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Transcription factor snail1 expression and poor survival in pharyngeal squamous cell carcinoma

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Summary. Snail1, a key regulator of epithelial-mesenchymal transition (EMT), plays an important role in tumour progression. Previous studies of snail1 have mainly focused on the epithelial tumour cells. The objective of this study was to evaluate the expression of snail1 protein in endothelial cells, stromal myofibroblasts and malignant epithelial cells of pharyngeal squamous cell carcinomas (PSCC), as well as its relation to clinicopathological features and survival. One hundred and ten tissue microarray samples were analyzed for snail1 expression using immunohistochemistry. In endothelial cells snail1 expression was observed in 51 (48%) of 107 cases and it predicted reduced disease specific survival (DSS) (p=0.009). In 49 (46%) tumour samples snail1 immunostaining was detected in stromal myofibroblasts and there was a tendency to poorer DSS in that group (p=0.067). Snail1 expression in endothelial cells and stromal myofibroblasts is also associated with hypopharyngeal tumours (p=0.01 and p=0.038 respectively), increasing T category (T3-4) (p=0.005, p=0.037 respectively) and poorer general condition of the patient (Karnofsky performance status score <70; p=0.029, p=0.039 respectively). Moreover endothelial expression correlated with advanced stage (III-IV) (p=0.005) and poorer differentiation (grade 2-3; p=0.012). In malignant epithelial cells snail1 immunostaining was detected in 75 of 110 cases (68%). Expression of the protein was more common in hypopharyngeal tumours (p=0.044). Snail1 positive tumours associated with a lower Karnofsky performance status score (p=0.039) and regional failure (p=0.042). Our findings indicate that snail1 protein expression in endothelial cells and to some extent also in tumour stromal myofibroblasts seems to be a predictor of poor survival in PSCC. The presence of snail1 protein in tumour microenvironment rather than in malignant epithelial tumour cells may induce tissue remodelling and tumour progression.

Key words: Pharyngeal squamous cell carcinoma, Endothelial cell, Stromal myofibroblast, Snail1 protein, Prognosis, Epithelial-mesenchymal transition

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Introduction

Head and neck cancer, a set of malignant tumours originating in several anatomic sites, is estimated to be the sixth commonest type of cancer. About 90% of these malignancies are histologically squamous cell carcinomas (SCC) (Argiris et al., 2008). The world wide incidence of pharyngeal squamous cell carcinoma (PSCC) including oropharyngeal and hypopharyngeal subsites, accounts for 130 000 new cases and 83 000 deaths per year (Parkin et al., 2005). Its prognosis is one of the poorest of any head and neck site.

Epithelial-mesenchymal transition (EMT) is a complex cellular process by which cells lose their

Abbreviations: DSS: disease specific survival; EMT: epithelial-mesenchymal transition; EndMT: endothelial-mesenchymal transition; HNSCC: head and neck squamous cell carcinoma; PBS: phosphate buffered saline; PSCC: pharyngeal squamous cell carcinoma; SCC squamous cell carcinoma

epithelial characteristics and gain mesenchymal features (Thiery, 2002). It is a normal physiological process in embryogenesis and wound healing but also an important mechanism during tumour progression where tumour cells disseminate from the primary location, invade and metastase (Thiery, 2002). Transcription factor snail1 is a key regulator of EMT mainly due to direct repression of E-cadherin (Cano et al., 2000). E-cadherin is an extracellular adhesion molecule present in adherent junctions required for maintenance of normal epithelial cell architecture (Cano et al., 2000). Additional cellular functions of snail1 in tumourigenesis include blocking of the cell cycle, protecting cells from apoptosis (Vega et al., 2004) and regulating cell polarity (Moreno-Bueno et al., 2008). Snail1 may also accelerate tumour metastasis through immunosuppression (Kudo-Saito et al., 2009).

There are two recently developed monoclonal anti-snail1 antibodies that are well-characterised and show evident nuclear staining (Franci et al., 2006; Rosivatz et al., 2006; Takkunen et al., 2006). In head and neck squamous cell carcinoma (HNSCC) only a few studies on snail1 expression have been reported. In a cohort of laryngeal SCC, either nuclear or cytoplasmic staining of snail1 was detected in 16% (40/251) of tumours and it is associated with local recurrence (Peinado et al., 2008). Zidar et al. detected positive nuclear snail1 staining in 4 of 30 HNSCC (Zidar et al., 2008). Additionally they found positivity in endothelial cells and inflammatory cells within the tumours. In oral SCC snail1 is mainly detected in stroma of the tumour invasive front and in some endothelial cells (Franz et al., 2009). Proinflammatory mediators were recently shown to increase snail1 expression in HNSCC (St John et al., 2009). In a recent study of 162 colon adenocarcinomas, stromal immunoreactivity of snail1 correlated with shortened disease specific survival. Endothelial staining was also detected (Franci et al., 2009). To date, there are no previous reports on association between snail1 expression and survival in HNSCC.

The aim of the present study was to evaluate the expression of snail1 in malignant epithelial cells, stromal myofibroblasts and endothelial cells in PSCC, and to investigate their association with clinicopathological features as well as survival.

Materials and methods

Patients

The present study included 110 patients diagnosed with squamous cell carcinoma of the oropharynx or hypopharynx in Eastern Finland between 1971 and 1997. Previously untreated patients with sufficient tumour material available for immunohistochemistry were gathered from PSCC study cohort (n=138). The representativeness of the patient group with the original cohort was confirmed by χ^2-test (Pukkila et al., 2001). The tumours were staged according to the International Association Against Cancer (UICC) classification (Sobin and Fleming, 1997). The performance status was coded according to the Karnofsky scale at the time of the diagnosis (Schag et al., 1984). Histological grade was evaluated according to WHO (Shanmugaratnam and Sobin, 1991). The patients were followed up until death or April 2009. None of the patients were lost from the follow-up. The research project was approved by the ethical committee of Kuopio University and Kuopio University Hospital.

Tissue microarray and immunohistochemistry

For each case two representative areas of PSCC were marked on the paraffin-embedded tissue block. Tissue microarrays (1.0 mm core) were constructed using manual tissue arrayer I (Beecher Instruments, Silver Spring, MD, USA). Four-μm-thick paraffin-embedded tissue sections were stained immunohistochemically. After deparaffinisation and rehydration, the sections were heated in a microwave oven for 2 x 5 min in Tris-EDTA buffer (pH 9.0), incubated in a Tris-EDTA buffer for 20 min and washed twice for 5 min in phosphate buffered saline (PBS). Hydrogen peroxide (5%, 5 min) was used to block endogenous peroxidase, followed by washing with water 2 x 5 min and with PBS for 2 x 5 min. Non-specific binding was blocked with 1,5% normal serum in PBS for 35 min at room temperature. The sections were incubated overnight at 4°C with the mouse monoclonal anti-snail1 antibody (1:750 dilution) (Franci et al., 2006; Takkunen et al., 2006). In negative controls primary antibody was omitted. The slides were then rinsed in PBS for 2 x 5 min and incubated with the biotinylated secondary antibody (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 45 min at room temperature. After this, the slides were washed with PBS for 2 x 5 min, incubated for 50 min in preformed avidin-biotinylated peroxidase complex (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) and washed in PBS for 2 x 5 min. The colour was developed with diaminobenzidine tetrahydrochloride (DAP) (Sigma, St. Louis, MO, USA). The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). Ovarian tumour tissue with known positive snail1 expression was used as a positive control.

Evaluation of the expression pattern

Snail1 expression was analysed by four observers (HT, AJ-M, YS, RS) unaware of the clinical data. The stained nuclei of malignant epithelial cells, stromal myofibroblasts, as well as endothelial cells, were counted in array spots (Soini et al., 2003). Stromal and endothelial expression was determined from one spot per tumour and malignant epithelial cell expression from two different spots taken from the same tumour. Stromal

myofibroblasts and endothelial cells were separated from each other according to cell morphology. The proportion of positive cell nuclei was counted on a continuous scale. Thereafter, the tumours were determined as negative or positive according to median (2 nuclei in malignant epithelial cells, 3 in stromal myofibroblasts and 7 in endothelial cells).

Statistical analysis

Mann-Whitney test was used to examine the associations between continuous variables. A chi-squared test was used in analyzing frequency tables. Interobserver agreement for the positivity of the immunoreactivity was evaluated by Spearman test. Univariate survival analyses were evaluated using the Kaplan-Meier method. The statistical differences between the curves were analyzed using the log-rank test. Multivariate survival analysis was performed using Cox's proportional hazards model. Disease specific survival was defined as the time period between the date of primary diagnostic biopsy and the date of death due to pharyngeal cancer. The statistical analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

Results

Cohort

The clinicopathological data are summarized in Table 1. The mean age at presentation of PSCC was 64 years with male predominance. Two thirds of the patients had stage III or IV disease. The main treatment modality used was radiotherapy either alone (64%) or postoperatively as adjuvant therapy (28%).

Snail1 expression

Snail1 expression in cell nuclei was detected in malignant epithelial cells, stromal myofibroblasts and endothelial cells in PSCC (Fig. 1). Altogether, there were less snail1 positive nuclei compared to ovarian tumour tissue used as a positive control. In malignant epithelial cells 75 (68%) tumours were graded positive. In stromal myofibroblasts positive immunoreactivity was detected in 49 (46%) and in endothelium in 51 (48%) of 107 samples. Excellent agreement of the immunohistochemical evaluation was reached between the observers (Spearman's correlation coefficient 0.73). Stromal myofibroblasts expression significantly associated with expression in endothelium and malignant epithelial cells (p-values <0.001) and expression in endothelium was related to immunoreactivity in malignant epithelial cells (p=0.002). In less than ten samples snail1 expression was detected adjacent to necrotic and inflammatory areas. Cytoplasmic snail1 expression in malignant epithelial cells was present in only 4 (4%) of 110 samples.

Clinicopathological variables and snail1 expression

Snail1 protein expression in malignant epithelial cells was statistically significantly associated with hypopharyngeal origin (p=0.044) and low Karnofsky performance status score (p=0.039). Positive snail1 immunoreactivity in malignant epithelial cells predicted earlier local relapse (p=0.042). Stromal myofibroblast snail1 expression was more common in hypopharyngeal tumours (p=0.038), with increasing T category (T3-4) (p=0.037), and in samples of patients with poorer

Table 1. Clinicopathological factors of the pharyngeal squamous cell carcinoma cases.

Variable	n (%)
Mean age at the time of presentation, years[a]	64 [40-89]
Median duration of the symptoms, months	3 [0-76]
Sex	
Male	83 (76)
Female	27 (24)
Site of primary tumour	
Oropharynx	70 (64)
Hypopharynx	40 (36)
T category	
T1	13 (12)
T2	40 (36)
T3	21 (19)
T4	36 (33)
N category	
N0	64 (58)
N1	16 (15)
N2	27 (24)
N3	3 (3)
M category	
M0	103 (94)
M1	7 (6)
Stage	
S I	9 (8)
S II	25 (23)
S III	21 (19)
S IV	55 (50)
Differentation, grade	
1	26 (24)
2	49 (45)
3	35 (32)
Karnofsky performance status score	
≥70%	73 (66)
<70%	37 (34)
Primary treatment	
Radiotherapy	70 (64)
Surgery and radiotherapy	31 (28)
Surgery	5 (4)
No cancer specific treatment	4 (4)
Recurrence	
No	37 (34)
Yes	42 (38)
No response	31 (28)
Second primary tumour	
No	100 (91)
Yes	10 (9)
Median OS, months	20.7 [1.1-401.3]

[a]: Values in square brackets indicate range. n=110.

general condition (Karnofsky performance status score <70; p=0.039). In endothelial cells positive snail1 immunoreactivity was related to hypopharyngeal primary site (p=0.01), increasing T category (p=0.005), advanced stage (III-IV) (p=0.005), high grade (2-3) (p=0.012) and low Karnofsky performance status score (p=0.029). No association was observed between snail1 immunoreactivity and age, gender or neck lymph node metastasis.

Survival analyses

Positive immunoreactivity of snail1 in endothelial cells strongly predicted poor five-year DSS (p=0.009; Fig. 2). Additionally, a trend was seen between stromal myofibroblast snail1 expression and poor DSS (p=0.067). Malignant epithelial cell expression was not related to prognosis. In multivariate analysis tumour T category and stage, as well as Karnofsky performance status score, were independent prognostic factors for survival (p<0.001 in all).

Discussion

Transcription factor snail1 expression is one of the major regulators of EMT (Zeisberg and Neilson, 2009). In the present study snail1 expression in endothelial cells was significantly associated with shortened survival in PSCC. Also stromal myofibroblast expression had a trend for poor survival. Immunoreactivity of snail1 has been detected previously in epithelial cancer cells in HNSCC (Peinado et al., 2008; Zidar et al., 2008; Franz et al., 2009) and several other cancer types (Franci et al., 2006; Rosivatz et al., 2006; Blechschmidt et al., 2007,

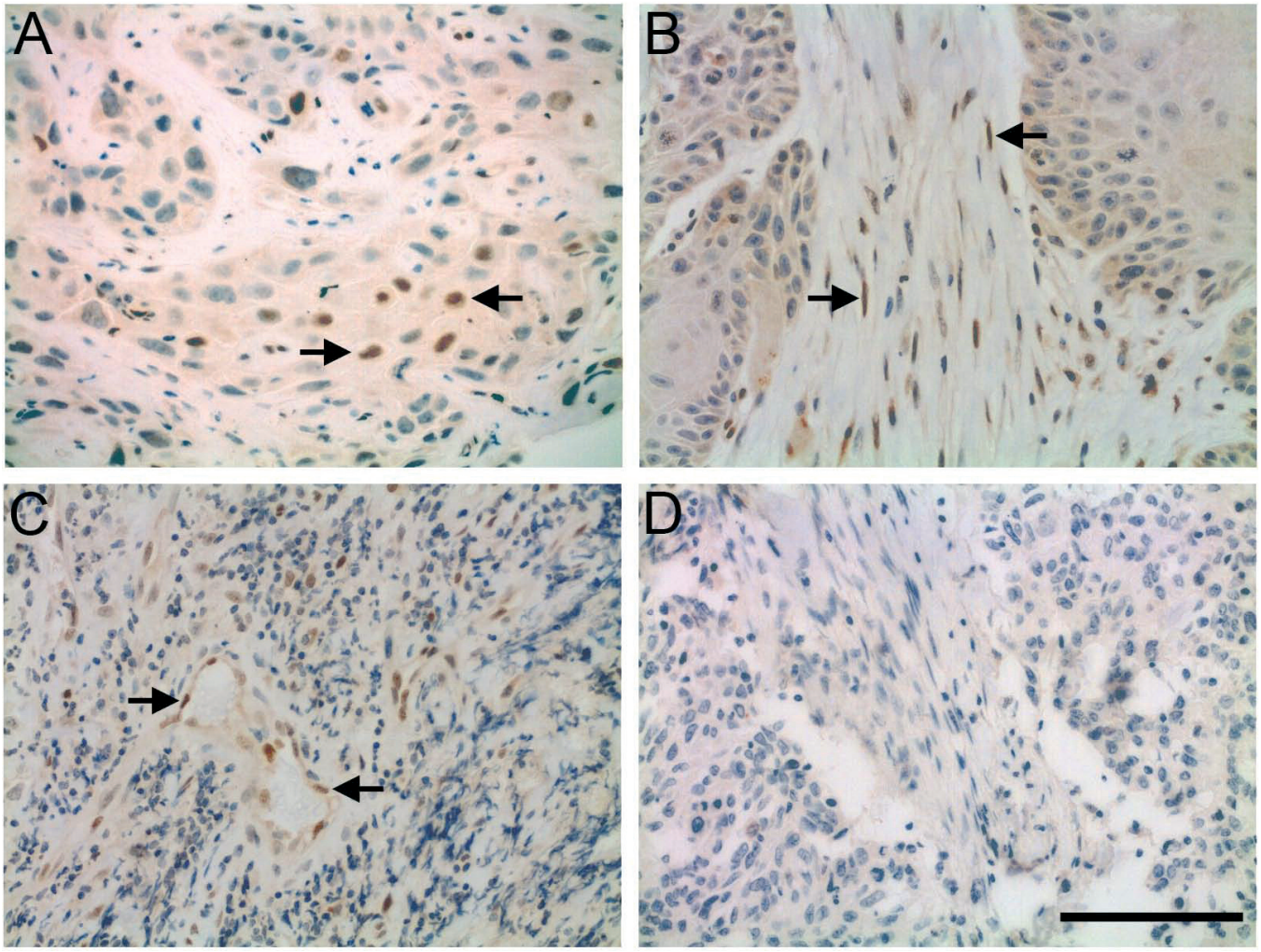

Fig. 1. Expression of snail1 nuclear transcription factor in pharyngeal squamous cell carcinoma. Positive immunostaining in the malignant epithelial cells **(A)**, stromal myofibroblasts **(B)** and in the vascular endothelium **(C).** Lack of expression is also illustrated **(D)**. Scale bar: 100 μm.

2008; Franci et al., 2009; Tuhkanen et al., 2009; Yanagawa et al., 2009). Snail1 expression has also been observed in stromal and endothelial cells in various malignant tumours (Franci et al., 2006, 2009; Rosivatz et al., 2006; Blechschmidt et al., 2007, 2008; Zidar et al., 2008; Franz et al., 2009; Tuhkanen et al., 2009).

In the present study snail1 expression was detected in a subset of endothelial cells and it was significantly associated with poor DSS in PSCC patients. This is the first study to show that endothelial cell staining is related to poor survival as well as to increasing T category, advanced stage, high grade, hypopharyngeal tumour location and low Karnofsky performance status score. Previously endothelial snail1 expression has been reported in several types of cancer and in non-malignant tumours (Rosivatz et al., 2006; Zidar et al., 2008; Franci et al., 2009; Franz et al., 2009; Rowe et al., 2009). Hypoxic activation of snail1 may take place in large poorly vascularised PSCC tumours (Peinado et al., 2007). Staining in or close to necrosis and inflammatory cell areas could reflect anti-apoptotic functions of snail1 (Barrallo-Gimeno and Nieto, 2005) or inflammation induced factors possibly stabilize the protein (Wu et al., 2009). Proinflammatory mediators are shown to upregulate snail1 and thus inflammation is supposed to promote EMT and tumour progression (St John et al., 2009). Snail1 expression in endothelial cells may indicate that the protein takes part in endothelial-mesenchymal transition (EndMT) which has been shown to be an important source of cancer-associated fibroblasts (Zeisberg et al., 2007). EndMT has been suggested to play a role in angiogenesis (Potenta et al., 2008) potentially mediated by snail1 (Kokudo et al., 2008).

Stromal expression of snail1 in epithelial tumours has recently gained interest. In colon carcinomas it correlates with poor survival (Franci et al., 2009). In the present PSCC cohort association was seen, although not statistically significant, between snail1 expression in stromal myofibroblasts and poor DSS. Stromal immunoreactivity was related to hypopharyngeal tumours with increasing T category and patients with lower Karnofsky performance status score. Our findings show that snail1 is expressed in the stromal myofibroblasts of locally advanced PSCC tumours where it may modify the tumour microenvironment, facilitating further cancer progression. Indeed, snail1 has been suggested to play a crucial role in mesenchymal cells by stimulating invasion and angiogenesis (Rowe et al., 2009). Stromal myofibroblasts expressing snail1 might also partly represent epithelial tumour cells that have undergone EMT (Franci et al., 2006). Furthermore, interaction between epithelial cells and stromal myofibroblasts during tumourigenesis may activate myofibroblasts to express snail1 (Franz et al., 2009).

Malignant epithelial cell nuclear expression of snail1

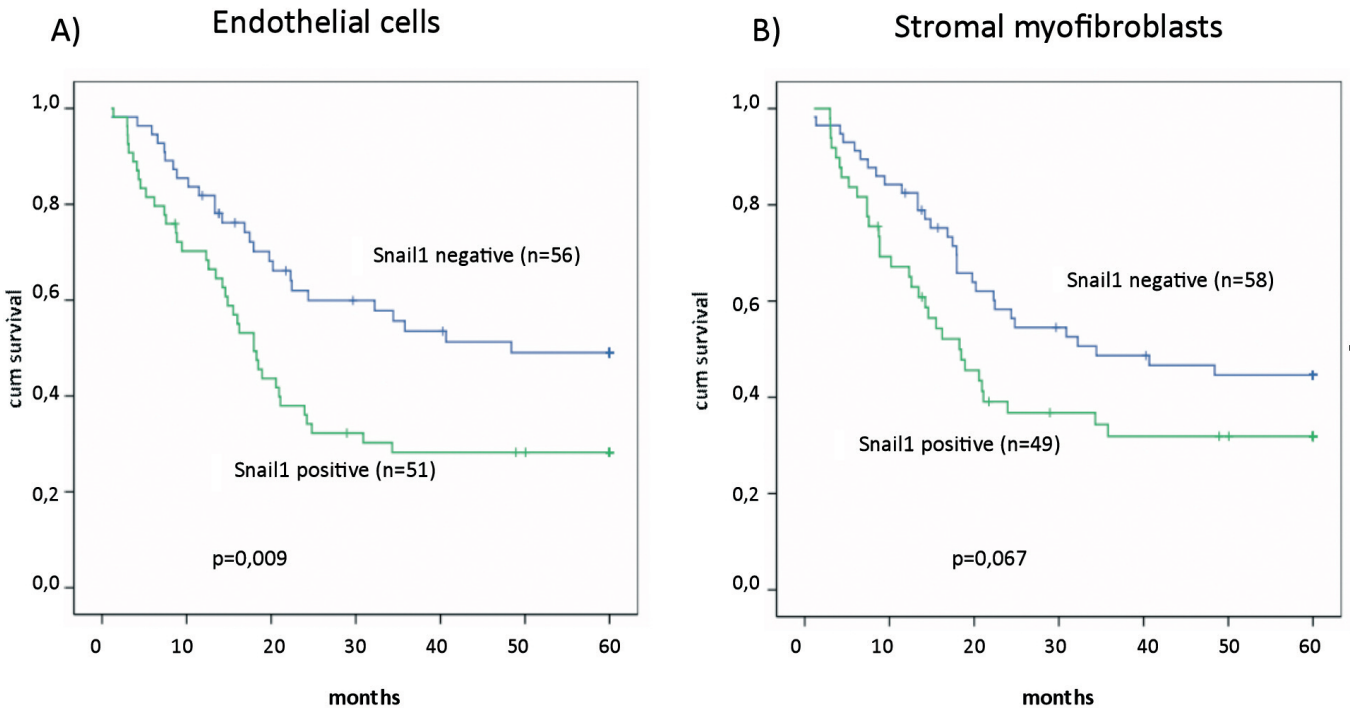

Fig. 2. Kaplan-Meier univariate 5-year disease specific survival analysis in pharyngeal squamous cell carcinoma. **A)** Snail1 expression in endothelial cells correlated significantly with poor survival. **B)** Snail1 expression in stromal myofibroblasts showed a trend with poor DSS.

protein has not previously been found to relate with survival in carcinomas (Rosivatz et al., 2006; Blechschmidt et al., 2007; Peinado et al., 2008; Zidar et al., 2008; Franz et al., 2009), except in a very recent study, where malignant epithelial cell snail1 overexpression significantly decreased survival for patients in lung adenocarcinoma but not in lung SCC (Yanagawa et al., 2009). Thus, our PSSC results are in line with previous findings. In our study snail1 was not an independent prognostic factor in multivariate analysis. The clinical variables seem to be more significant to predict the patients' outcome. An association with malignant epithelial cell snail1 expression and hypopharyngeal tumours, as well as low Karnofsky performance, was established. Expression of snail1 in malignant epithelial cells probably occurs already in early phases of tumour development. In our previous study we examined snail1 protein expression during ovarian tumour development from precursor lesions into carcinomas (Tuhkanen et al., 2009). In benign tumours no epithelial or stromal staining was observed, in contrast to borderline tumours and especially in carcinomas supporting a role for snail1 in early tumourigenesis. Cytoplasmic staining was seen in only 4 samples of the current material. However, the meaning of cytoplasmic reactivity is indefinite and only nuclear snail1 is considered to be active and stable (Zhou et al., 2004).

In conclusion, snail1 protein expression in the tumour microenvironment of PSCC seems to be related with poor disease specific survival and may indicate tissue remodelling, angiogenesis and EndMT, in addition to EMT leading to cancer progression.

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