Expression pattern of squamous cell carcinoma antigen in oesophageal dysplasia and squamous cell carcinoma

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Summary. The aim of the present study was to evaluate the tissue expression of squamous cell carcinoma antigen (SCCA) in oesophageal dysplasia and squamous cell carcinoma (SCC) with reference to its clinico-pathologic and prognostic significance. Immuno-histochemistry using SCCA polyclonal antibody was performed on SCCs from 61 surgical oesophagectomies. Fifteen cases of low-grade dysplasia (LGD) and 37 non-coexistent high-grade dysplasia (HGD) were also sampled from these materials, together with sixteen chronic cases of oesophagitis. SCCA immunoreactivity was present in the maturative compartments of all normal epithelia and oesophagitis. LGDs showed no SCCA immunoreactivity in the dysplastic proliferative component but only in the superficial normal layers. In 94.6% of HGDs, no SCCA immunoreactivity was detected throughout the thickness of the epithelium. In SCCs, SCCA expression higher than 25% was found in 54% of cases. SCCA positivity showed an inverse correlation with histological grade, whereas no statistically significant correlation was found with TNM classifications, stage, or survival. SCCA is not expressed in early oesophageal carcinogenesis but, in SCC, it represents an indicator of histologic differentiation. In differentiated SCC, SCCA may represent a negative factor for cancer invasiveness, through inhibition of proteases.

Key words: Squamous cell carcinoma antigen, Oesophageal dysplasia, Immunohistochemistry

Introduction

Oesophageal squamous cell carcinoma (SCC) is a major cause of cancer-related mortality (Lara and Cuyas, 1995; Ries et al., 1998) and its mortality rates are often similar to incidence rates, due to the relatively late stage of diagnosis, aggressive behaviour, and paucity of effective treatment (Kohn and Liotta, 1995). The clinical course of oesophageal SCC and its response to chemotherapy and radiotherapy vary, and may be partly associated with its biological heterogeneity. Useful markers associated with biological aggressiveness are needed to predict the outcome of the disease. Invasive carcinoma evolves from low- to high-grade dysplasia (Shirakawa et al., 2000; Ohbu et al., 2001; Shibata and Matsubara, 2001), but little is known about the biological markers involved in the progression from dysplasia to invasive oesophageal SCC.

Squamous Cell Carcinoma Antigen (SCCA) is a tumour-associated protein, which was first isolated biochemically from SCC tissue of the uterine cervix (Kato and Torigoe, 1977). The level of serum SCCA has been used as a tumour marker for patients with gynecological (Maruo et al., 1985; Brioschi et al., 1991), head and neck (Lara and Cuyas, 1995; Molina et al., 1996; Wollenberg et al., 1996), lung (De Cos et al., 1994) and oesophageal SCCs (Matsuda et al., 1990; Shimada et al., 2003). Serum SCCA levels were also found to be elevated in subjects with skin pathologies, such as psoriasis and eczema (Duk et al., 1989; Numahara et al., 1989; Campbell and De'Ambrosis, 1990) and with lung pathologies, such as bronchitis and pneumonia (Molina et al., 1990; Kato, 1992).

SCCA belongs to the superfamily of high molecular weight serine proteinase inhibitors (serpins) (Suminami et al., 1991). Analysis by chromatofocusing shows the presence of two antigenic fractions: neutral (pI 6.3-6.6) and acidic (pI 5.9-6.2) (Kato et al., 1984). Molecular cloning reveals two genes: the more telomeric, called

SCCA1, and the more centromeric, SCCA2. Nucleotide and amino acid homology between the two forms is 98% and 92%, respectively. SCCA1 corresponds to the neutral fraction and SCCA2 to the acidic one. The two SCCAs inhibit different classes of proteinases: SCCA1 inhibits papain-like cysteine proteinases, such as cathepsins L, S, and K; SCCA2 is an inhibitor of chymotrypsin-like serine proteinases, such as cathepsin G and mast cell chymase (Schick et al., 1997, 1998).

The majority of studies concerning SCCA consider serum levels more than local expression detected by immunohistochemistry. In particular, expression of SCCA in the first stages of oesophageal squamous cell carcinogenesis has not been studied previously. The aim of the present study was to evaluate the expression of SCCA in oesophageal dysplasia and SCC by immunohistochemistry, also with reference to its clinico-pathologic and prognostic significance.

Materials and methods

Selection of cases

Sixty-one consecutive oesophagectomies (40 males, 21 females; age range: 50-78, mean age: 62.8 years) collected in a 5-year period in the Department of Pathology, University of Padova, formed the material for this study. Patients underwent radical oesophagectomy for SCC. No patient had received irradiation or chemotherapy prior to surgery.

All surgical specimens were fixed in 10% buffered formalin. Multiple samples were collected from both tumours and mucosa without macroscopic evidence of tumor, with the aim of identifying foci of chronic oesophagitis and low- (LGD) or high-grade (HGD) dysplasia. LGD was found in 15 cases and HGD in 37 other cases. Oesophagitis was found in 16 cases; coexistence with LGD and HGD was found in 3 and 6 cases, respectively. Tumours were classified using the International Union Against Cancer (UICC) TNM system; histotype and grading were assessed according to the World Health Organization criteria. For control purposes, samples of normal oesophageal mucosa were also examined. Histological classification of lesions and expression of SCCA were carried out by two of the authors (An.Pa., S.P.).

Immunohistochemistry

Five μm-thick sections, obtained from the paraffin blocks used for histological diagnosis, were subjected to immunohistochemistry with anti-SCCA. Sections were hydrated gradually through decreasing concentrations of ethanol and then washed in deionised H_2O. They were incubated in 15% hydrogen peroxide in 0.01 M phosphate buffered saline (PBS), pH 7, to remove endogenous peroxidase activity and enhance antibody penetration into the tissue. Antigen retrieval was performed by treating sections in sodium citrate buffer

10 mM, pH 6, in a microwave oven at 90°C for 20 min. Sections were incubated for 10 min in blocking serum (Super block UltraTech HRP, Scytek Laboratories) to eliminate non-specific binding, and then incubated for 45 min at room temperature with primary polyclonal antibody recognising both isoforms of SCCA (HEPA-Ab SCCA variant rabbit polyclonal antibody, XEPTAGEN Life Biotechnology, Italy), diluted 1:50 in Bovine Serum Albumin. Sections were then washed three times for 3 min in PBS, revealed with biotinylated goat anti-rabbit serum (UltraTech HRP) for 10 min and a horseradish peroxidase complex (Super Sensitive Ready-to-Use, Biogenex, Menarini) for 10 min. Sections were developed in 3-3'-diaminobenzidine (DAB) for 1 min and then washed in PBS, counterstained using haematoxylin for 15 sec, and mounted. Similarly processed sections from normal oesophageal mucosa were used as positive controls. As negative controls, the primary antibody was replaced by PBS in serial sections of each sample.

Assessment of immunohistochemical staining

SCCA immunohistochemical staining was quantified by two independent observers. In each case of SCC, the mean percentage of positive cells over total cancer cells was determined in 20 fields at 40x, in 2 sections representative of the tumour, and assigned to one of the following five categories: 0, no staining; 1, 0-25%; 2, 25-50%; 3, 50-75%; 4, >75%. Cases with weighted scores of 0 or 1 were defined as negative. In cases of oesophagitis and dysplasia, positive cells were topographically assessed within the thickness of the oesophageal squamous epithelium, distinguishing whether their location was restricted to the proliferative or maturative compartment, or extended to both.

Data analysis

Chi-square statistics were used to compare SCCA positivity with clinico-pathologic features. The Kaplan-Meier method was used for analysis of survival data. The significance of differences of survival plots was analysed by the log-rank test. Statistical calculations were performed using Statigraphic 4.0 software (STSC Inc., Rockville, MD, USA). P-values <0.05 were considered as statistically significant.

Results

SCCA staining was cytoplasmic. Immunoreactivity for SCCA was present in all samples of normal oesophageal mucosa and oesophagitis, in which it was located in the maturative compartment of the epithelium, although SCCA immunoreactivity in the most superficial layers of normal oesophageal mucosa was slighter. No cases of LGD showed immunoreactivity for SCCA in the dysplastic component, correspondent in the majority of cases to the basal proliferative compartment, but only
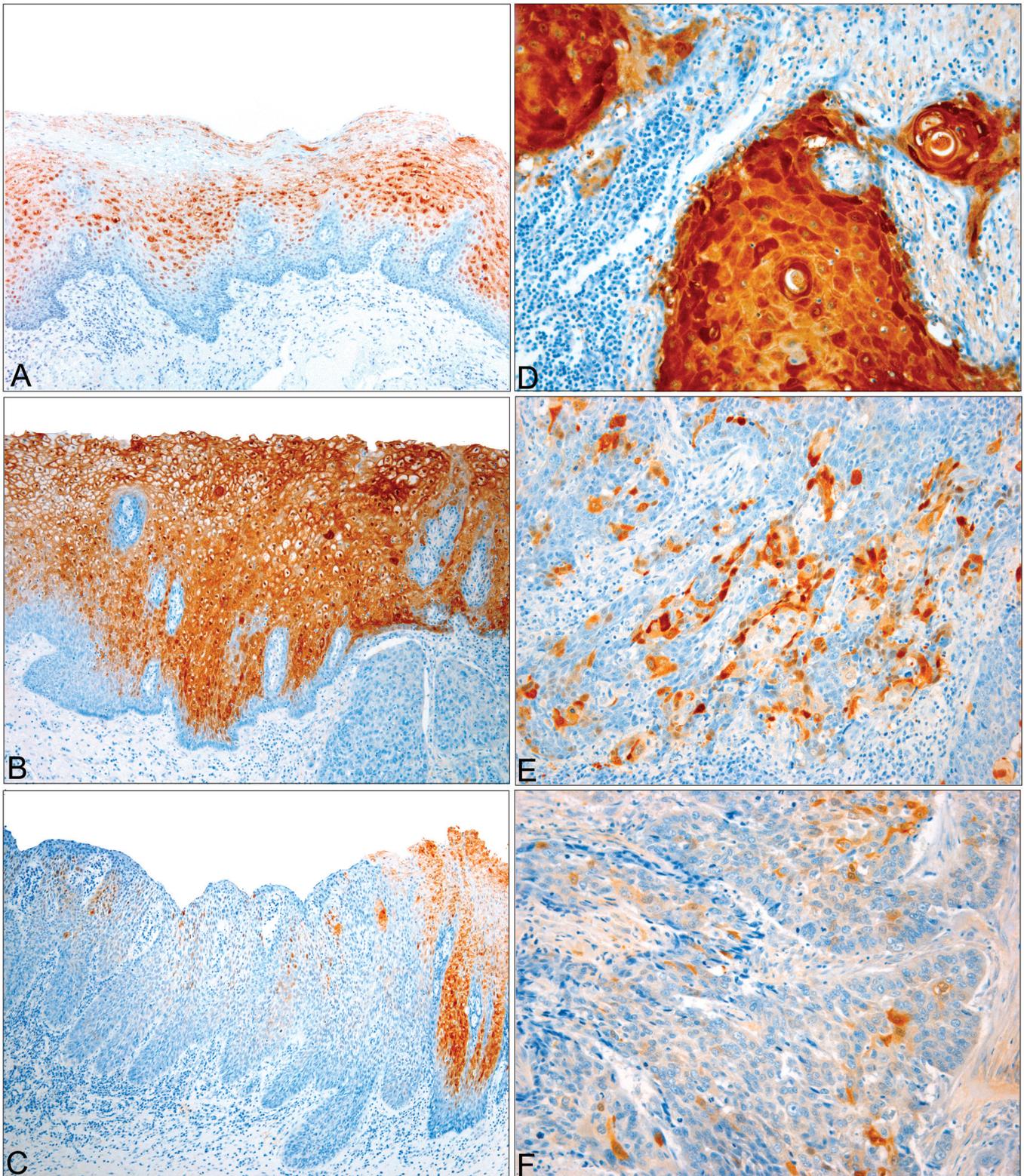
Fig. 1. A. Normal oesophageal epithelium, showing SCCA immunoreactivity in suprabasal layers. **B.** Low-grade dysplasia, with no SCCA positivity in the basal dysplastic component. **C.** High-grade dysplasia, showing no SCCA immunoreactivity throughout thickness of epithelium. **D-F.** SCCs showing higher SCCA expression in G1 **(D)** than in G2 **(E)** and G3 **(F)**.

in the superficial normal layers. The most superficial layers of the LGD showed higher SCCA immuno-reactivity than normal oesophageal mucosa (Fig. 1). Instead, 35/37 (94.6%) cases of HGD did not express SCCA throughout the epithelium thickness.

In oesophageal SCCs, SCCA positivity (i.e. >25% expression) was found in 33/61 cases (54%). The mean SCCA weighed score was 1.64.

Both the prevalence of SCCA positivity and the SCCA score showed inverse correlation with grading (P=0.001 and P=0.007, respectively, Chi-square statistics) (Table 1). The prevalence of SCCA positivity and the SCCA score did not correlate with tumour depth (pT) (P=0.10 and P=0.16), lymph node metastasis (pN) (P=0.60 and P=0.96), distant metastasis (pM) (P=0.44 and P=0.058) or stage (P=0.42 and P=0.26).

Seven subjects died of non-cancer-related causes. Thirty-eight subjects died of cancer, with a range of survival of 7-56 months and a median of survival of 23 months. Sixteen subjects were still alive, with a follow-up range of 60 months. Subjects with SCCA scores of 0-1 (n: 26; median of survival: 50 months) did not show different survival with respect to subjects with scores of 2-4 (n: 28; median of survival: 32 months) (P>0.05) (Fig. 2).

Discussion

SCCA1 and SCCA2 are co-expressed in normal squamous epithelium of the tongue, tonsil, oesophagus, uterine cervix and vagina, Hassall's corpuscle and skin (Cataltepe et al., 2000). In these epithelia, SCCA immunoreactivity was observed in the suprabasal layer (Cataltepe et al., 2000). As regards the role of SCCA in normal epithelia, it has been suggested that SCCA may protect from bacterial and viral cysteine proteases (Suminami et al., 1998) and SCCA2 may protect the epithelial barrier from mast cell chymase (Schick et al.,
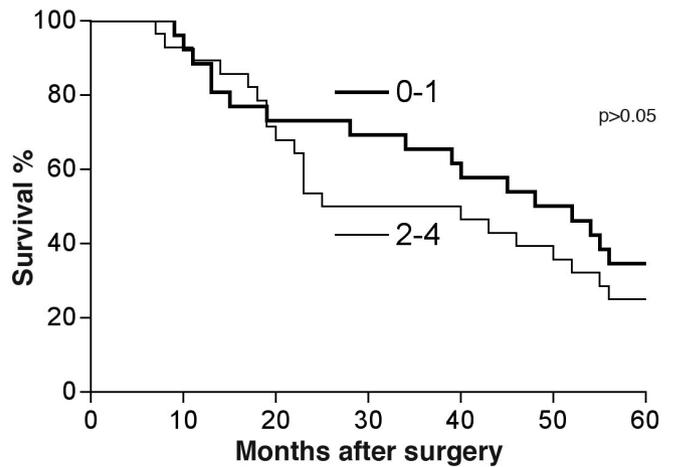

Fig. 2. Kaplan-Meier plot of SCCA scores (0-1 vs 2-4) in relation to overall survival period.

Table 1. Correlations between expression of SCCA and clinico-pathological factors in oesophageal carcinoma.

Factors	Cases	Mean SCCA expression score (% positivity)	P-value
Histological grade			
G1	14	2.57 (92.9)	
G2	34	1.53 (50.0)	
G3	13	0.92 (23.1)	0.007 (0.001)
Size (T)			
1	13	1.23 (30.8)	
2	15	1.53 (46.7)	
3	31	1.74 (64.5)	
4	2	3.5 (100)	0.16 (0.10)
Lymph node metastasis			
Negative	34	1.56 (50.0)	
Positive	27	1.74 (59.3)	0.96 (0.60)
Metastasis			
Negative	54	1.52 (51.9)	
Positive	7	2.57 (71.4)	0.058 (0.44)
Stage			
I	12	1.25 (33.3)	
IIA	21	1.62 (57.1)	
IIB	5	1.40 (40.0)	
III	16	1.63 (62.5)	
IV	7	2.57 (71.4)	0.26 (0.42)
Stage			
Early (Stage I)	12	1.25 (33.3)	
Advanced (Stages II + III + IV)	49	1.73 (59.2)	0.37 (0.20)

1997). Moreover, SCCA expression in the spinous and granular layers of squamous epithelia is retained to prevent typical cellular apoptosis of the cornified layer (Suminami et al., 1998). Our findings confirm the expression of SCCA in the suprabasal layers of oesophageal squamous epithelium, with slight immunoreactivity in the most superficial layers.

Balance between proteinases and inhibitors can affect tumour cell motility, invasiveness, proliferation and death. The specific biological role of SCCA on cancer cells is still unclear. In general, protease inhibitors are negative factors for cancer invasion and metastasis. It has been suggested that the inhibition of cathepsins by SCCA may reduce the invasive potential of head and neck SCC (Nakashima et al., 2006), in that cathepsin L is highly expressed in these cancer cells and is involved in their invasive potential (Kos et al., 1995; Strojan et al., 2000). It has been demonstrated that transcriptional activation of SCCA inhibits the invasiveness of SiHa cervical cancer cells, whereas antisense SCCA transfection enhances their invasive behaviour (Iwasaki et al., 2004). However, many authors have proposed distinct effects of cytosol SCCA and secreted SCCA on cell invasiveness (Sueoka et al., 2005). For instance, it has been reported that extracellular SCCAs can promote cell invasion in squamous carcinoma cells by inhibiting the infiltration of NK cells (Suminami et al., 2001a) and stimulating the production of pro-matrix metalloproteinases 9 (MMP-9) (Sueoka et al., 2005). The property of stimulating MMP-9 production requires the structure of the SCCAs to be intact. Recent non-denaturing polyacrylamide gel electrophoretic analysis can distinguish intact forms from cleaved ones and allows the different expression pattern of functional SCCAs to be studied (Nawata et al., 2006). It has been demonstrated that both SCCA1 and SCCA2 prevent apoptosis, inhibiting caspase-3 activity and upstream proteases (Suminami et al., 2000, 2001a), and recent studies have also shown that SCCAs play a role in the prevention of TNFα-induced cell death by inhibiting release of mitochondrial cytochrome c (Hashimoto et al., 2005). SCCA1 can also promote tumour growth (Suminami et al., 2000, 2001a). Moreover, it has been shown that an elevated SCCA2/SCCA1 mRNA expression ratio indicates a more aggressive squamous cell cancer, suggesting the positive and negative regulatory role played by SCCA2 and SCCA1 in cancer cells, respectively (Hamada et al., 2001; Stenman et al., 2001). Both isoforms are also expressed in moderately and well-differentiated SCC of the lung and head and neck (Cataltepe et al., 2000). It has been found that SCCA2, corresponding to the acidic isoform, is expressed more than SCCA1 in cancer cells, with respect to normal squamous epithelium (Suminami et al., 1998), and is the main circulating isoform in neoplastic patients (Kato et al., 1984, 1987).

In the literature, this is the first study to investigate the pattern of SCCA expression in early oesophageal carcinogenesis. In oesophageal epithelium with LGD, SCCA was still detectable at the level of the superficial non-dysplastic layers but, with respect to normal oesophageal epithelium, the most superficial layer showed higher immunoreactivity, indicating extension of apoptosis inhibition as far as the epithelial surface. In both LGD and HGD, dysplastic cells did not express SCCA. In HGD, the absence of SCCA immunoreactivity throughout the epithelium was due to superficial spreading of the neoplastic clone. Dysplastic cells share the absence of SCCA immunoreactivity with the basal layer of normal epithelium, supporting the origin of the neoplastic clone from this layer.

In cervical SCC, immunohistochemical studies have demonstrated that SCCA expression is related to the grade of differentiation (Hoshina et al., 1986; Suehiro et al., 1986). As regards oesophageal SCC, the correlation found between SCCA immunoreactivity and histological grade is in accordance with the results of Matsuda et al. (1990) and confirms that SCCA is an indicator of differentiation of SCC of the oesophagus. The higher expression of SCCA in G1 SCC than in dysplastic cells may be due to the development of clones with characteristics of major differentiation. This finding may be also explained by the different involvement of the two isoforms of SCCA and of the intact or cleaved forms – the form overexpressed in highly differentiated SCC is probably different from that expressed in normal epithelia, as was also found in other forms of carcinogenesis, in terms of isoforms (Kato et al., 1984; Murakami et al., 2000; Hamada et al., 2001), splicing variants (Suminami et al., 2001b) or intact/cleaved forms (Nawata et al., 2006). We cannot exclude that the SCCA expressed in SCC may be mutated and non-functioning. If functioning, it may represent an initial negative factor for cancer invasiveness, through inhibition of proteases, which is then lost in less differentiated carcinomas.

Serum SCCA has been ascribed to direct release from tumour cells, with particular reference to the SCCA2 isoform (Crombach et al., 1989), but some authors have found that elevated serum SCCA1 concentrations in SCC of the tongue did not correlate with SCCA1 expression in cancer cells, but with expression in T-lymphocytes peripheral to the cancer cells (Yasumatsu et al., 2001). In oesophageal SCC, serum SCCA positivity rates and high serum SCCA concentrations have been found to correlate with tumour size, tumour depth, lymph node status and distant metastasis (Shimada et al., 2003). High serum SCCA concentration is also a significant prognostic factor (Shimada et al., 2003). In the literature, the serum SCCA concentration of patients with differentiated tumours was also higher than that of the poorly differentiated type, but the difference was not statistically significant (Shimada et al., 2003). In the study by Matsuda et al. (1990) and in our study, SCCA immunoreactivity correlated only with histological grade. This discrepancy between the prognostic importance of serum and tissue SCCA may be due to the above-mentioned distinct biological effect of intracellular and extracellular SCCA

on the invasive tumour phenotype. The different tissue expression pattern of the SCCAs, i.e., intact or cleaved forms, may also play a role.

In conclusion, immunohistochemical detection of SCCA gives different types of information with respect to serum SCCA. Our study demonstrates that SCCA is not expressed in early oesophageal carcinogenesis but in SCC represents an indicator of histologic differentiation. Further studies should also consider the different expression patterns of intact and cleaved SCCAs between normal and malignant oesophageal epithelial tissues.

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