	59% (26)	47% (30)	0.21	61% (23)	48% (31)	0.21
S I-II	18% (8)	39% (25)		24% (9)	34% (22)	
S III-IV	82% (36)	61% (39)	0.02	76% (29)	66% (43)	0.28
N0	45% (20)	66% (42)		53% (20)	60% (39)	
N1-3	55% (24)	34% (22)	0.04	47% (18)	40% (26)	0.47
M0	93% (41)	98% (63)		97% (37)	97% (63)	
M1	7% (3)	2% (1)	0.16	3% (1)	3% (2)	0.90
Oropharyngeal origin	45% (20)	75% (48)		47% (18)	69% (45)	
Hypopharyngeal origin	55% (24)	25% (16)	0.002	53% (20)	31% (20)	0.03
Remission	25% (11)	39% (25)		16% (6)	42% (27)	
No remission or relapse	75% (33)	61% (39)	0.13	84% (32)	58% (38)	0.007
No second primary	84% (37)	95% (61)		87% (33)	92% (60)	
Necond primary tumor	16% (7)	5% (3)	0.048	13% (5)	8% (5)	0.37

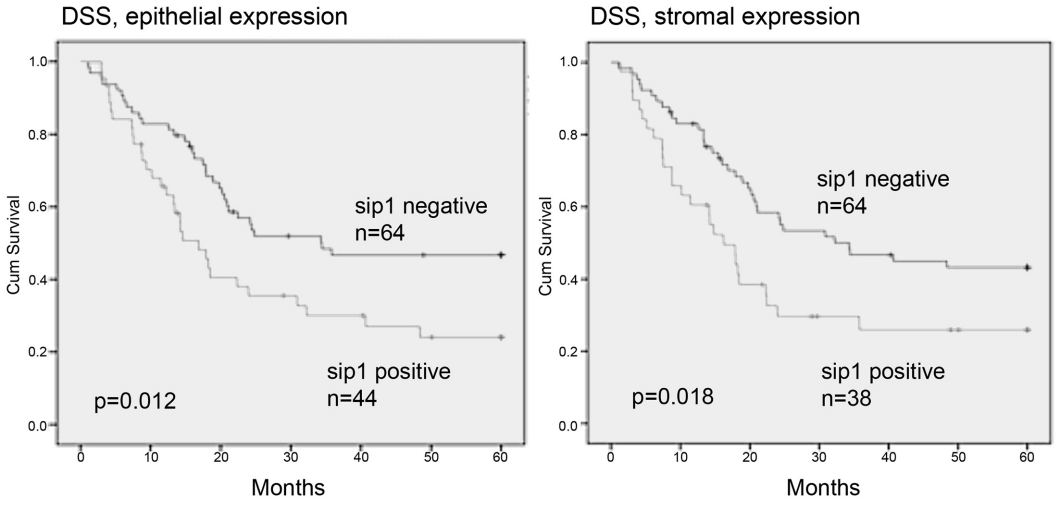

Fig. 2. Kaplan-Meier univariate 5-year survival analysis of patients with pharyngeal squamous cell carcinoma. SIP1 expression in tumor epithelial or stromal cell nuclei predicts poorer disease-specific survival.

Tumors with positive SIP1 immunostaining in epithelial cell nuclei were more advanced (SIII-IV) (p=0.02) and more often had lymph node metastases (N1-3) (p=0.04) than SIP1-negative tumors. There were also more second primaries diagnosed in the patient group with SIP1-positive tumors (p=0.048). Hypopharyngeal tumors were SIP1-positive more often than oropharyngeal tumors (epithelial nuclei p=0.002, stromal nuclei p=0.03, all cytoplasm p<0.001). Better-differentiated tumors (grade 1-2 vs. 3) often exhibited positive SIP1 immunostaining in epithelial cell nuclear (n=36, p=0.006), stromal cell nuclear (n=30, p=0.048), endothelial cell (n=51, p<0.001), or cytoplasmic compartments (n=35, p=0.02) of all cell types. Tumors with SIP1-positive stromal cell nuclei showed local locoregional or distant recurrences significantly more often than SIP1-negative tumors (n=32, p=0.007; Table 3).

Tumors with SLUG-positive epithelial nuclei were located in the hypopharynx more often than in the oropharynx (n=27, p=0.02). In addition, SLUG-positive tumors were more often well or moderately differentiated (epithelial nuclei n=45, p=0.004; endothelial nuclei n=41, p=0.049; cytoplasm of all cell types n=43, p=0.01). Patients younger than 65 years of age lacked SLUG expression in the stromal compartment more often than patients aged 65 years or older (n=32, p=0.007). ZEB1 immunoreactivity did not correlate with any of the clinicopathological variables.

Co-expression of transcription factors

There was a distinct association between tumor epithelial cell staining, and especially stromal cell nuclear staining, of SIP1, SLUG, and ZEB1. The co-

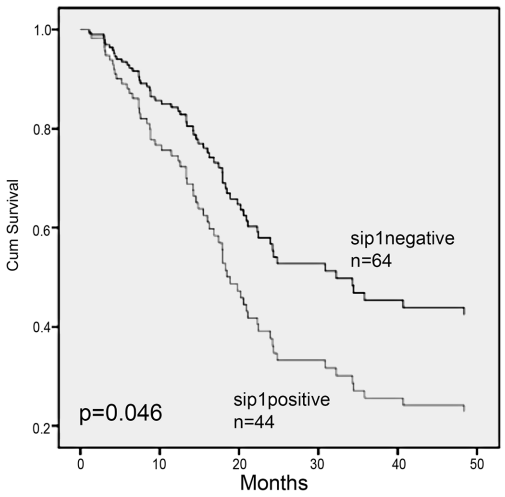

Fig. 3. Cox proportional hazards model of 5-year survival analysis of patients with pharyngeal squamous cell carcinoma. SIP1 expression in tumor epithelial cell nuclei predicts poorer disease-specific survival (p=0.046).

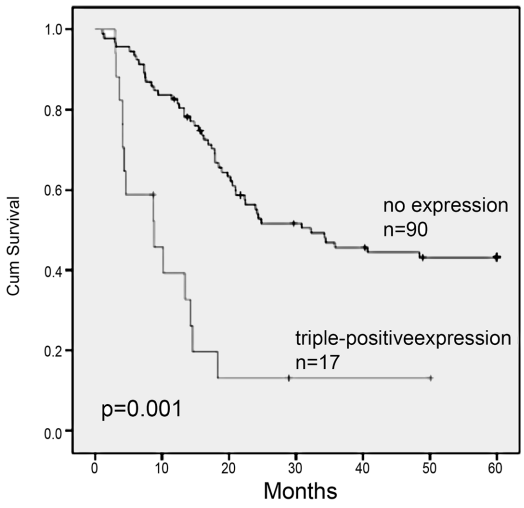

Fig. 4. Kaplan-Meier univariate 5-year survival analysis of patients with pharyngeal squamous cell carcinoma. Disease-specific survival is significantly poorer among patients in which SNAI1, TWIST, and SIP1 are all co-expressed in tumor epithelial cell nuclei (triple-positive expression) than among patients in which epithelial cell nuclei are negative for at least one EMT marker (p<0.001).

Table 4. Percentage of samples with positive co-expression of two EMT transcription factors in tumor epithelial and stromal cell nuclei.

	SIP1	SLUG	ZEB1	SNAI1
SLUG	27% e 30% s			
ZEB1	9% e 20% s	16% e 28% s		
SNAI1	33% e 25% s	44% e 35% s	23% e 24% s	
TWIST	20% e 23% s	20% e 30% s	13% e 27% s	27% e 30% s

e, epithelial cell nuclei; s, stromal cell nuclei

Table 5. Multivariate analysis of prognostic factors in PSCC.

Independent factors	p	Hazard ratio	95% Confidence interval
Age <65/>65	0.703		
KPS score <70/>70	0.001	2.406	1.434-4.036
T1-2/T2-3	0.000	3.616	2.048-6.383
N0/ N1-3	0.539		
Histological grade 1-2/3	0.342		
SIP1 +/-	0.045	0.600	0.365-0.989
SNAI1, TWIST, and SIP1 one or all negative/all positive	0.002	2.649	1.431-4.902

expression of all five transcription factors involved in EMT is detailed in Table 4 (Jouppila-Matto et al., 2011a,b). We stratified a subgroup of tumors (n=17, 16%) in which SNAI1, TWIST, and SIP1 were all co-expressed in tumor epithelium (triple-positive tumors). All tumors in this subgroup were at least stage III (p=0.003). The tumors were generally located in the hypopharynx (n=11, p=0.009), were more often T3-4 (n=13, p=0.02), and had more lymph node metastases (N1-3, n=11, p=0.04). Almost all of the triple-positive tumors were well or moderately differentiated (n=15, p=0.04). There were 19 samples in which none of the three transcription factors were expressed in tumor epithelial cell nuclei; however, there was no significant correlation with clinicopathological variables in this subgroup. In 17 samples (15.6%), SNAI1, TWIST, and SIP1 were all expressed in the nuclei of tumor stromal cells. Only two patients in that subgroup recovered; 15 relapsed or did not attain remission (p=0.05). In 31 samples, none of those transcription factors were expressed in tumor stromal cell nuclei (28.4%) (triple-negative tumors); most of the tumors in that group were T1-2 (n=21, p=0.007).

Survival analyses

In Kaplan-Meier univariate analysis, tumor epithelial cell nuclear SIP1 immunoreactivity correlated significantly with 5-year DSS and overall survival (OS). The median survival time was 34 months for patients with SIP1-negative tumors and 17 months for SIP1-positive tumors (DSS p=0.012, OS p=0.003). Stromal cell nuclear SIP1 positivity was associated with DSS and OS (p=0.018 and p=0.003, respectively; Fig. 2). The Cox proportional hazards model was run with the following variables: age; KPS score; T class; N class; histolopathological grade; and epithelial, stromal, endothelial, and cytoplasmic expression of SNAI1, TWIST, SIP1, SLUG and ZEB1. SIP1-positive tumor epithelial staining was an independent prognostic factor for DSS and OS, together with KPS score and T class (DSS p=0.046, p=0.001, p<0.001 and OS p=0.023, p<0.001, p<0.001, respectively; Table 5 and Fig. 3). As already suggested by univariate survival analysis, SLUG or ZEB1 immunostaining did not associate with survival in Cox multivariate model analysis (DSS, p > 0.16).

In the group in which SNAI1, TWIST, and SIP1 were all co-expressed in the nuclei of tumor epithelial cells (triple-positive expression), both DSS and OS were ominous (p<0.001; Fig. 4). None of these patients was still alive after 5 years, and the mean survival time was only 12 months. This triple-positivity also remained significant in the previously mentioned Cox proportional hazards model, meaning that the epithelial co-expression of these three transcription factors is an independent prognostic factor for DSS and OS (p=0.002 and p<0.000) in PSCC, together with KPS and T class (p=0.002 and p<0.001, respectively; Table 5). DSS and OS were significantly better if no expression of SNAI1, TWIST, or SIP1 was detected in the nuclei of tumor stromal cells (p=0.05 and p=0.02, respectively; Fig. 5). However, this triple-negativity was not an independent prognostic factor in the Cox proportional hazards model.

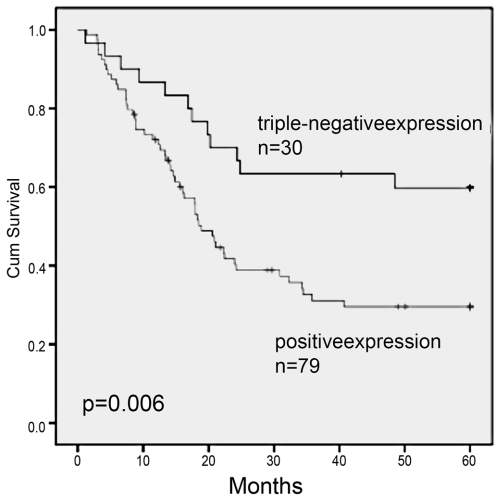

Fig. 5. Kaplan-Meier univariate 5-year survival analysis of patients with pharyngeal squamous cell carcinoma. Disease-specific survival is significantly better among patients whose tumor stromal cell nuclei lack SNAI1, TWIST, and SIP1 expression (triple-negative expression) than among patients whose tumor stromal cell nuclei are positive for at least one EMT marker (p=0.006).

Discussion

This study was undertaken to analyze the expression and role of transcription factors SIP1, SLUG, and ZEB1 in PSCC. For the first time in PSCC, this study shows that SIP1 enhances tumor progression both alone and together with other transcription factors. Epithelial expression of SIP1 in the nuclei of carcinoma cells was associated with advanced stage and lymph node status. SIP1 expression also had a major impact on patients' DSS. This may partly be caused by advanced stage and lymph node metastases, but epithelial expression of SIP1 also remained an independent prognostic factor in a Cox proportional hazards model together with KPS and T class. A similar association of increased SIP1 expression and decreased survival has been demonstrated in oral SCC (Maeda et al., 2005). Furthermore, SIP1 negatively affects survival in urothelial and non-small cell lung cancers (Sayan et al., 2009; Miura et al., 2009). This finding implicates SIP1 as a potential marker of the aggressiveness of these types of carcinomas. In our material, SIP1 expression was increased in less-differentiated carcinomas, a phenomenon also observed in oral SCC by Maeda et al. (2005). Histological grading alone had no effect on survival in either of the analyses. Thus, it is possible that SIP1 directly enhances tumor progression and metastasis independently of cellular

differentiation.

The stromal nuclear expression of SIP1 was correlated with local, locoregional or distant tumor relapses. SIP1 overexpression predicted delayed neck metastases in oral SCC (Sakamoto et al., 2011). It has been suggested that SNAI1 implicates early EMT alterations, and other transcription factors (e.g., SIP1) might be responsible for the maintenance of migratory cell behavior (Peinado et al., 2007). Accordingly, the immunostained stromal cells might, at least partially, represent transformed tumor cells that have undergone EMT. These motile cells would thus be capable of invading adjacent tissues and vessels to promote metastases and recurrences. On the other hand, non-neoplastic stromal fibroblasts may express transcription factors through interactions with adjacent epithelial cancer cells. The significance of stromal tissue in tumor progression has been studied and emphasized in recent years (Bhowmick et al., 2004; van der Horst et al., 2012; Celesti et al., 2013). These modified stromal fibroblasts might be not just enablers, but potential inducers of malignancy.

SIP1 expression varies remarkably in human tissues and tumors, and both nuclear and cytoplasmic expression have been described at various sites (Oztas et al., 2010). In the present study, SIP1 was expressed abundantly in both tumor epithelial nuclei cells and cytoplasm. There was also profuse stromal and endothelial staining in these tissue samples. SIP1 was detected in tumor cell nuclei, stromal fibroblasts, or cytoplasm in 28% of oral SCC samples (Maeda et al., 2005) and in cell nuclei of 40% of head and neck spindle cell carcinoma samples (Kojc et al., 2009). Our results are in line with these previous findings. SNAI1 is very unstable; therefore, only the nuclear protein is considered active (Zhou et al., 2004). The half-life of SIP1 has not been reported. Cytoplasmic expression of SIP1 was as abundant as nuclear staining, although it did not correlate with tumor progression or survival. Therefore, only nuclear expression of SIP1 appears to be clinically significant.

In addition to being a part of normal embryogenesis, EMT occurs in pathological situations such as wound healing, fibrosis, and acquisition of invasive phenotype in epithelial tumors (Thiery, 2002). E-cadherin downregulation has been considered a principal landmark of EMT. SIP1 binds to the promoter area of E-cadherin, inducing its downregulation (Comijn et al., 2001). However, the association between E-cadherin and SIP1 is not quite unambiguous. In OSCC, there was no significant inverse correlation between them (Sakamoto et al., 2011). This situation raises the possibility that SIP1 has functions other than the downregulation of E-cadherin. SIP1 expression also causes the downregulation of other major constituents of tight junctions, adherens junctions, desmosomes, and gap junctions at the transcriptional level (Vandewalle et al., 2005). In SCC, SIP1 protects cells from DNA-damage-induced apoptosis independently of cell cycle arrest (Sayan et al., 2009). Different levels of regulation influence the spatio-temporal expression of SIP1 protein and may point to its ability to play diverse roles in different contexts (Gheldof et al., 2012).Although several studies explore the role of SIP1 in the development of multiple cancers (Elloul et al., 2005; Miura et al., 2009; Sayan et al., 2009), to our knowledge there are no previous studies about SIP1 in PSCC.

SIP1 and SLUG expression were more frequent in hypopharyngeal tumors than in oropharyngeal tumors. The same phenomenon was also observed with TWIST in our previous study (Jouppila-Matto et al., 2011a). Carcinomas of various origins feature different expression patterns of EMT-related transcription factors (Alves et al., 2009; Oztas et al., 2010). On the other hand, SIP1, SLUG, and ZEB1 facilitate tumor growth by triggering EMT, inhibiting apoptosis, and enhancing angiogenesis and their expression often implies advanced tumors (Comijn et al., 2001; Yang et al., 2006; Sayan et al., 2009).

Concequently, it still remains unclear whether these different expression patterns are due to more advanced disease stage at the time of diagnosis or whether it represents a true feature of the hypopharyngeal tumors.

Even though SLUG has been associated with cancer progression in colorectal carcinoma and esophageal SCC (Uchikado et al., 2005; Shioiri et al., 2006), in the present study the expression of SLUG did not correlate with prognosis. Also, SLUG expression in oral SCC has not previously been associated with clinicopathological variables or survival (Wushou et al., 2011). That finding implies that EMT-related transcription factors have different roles in distinct tumors. To our knowledge, there are no previous studies about ZEB1 in head and neck carcinoma. In the present study we observed no association between ZEB1 expression and clinicopathological variables or survival.

Our previous research demonstrated the co-expression of SNAI1 and TWIST in PSCC (Jouppila-Matto et al., 2011a). In the present study, we observed moderate co-expression of all of the tested transcription factors in tumor epithelial cell nuclei, and SNAI1 and SLUG expression were clearly correlated. In stromal cell nuclei, the expression of all five transcription factors was strongly associated with each other. During embryogenesis, many EMT-related transcription factors are often activated simultaneously (Casas et al., 2011). However, in distinct tumors these transcription factors also appear to work separately. There is also a certain hierarchy among the factors, as TWIST requires direct induction by SLUG to induce EMT (Casas et al., 2011). In diffuse-type gastric carcinomas, SLUG, SNAI1, and SIP1 appear to complement each other (Castro Alves et al., 2007). In our material, tumors that expressed three transcription factors simultaneously were larger and more advanced, and were associated with significantly poorer prognosis, than tumors that lacked the expression

of at least one transcription factor. Thus, the transcription factors studied also appear to cooperate and intensify each other's function in PSCC. We used the GeneSapiens database (http://www.genesapiens.org) to analyze previously published data regarding the correlations of the expression of the genes that encode EMT-related transcription factors. We found 19-34 analyses of head and neck carcinoma that revealed significant positive correlations between the expression levels of SIP1 and SLUG ($p=0.047$), and between ZEB1 and TWIST ($p<0.001$), as well as a trend toward correlation between SNAI1 and SIP1 ($p=0.056$). No correlation was observed between transcription factors in normal oral or pharyngeal tissue samples; this finding implies that the cooperative work of the transcription factors takes place in malignant tissue in particular, as also reported by Kilpinen et al. (2008). In the present study, we selected a subgroup in which SNAI1, TWIST, SIP1, and SLUG were all co-expressed in tumor epithelial cell nuclei. However, the number of positive cases remained too small to support any statistical analyses.

It was recently discovered that human papilloma virus (HPV) is involved in the pathogenesis of many oropharyngeal and hypopharyngeal carcinomas and its incidence is increasing. These HPV-positive tumors are more sensitive to chemoradiotherapy and also have better prognosis (Hafkamp et al., 2008; Goon et al., 2009; Ang et al., 2010). However, the prognosis of pharyngeal SCC remains poorest of all head and neck SCCs (Goon et al., 2009). As our cohort is old, dating back to 1971, HPV associated tumors were quite uncommon. P16-immonohistochemistry, a widely used method for detecting HPV (Hafkamp et al., 2008; Allen et al., 2010), showed positive staining in only 17 cases (16 %) and it did not associate with any of the studied EMT transcription factors (data not shown). Thus, our material seems to represent a subset of PSCCs with other etiopathogenesis than HPV.

In spite of recent breakthroughs in some human cancer treatments and improved survival for some, the prognosis remains poor for patients with PSCC. One explanation is that tumors are already at advanced stages at the time of diagnosis (Argiris et al., 2008). However, there is clear demand to detect these carcinomas, which must be treated more aggressively and monitored more carefully. SIP1 may be a potential candidate to assist in the identification of these more aggressive carcinomas. However, additional studies are needed to confirm the role of this and other transcription factors as prognostic predictors in cancer.

Conclusions

We have demonstrated a significant correlation between positive SIP1 expression and tumor progression and poorer prognosis in PSCC. SNAI1 and TWIST co-expression further enhances the effect, which could be a sign of collaboration. According to these results, SIP1 may have a role as a novel biomarker to indicate aggressive tumors with poor prognosis.

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