

Squamous cell carcinoma antigen 1 and 2 expression in cultured normal peripheral blood mononuclear cells and in vulvar squamous cell carcinoma

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Abstract Squamous cell carcinoma antigen (SCCA) is expressed in normal squamous cell epithelia and in squamous cell carcinomas (SCC). Two nearly identical genes encode the inhibitory serpins SCCA1 (SERPINB3) and SCCA2 (SERPINB4). Serum levels of SCCA are elevated in patients with benign skin diseases and in patients with SCC. SCCA, used for the monitoring of SCC patients, presents no satisfactory diagnostic specificity. As we have shown previously, the reverse transcription polymerase chain reaction (RT-PCR)-based SCCA messenger RNA (mRNA) testing aimed at detecting disseminated cancer cells may be hampered by the false-positive results due to SCCA expression in activated peripheral blood mononuclear cells (PBMC). The aim of this study was to

assess the expression of SCCA at mRNA and protein levels in cultured normal PBMC, compared to that in vulvar SCC (VSCC) samples. High SCCA concentrations were found in vulvar tumours and in metastatic lymph nodes, while negative inguinal lymph nodes from the same patients often presented significantly less SCCA. In normal activated PBMC, the level of SCCA protein was the lowest. At the mRNA level SCCA was detectable in normal PBMC even in cultures with no mitogen stimulation, but only by the nested RT-PCR, contrary to VSCC samples found to be SCCA positive already in one-step PCR. Both SCCA1 and SCCA2 transcripts were present in cultured PBMC; SCCA1 was expressed at a higher level than SCCA2. In conclusion, both SCCA forms are detectable in normal

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PBMC cultured in vitro. SCCA expression level in normal PBMC is much lower than in the squamous epithelium-derived cells. In VSCC, in addition to tumour itself, metastatic lymph nodes seem also to be a potential source of serum SCCA.

Keywords SCC · SERPINB3 · SERPINB4 · Tumour marker · PBMC · Vulvar carcinoma

Introduction

Squamous cell carcinoma antigen (SCCA), first discovered in the squamous cell carcinoma of the cervix [1], is expressed in different squamous cell carcinomas (SCC) and in normal squamous cell epithelia. SCCA belongs to the family of inhibitory serine protease inhibitors (serpins), involved in apoptosis, cell migration and invasiveness [2].

Elevated levels of serum SCCA proteins are common in patients with benign, inflammatory skin diseases, such as psoriasis and atopic dermatitis, as well as in patients with squamous cell carcinomas, especially of the cervix, head and neck, lung, oesophagus and vulva, where SCCA concentrations may correlate with clinical stage, histological grade and tumour burden [1, 3–7]. Serum SCCA has an independent prognostic value in patients with cervical carcinoma and with head and neck malignancies [6, 8] and is used for the diagnosis and monitoring of patients with SCC [9]. There are two SCCA proteins, encoded by two, nearly identical genes, *SCCA1* (SERPINB3) and *SCCA2* (SERPINB4). *SCCA1* and *SCCA2* proteins, although they present 92% amino acid sequence homology and target different proteases. The neutral *SCCA1* inhibits papaine-like lysosomal cysteine proteases, cathepsin S, L and K, and the acidic *SCCA2* inhibits the serine proteases, cathepsin G and mast cell chymase [10, 11].

Both SCCA proteins are colocalised in normal and malignant squamous epithelial cells, but the dominating serum form in patients with SCC is *SCCA2* [12–14]. Elevated *SCCA2*/*SCCA1* messenger RNA (mRNA) ratio in primary SCC of the head and neck and of cervix was found to be an independent predictor of tumour recurrence [15, 16]. SCCA is also regarded as a molecular tumour marker for disseminated SCC cell detection [17]. However, as we have previously shown while studying the question of false-positive reverse transcription polymerase chain reaction (RT-PCR) results of disseminated cancer cell detection [18], there is an expression of a number of the so-called tumour-specific molecular markers, including SCCA, in activated peripheral blood mononuclear cells (PBMC).

Here, we aimed to study the total SCCA as well as *SCCA1* and *SCCA2* expression at both the mRNA and protein levels, in normal unstimulated and PHA-stimulated

PBMC, and to compare the obtained total SCCA protein expression to that in vulvar squamous cell carcinoma (VSCC), one of many types of SCCs.

Material and methods

PBMC and cell lines

PBMC of six healthy donors were obtained by standard Ficoll-Paque (Pharmacia) gradient centrifugation; samples of uncultured cells were taken, and the remaining cells were cultured in a standard medium supplemented with 10% fetal calf serum (FCS) and gentamycin, for 1 day without mitogen, and in parallel, for 1–6 days in the presence of PHA (1 µg/ml). Cultures were centrifuged, supernatants were collected and frozen, cells were washed twice in PBS and the cell pellet was kept frozen at –70°C until RNA was isolated.

Squamous epithelial cell lines, the epidermoid vulvar carcinoma cell line, A431 (Deutsche Sammlung von Zellkulturen und Mikroorganismen, Braunschweig, Germany), the human cervical carcinoma cell line, SKG-IIIa (RINKEN BRC, National Bio-Resource Project of the MEXT, Japan), the supraglottic larynx carcinoma cell line, UT-SCC-8 (The University of Turku, Finland), and the human keratinocyte cell line, HaCat [19], as well as the cervix adenocarcinoma cell line, HeLa (Warsaw Medical University, Warsaw Poland), were cultured at 37°C, 5% CO₂ in the standard media supplemented with 10% FCS and gentamycin.

Post-surgical samples from vulvar carcinoma patients

We studied the samples obtained from surgical specimens of 41 patients treated in the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology for VSCC (pT1-2, N0-2, M0). All patients gave their informed consent. Samples including 39 tumours, 15 regional lymph nodes and 36 uninvolved adjacent tissues were snap-frozen and stored at –70°C until they were pulverised on liquid nitrogen, using a Microdismembrator II (B Braun Biotech International, Melsungen, Germany). The uninvolved tissues comprised cross-sections of skin next to the surgical margin. Histologically, they were composed of dermis and the epidermal layer.

RNA isolation and RT-PCR

Total RNA was isolated from PBMC at each time point (0, before culture; unstimulated 1-day cultures; and PHA-stimulated 1–6-day cultures) and from pulverised post-surgical samples from VSCC patients with the use of

Quiagen RNeasy kits. The quantity and quality of the RNA that was obtained were examined by spectrophotometry (Nanodrop, ThermoScientific) and by denaturing gel electrophoresis with ethidium bromide. Two micrograms of the total RNA was reverse transcribed with random hexamer primers, in the final volume of 20 μ l, using SuperScript reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The quality of complementary DNA preparations was controlled by the PCR of DNA polymerase β , as described previously [18]. Total SCCA, SCCA1 and SCCA2 expression was analysed in PBMC by the nested RT-PCR with specific primers. Tissue samples obtained from VSCC patients were examined for total SCCA expression by one-step RT-PCR.

Measurements of SCCA protein expression

Total cell protein was extracted from unstimulated and stimulated PBMC of four normal donors and from pulverised post-surgical samples of seven patients with vulvar cancer, according to the procedure described elsewhere [13].

Total SCCA expression in the protein extracts of PBMC and vulvar tumours, the concentrations of SCCA (total) in culture media and 22 patients' sera were measured using the Architect SCCA kit of Abbott, and SCCA in protein extracts were expressed in nanogram per milligram of cell protein (CP), while in the sera and culture media in nanogram per millilitre.

Immunocytochemical staining was performed using primary anti-SCCA1, anti-SCCA2 MAb (Santa Cruz, 8H11 and 10C12, respectively) and secondary anti-mouse IgG rabbit antibody Alexa 633 conjugated (Invitrogen). Nuclei were counterstained with chromomycin A₃ (Sigma), and the results were imaged with confocal microscope (Axiovert 100 M, Zeiss) and LSM 510 META application.

Immunohistochemical staining was performed with anti-SCCA1 (8H11), anti-SCCA2 (10C12) and anti-total SCCA (B-9) MAbs (Santa Cruz). Nuclei were counterstained with haematoxylin and eosin, and the results were imaged under a light microscope.

Results

The specific primers for total SCCA, SCCA1 and SCCA2 amplified the correct sequences, as confirmed by sequencing. As shown by the nested RT-PCR, total SCCA was expressed in mitogen-stimulated PBMC, where it persisted for all 6 days of culture, as well as in unstimulated PBMC following 1-day culture without mitogen (Fig. 1 and Table 1). SCCA was undetectable in the freshly isolated PBMC.

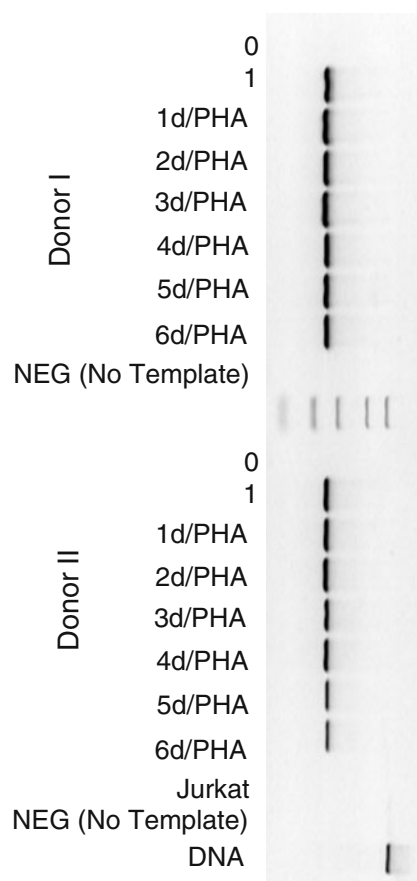


Fig. 1 Expression of total SCCA mRNA in PBMC from donor I (0—before culture, 1—unstimulated 1-day culture and PHA stimulated 1–6 day cultures)

The *SCCA1* and *SCCA2* genes usually co-expressed in PBMC, and this was also observed in cultures with no mitogen stimulation. In PBMC from donor I, the level of *SCCA1* expression was constant up to 3 days of culture, but in the subsequent 3 days, PBMC presented no *SCCA1* expression, in contrast to *SCCA2*, which was detectable throughout the whole culture period (Fig. 2 and Table 1). The level of *SCCA2* expression assessed semiquantitatively in 1-day cultures was found to be lower than that of *SCCA1*, in both stimulated and unstimulated PBMC of donor I (Fig. 3). The total SCCA was found to be consequently expressed in PBMC of all donors throughout the culture, while the *SCCA1* and *SCCA2* gene expression, although found to be expressed in all donors, was identified in different time points in different donors (Table 1).

The SCCA mRNA expression in post-surgical samples from patients with VSCC was also examined. Already in one-step PCR, all 39 tumour samples were SCCA positive, while the squamous cell vulvar carcinoma cell line A431 was SCCA negative (A431 was positive in two-step PCR). The SCCA transcripts were also detected in 31 out of 36 uninvolved adjacent tissues (86%).

Table 1 Total SCCA, SCCA1 and SCCA2 expression, assessed by the nested RT-PCR

Donor	Total SCCA										SCCA1						SCCA2									
	I	II	II	II	III	III	IV	V	VI		I	II	II	III	IV	V	VI	I	II	II	III	III	IV	V	VI	
Normal	0	-	-	-	n.d.	-	-	n.d.	-	n.d.	-	-	-	-	n.d.	-	-	-	-	-	-	-	-	n.d.	-	-
PBMC	1 d	+	+	+	n.d.	+	+	n.d.	n.d.	n.d.	+	-	-	+	n.d.	n.d.	n.d.	+	+	-	+	-	n.d.	n.d.	n.d.	
Days of culture	1 d/PHA	+	+	+	n.d.	+	+	n.d.	+	+	+	-	-	-	n.d.	+	+	+	-	-	-	+	n.d.	+	+	
	2 d/PHA	+	+	+	n.d.	+	+	n.d.	n.d.	n.d.	+	+	-	-	n.d.	n.d.	n.d.	+/-	-	-	-	-	n.d.	n.d.	n.d.	
	3 d/PHA	+	+	+	n.d.	+	+	n.d.	n.d.	n.d.	+	-	+	-	n.d.	n.d.	n.d.	+/-	-	-	-	-	n.d.	n.d.	n.d.	
	4 d/PHA	+	+	+	n.d.	+	+	n.d.	n.d.	n.d.	-	-	-	-	n.d.	n.d.	n.d.	+/-	-	-	-	-	n.d.	n.d.	n.d.	
	5 d/PHA	+	+	-	+	-	+	n.d.	n.d.	n.d.	-	-	-	-	n.d.	n.d.	n.d.	+/-	-	-	-	-	n.d.	n.d.	n.d.	
	6 d/PHA	+	+	+	n.d.	+	-	+	n.d.	n.d.	-	-	-	-	+	n.d.	n.d.	+	-	-	-	-	-	n.d.	n.d.	

In donors II and III repeated assessments were performed

(+) present, (-) absent, (+/-) trace expression, *n.d.* not determined

The SCCA concentration in the protein extract of activated PBMC was found to reach 2.7 ng/mg of CP (mean, 2.15 ng/mg; Table 2). In the post-surgical samples from VSCC patients, SCCA concentrations were two to four orders of magnitude higher than those in cultured PBMC. The SCCA protein concentrations were usually higher in the tumour than in the uninvolved adjacent tissue (Table 2). Metastatic inguinal lymph nodes expressed higher levels of SCCA protein than uninvolved inguinal lymph nodes derived from the same patients. Interestingly,

in patients 12 and 30, the lymph nodes that had been classified histopathologically as uninvolved (Table 2) presented relatively high SCCA levels. This might suggest that these lymph nodes did contain micrometastases.

Immunocytochemical analysis under confocal microscope has shown traces of SCCA 1 and 2 proteins in cultured PBMC (Fig. 4). As shown in donor III, the SCCA1 and 2 expression at the protein level was detected in PBMC later after stimulation than at the mRNA level (Table 3).

The immunohistochemical analysis of tumour and inguinal lymph node from a patient with VSCC confirmed the evidently stronger SCCA staining in the metastatic lymph node than in the SCC sample (Fig. 5) and showed that not only cancer cells express SCCA protein (Table 4).

SCCA secretion was examined in cell cultures. SCCA was undetectable in the spent media from PBMC cultures, as well as from HaCat keratinocyte cell line (<0.3 ng/ml), while it was secreted in relatively high amounts by the squamous cervical cancer cell line, SKG-IIIa (8 ng/ml), known for high SCCA expression level.

The median SCCA level in the sera of 22 patients with early VSCC was 1.35 ng/ml (7.4–0.6). Only four (18%) of

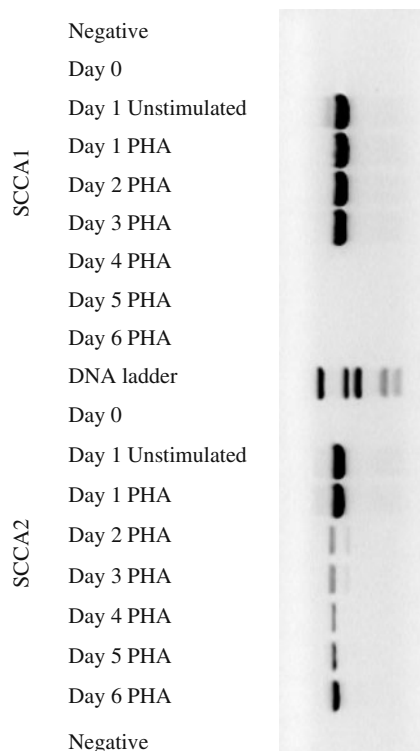


Fig. 2 SCCA1 and SCCA2 mRNA expression kinetics in stimulated PBMC from donor I

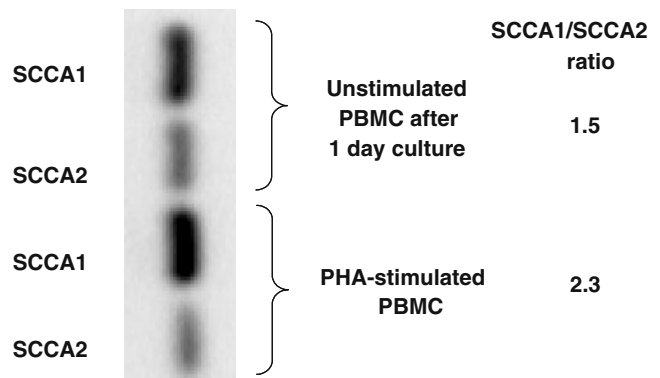


Fig. 3 Semiquantitative assessment of SCCA1/SCCA2 expression ratio in PBMC

Table 2 SCCA concentrations (Abbott IMX SCCA assay) in the protein extracts of normal unstimulated (day 0) and stimulated (day 3) peripheral blood mononuclear cells (PBMC) and of tissue samples as well as in the sera from patients with VSCC

	Donor number				Patient number							
	I	II	III	IV								
Healthy donor PBMC												
Unstimulated	0	0	0	0								
Stimulated	2.7	2.7	1.4	1.8								
Samples of VSCC					12	13	15	30	34	38	53	
Tumour					850	4890	19000	1850	6000	481	1600	
Adjacent uninvolved tissue					2920	290	2000	946	1650	194	818	
Metastatic lymph node					x	x	35300	810	3600	609	579	
Uninvolved lymph node					0.3 ^a	0 ^a	24	600	274	2.5	0	
					740	0.7						
						0						
Serum					n.d.	0.9	n.d.	n.d.	5.6	1.8	2	

Concentrations of SCCA in the cells and post-surgical samples are presented in nanogram per milligram of cell protein and in the sera in nanogram per millilitre of serum

x no metastatic nodes found, *n.d.* not determined

^a More than one lymph node of this patient was tested

those patients had elevated SCCA levels, and three had SCCA at the cut-off level (2 ng/ml).

Discussion

We were the first to show the SCCA expression to be inducible in normal PBMC [18]. Here, we studied the total SCCA expression along with the expression of its acidic and neutral forms in more detail. We confirmed our earlier results [18] and those of Catatlepe [12], who studied different normal tissues, that SCCA mRNA is not expressed

in uncultured normal PBMC. However, as we showed here, normal, activated PBMCs express SCCA1 and SCCA2 at both the mRNA and protein level. The two SCCA forms are often identified in different time points following stimulation, both in protein and mRNA studies. Interestingly, also unstimulated PBMCs following culture in the presence of FCS were found to express SCCA1 and SCCA2 transcripts. In addition, both in unstimulated (cultured for 1 day without mitogen) and in stimulated normal PBMC, the SCCA1 expression measured at the mRNA level was higher than the SCCA2 expression. Our semiquantitative assessment of the relative SCCA1 and SCCA2 expression revealed that

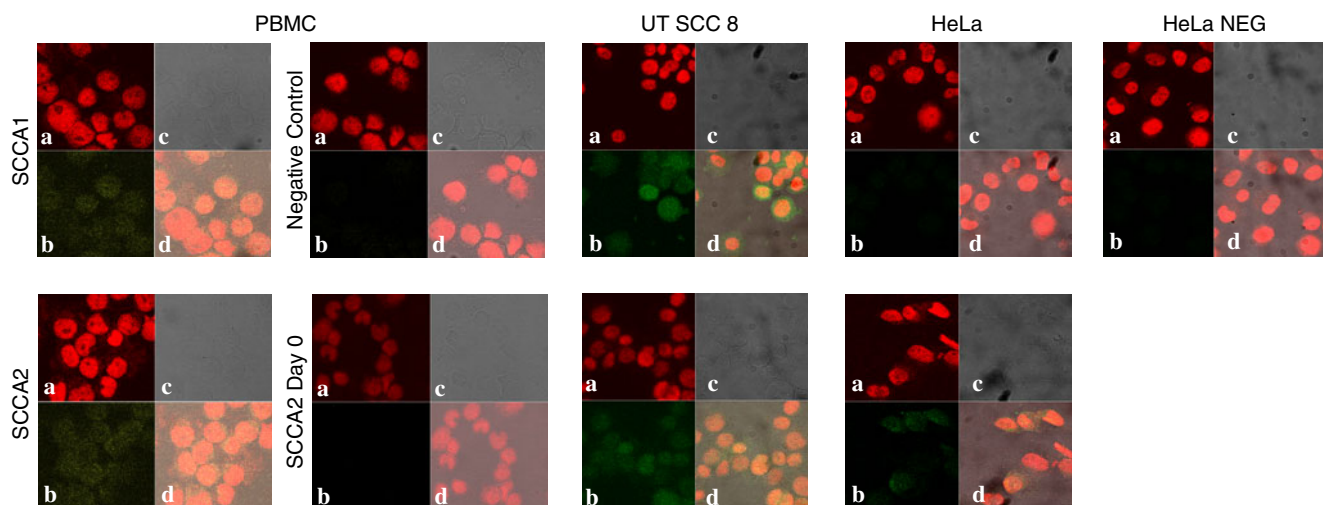


Fig. 4 SCCA1 and SCCA2 expression (*green*) in stimulated PBMC and UT-SCC-8 and HeLa cell lines. Confocal microscope images. **a** Chromomycin-stained nuclei (*red*), **b** SCCA 1 or SCCA 2 fluores-

cence, **c** transmitted light images, **d** an overlay of panels **b** on **a**, illustrating the cytoplasmic fluorescence

Table 3 A comparison of SCCA1 and SCCA2 expression in the peripheral blood mononuclear cells (PBMC)

	Donor III				
	Normal PBMC	SCCA1 mRNA	Anti-SCCA1	SCCA2 mRNA	Anti-SCCA2
	0	–	–	–	–
	1d	+	–	+	–
	1d/PHA	–	–	–	+
	2d/PHA	–	+/-	–	–
	3d/PHA	–	–	–	–
	4d/PHA	–	+	–	–
	5d/PHA	–	+/-	–	–
	6d/PHA	–	–	–	–

mRNA expression was evaluated by nested RT-PCR, protein expression was estimated by immunocytochemistry
 (+) present, (–) absent, (+/-) trace expression

the SCCA1/SCCA2 ratio increased 1.5 fold (1.54:2.38) upon PBMC stimulation. Similarly, squamous epithelium-derived cells, such as normal keratinocytes, normal non-keratinocyte cells, cervical squamous cell carcinoma and adenocarcinoma cells, have been shown to express higher mRNA levels of SCCA1 than of SCCA2 [20]. However, in squamous cell carcinomas of head and neck, Stenman et al. [15] observed a decrease in SCCA1/SCCA2 expression ratio in malignant squamous cells; that is, the SCCA1/SCCA2 mRNA ratios were lower in tumour tissue (median ratio <5) than in normal epithelium (median ratio >10). Those ratios were also lower in primary tumours that developed into recurrent disease and in recurrent secondary tumours than in non-recurring primary

tumours. Similarly, Hsu et al. [16] found that in the uterine, the SCCA1/SCCA2 mRNA ratios were progressively decreased in normal vs. dysplastic and dysplastic vs. cancer cells. In addition, the decreased SCCA1/SCCA2 ratio carried a higher risk for recurrence in early-stage uterine cervical cancer.

The published data on SCCA expression in normal human tissues are inconsistent. In the squamous cells of normal epithelia, some authors reported the SCCA immunoreactivity [21, 22], while others [23, 24] detected low or absent SCCA expression. The discrepancies might result from the different antibody specificities and tissue origins. Cataltepe et al. [12] have shown stronger SCCA1 and SCCA2 immunoreactivity in normal skin over areas of

Fig. 5 Expression of total SCCA and its forms (SCCA1 and SCCA2) in the paraffin sections of VSCC and metastatic lymph node sections. Immunohistochemical staining (Santa Cruz MAb)

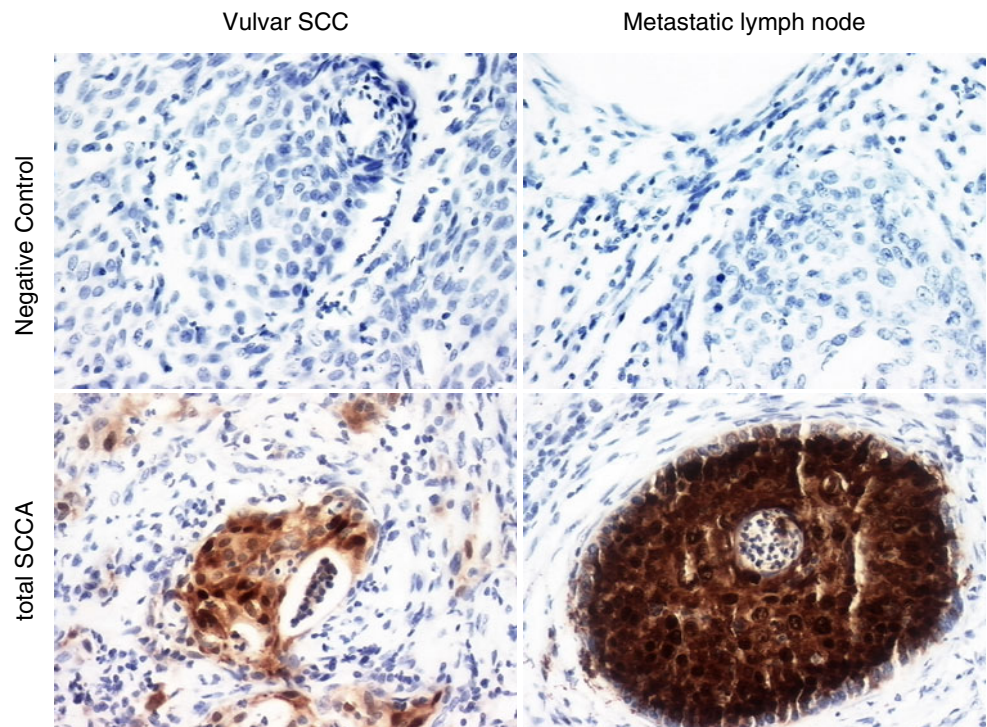


Table 4 Total SCCA, SCCA1 and SCCA2 expression according to cell types, identified by immunohistochemical examination of vulvar tumour and inguinal lymph node sections

	Cell types										
	Cancers		Granulocyte		Lymphocytes		Plasmocytes		Normal basal epithelial		
	T	N	T	N	T	N	T	N	T	N	
Total SCCA	+	+	–	–	–	–	–	–	–	–	0
SCCA1	–	–	+	0	–	–	–	0	–	–	0
SCCA2	+	+	+	0	–	–	–	0	+	–	0

T tumour, *N* inguinal lymph node, 0 no such cells identified, (+) present, (–) absent

inflammation. This observation indicates that the expression of SCCA in the skin can be induced by local inflammation. Indeed, SCCA has been recognised as a marker of inflammatory skin diseases [23, 25, 26].

Interestingly, in the squamous cell carcinomas of the tongue, Yasumatsu et al. [27] observed SCCA1 immunostaining in the T-lymphocytes peripheral to cancer cells and found this staining, but not SCCA1 expression in cancer cells, to significantly correlate with elevated serum SCCA concentrations. We showed here SCCA immunoreactivity in vulvar tumour-associated granulocytes. Thus, the source of serum SCCA may not necessarily be cancer cells but other tumour-associated cells, such as infiltrating immune cells. Elevated serum SCCA associated with several non-neoplastic dermatological diseases [23, 25] as well as with renal failure or lung diseases [28] support the possible role of different non-neoplastic cells in SCCA release. Furthermore, Van der Sijde et al. [29], who found serum SCCA assessment a useful tool for the early detection of recurrent VSCC, also observed 25% of false-positive (i.e. in patients with no cancer) SCCA results in patients with benign skin disorders. In addition, elevated concentrations of serum SCCA and/or C-reactive protein were reported to be independent unfavourable prognostic factors in head and neck squamous cell carcinomas [30], which links SCCA with systemic symptoms of cancer-related inflammation. Taken together, these data suggest that SCCA is not only a marker of squamous cell carcinomas but also of inflammatory conditions, and although high sensitivity of serum SCCA determination in cancer patients is generally appreciated, at the same time its limited specificity is evident.

Here, we also examined SCCA expression in VSCC patients. The levels of SCCA protein and mRNA induced in normal PBMC were several orders of magnitude lower than those found in the vulvar tumour cells and in the cells of tumour-adjacent tissues and lymph nodes. However, high levels of SCCA in the local tumour microenvironment and regional metastatic sites in patients with VSCC seem not to imply elevated serum SCCA levels, as few of those patients had serum SCCA concentrations above the normal level. In patients with early stage VSCC, consistently with our data, other researchers have shown mean

serum SCCA level to be below the cut-off value [4]. Serum SCCA levels were found not to be associated with lymph node involvement [4, 29, 31]. In advanced VSCC (FIGO stage IV), less than 40% of patients present elevated serum SCCA concentrations [4, 29].

The origin of serum SCCA in patients with SCC remains an open question. Uemura et al. [13] have shown the lack of SCCA secretion by the SCC cells, but other studies contradicted this result [32, 33]. Our analysis of SCCA concentrations in the spent media of stimulated PBMC, and of the squamous epithelia-derived cells of the HaCat and SKG-IIIa cervical cancer cell lines, showed SCCA in the culture media of SKG-IIIa cells but not of PBMC or HaCat cells. Thus, SCCA identified in body fluids may either be secreted by the cells and/or may derive from dying cells. In addition, SCCA expression and secretion is inducible by the Th2 cytokines, IL-13 and IL-4 in normal squamous epithelial cells [26, 33–35]. The Th1 to Th2 shift is regarded as typical of cancer; still, only SCC patients present elevated serum SCCA concentrations. Therefore, we hypothesise that an increase in the serum SCCA levels in patients with squamous cell carcinomas may be associated with SCCA induction by cytokines in normal squamous epithelial cells present in SCC microenvironment.

In summary, normal, activated PBMCs express SCCA1 and SCCA2 at both the mRNA and protein level, but the two forms of SCCA are not necessarily expressed concurrently. We could not demonstrate that cancer-related inflammatory cells are potentially a significant source of circulating SCCA in VSCC. In vulvar SCC patients, not only tumours but also metastatic lymph nodes are potential sources of SCCA protein. We think that the raised levels of serum SCCA observed by some researchers in the advanced stages of vulvar carcinoma [36] may result from secondary changes related to the disease progression, such as alterations induced by metastatic cancer cells in their microenvironment and/or an increase in proinflammatory cytokines. These changes create a feedback between local and systemic inflammation, a phenomenon that we have discussed elsewhere [37]. The exact mechanisms of releasing of SCCA into the body fluids remain to be resolved.

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