



ORIGINAL ARTICLE

Squamous cell carcinoma antigen 1 is associated to poor prognosis in esophageal cancer through immune surveillance impairment and reduced chemosensitivity

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Squamous cell carcinoma antigen-1 (SCCA1) overexpression is associated with poor prognosis and chemoresistance in several tumor types, however, the underlying mechanisms remain elusive. Here, we report SCCA1 in relation to the immune and peritumoral adipose tissue microenvironment in early and advanced esophageal adenocarcinoma (EAC). In our series of patients with EAC, free SCCA1 serum levels were associated with significantly worse overall survival, and SCCA1-IgM serum levels showed a trend to a worse overall survival. Serum SCCA1 and intratumoral SCCA1 were inversely correlated with immune activation markers. In agreement with these findings, SCCA1 induced the expression of the immune checkpoint molecule programmed death ligand-1 on monocytes and a direct correlation of these 2 molecules was observed in sequential tumor sections. Furthermore, SCCA1 mRNA expression within the tumor was inversely correlated with stem cell marker expression both within the tumor and in the peritumoral adipose tissue. In vitro, in EAC cell lines treated with different chemotherapeutic drugs, cell viability was significantly modified by SCCA1 presence, as cells overexpressing SCCA1 were significantly more resistant to cell death. In conclusion, poor prognosis in EAC overexpressing SCCA1 is due to reduced tumor chemosensitivity as well as intratumoral immunity impairment, likely induced by this molecule.

KEYWORDS

chemosensitivity, esophageal adenocarcinoma, immune surveillance, prognosis, SCCA1

1 | INTRODUCTION

Esophageal adenocarcinoma (EAC) is a rare tumor, but its frequency is rapidly increasing in developed countries.¹ This type of cancer is

one of the most fatal, with 5-year survival rates ranging from 15% to 39%.² Additionally, its pathogenesis is associated with reflux disease and Barrett's esophagus onset.³ Multimodal treatment that includes a combination of chemotherapy, radiotherapy, and surgery is warranted

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to obtain the best results in terms of survival.⁴ Recent advances in therapeutic approaches for gastroesophageal tumours have significantly improved the curative resection rates, and both the disease-free and the overall survival.⁵⁻⁷ Nevertheless, no biomarkers are yet available to reliably predict tumor chemosensitivity. Recent findings indicate that squamous cell carcinoma antigen-1 (SCCA1) is upregulated during Barrett's carcinogenesis and predicts EAC resistance to neoadjuvant chemotherapy.⁸ Squamous cell carcinoma antigen-1 is a serine protease inhibitor that belongs to the clade B subset of serpins and includes the 2 isoforms SCCA1-1 (also known as SerpinB3) and SCCA1-2 (also known as SerpinB4).⁹⁻¹¹ The first described isoform, SCCA1-1, was initially found to be significantly overexpressed in carcinomas with squamous differentiation, but recent evidence also extends to adenocarcinoma of the lung, breast, and pancreas, as well as hepatocellular carcinoma.¹²⁻¹⁴ The SCCA1-1 isoform confers to cancer cells resistance to induced apoptosis by different mechanisms, including inhibition of lysosomal cathepsin proteases, the JNK pathway, or p38 activation.⁹ More recently, the role of SCCA1-1 has been reported in inhibiting reactive oxygen species generation and cell death through its inhibitory interaction with respiratory complex I.¹⁵ In addition, these and other studies have highlighted a significant association between SCCA1-1 overexpression and poor prognosis, also associated with chemoresistance in different tumor types.^{9,14-17}

Cancer immune surveillance mechanisms might significantly influence the prognosis of EAC, as it does in the case of colorectal cancer.¹⁸ Several studies have indicated that in colorectal cancer the impairment of immune surveillance mechanisms plays a crucial role in cancer recurrence.^{19,20} Moreover, peritumoral adipose tissue plays a role in lymph node involvement in EAC.²¹ Adipose tissue-derived mesenchymal stem cells can differentiate into cancer-associated fibroblasts under the influence of specific tumor-derived factors²² and cumulative evidence supports the hypothesis that cancer-associated adipose tissue represents a key component in carcinogenesis.²³ On this basis, several studies investigated the relationship between the expression of specific stemness genes in tumor cells and their potential association to chemotherapy resistance.

In EAC, SCCA1 expression has never been investigated in relation to immune surveillance profile and to the peritumoral adipose tissue microenvironment. Therefore, the aim of our study was to investigate the potential mechanisms of SCCA1 expression leading to poor prognosis and chemoresistance in early and locally advanced EAC.

2 | MATERIALS AND METHODS

2.1 | Study design

Cancer tissue, peritumoral adipose tissue, and blood samples were collected at the time of surgery in 75 consecutive patients with EAC who underwent esophagectomy at the Esophageal and Digestive Tract Surgical Unit of the Veneto Institute of Oncology (Padua, Italy) and immediately frozen at -80°C for further analysis. This prospective study obtained the approval of the Ethical Committee of the

Veneto Institute of Oncology for the MICCE1 protocol. The study was carried out in accordance with the principles of the Declaration of Helsinki and all potential patients were asked to give written consent to have their data collected. Clinical characteristics of patients and tumor staging data are summarized in Table 1.

2.2 | Preoperative staging and neoadjuvant therapy protocols

Preoperative staging and neoadjuvant therapy protocols are described in Document S1.⁴

2.3 | Surgical resection procedures and tissue sample collection

Details concerning surgical techniques have been published elsewhere.²⁴ Briefly, esophagectomy was carried out using an Ivor Lewis procedure, by laparotomy or laparoscopy, and right thoracotomy for tumors of the mid-lower esophagus and the gastric cardia.²⁵ During surgical procedures, biopsies of periesophageal adipose tissue and of tumoral and nontumoral mucosa were collected for each patient. Fresh specimens were immediately frozen in liquid nitrogen, or fixed in formalin for subsequent analysis.

2.4 | Serological assays

2.4.1 | Free SCCA1 determination

The concentration of SCCA1 in serum was quantified by sandwich ELISA (Hepalisa kit) purchased from Xeptagen (Venice, Italy) following the manufacturer's instructions. Briefly, serum samples (100 μL) were incubated for 1 hour at room temperature on plates coated with rabbit anti-human-SCCA1 capture Ab (10 $\mu\text{g}/\text{mL}$ in PBS, pH 7.4). This Ab recognizes both SCCA1 and SCCA12 isoforms. Standard curve, obtained by dilution of recombinant SCCA1 from 20 to 0.25 ng/mL, was also included. All samples were tested in duplicate. After washing, SCCA1 was revealed by incubation with 100 μL HRP-conjugated streptavidin secondary anti-SCCA1 Ab (0.5 $\mu\text{g}/\text{mL}$). The plate was developed with a ready-to-use 3,3', 5,5'-tetramethylbenzidine (TMB) substrate solution. The reaction was stopped with 1 mol/L HCl (100 μL) and absorbance at 450 nm was measured on a microplate reader (Victor \times 3; Perkin Elmer, Waltham, MA, USA).

2.4.2 | Determination of SCCA1-IgM

Levels of SCCA1-IgM immune complexes were measured by HEPA-IC kit (Xeptagen), according to the manufacturer's instructions. Briefly, plates precoated with anti-human SCCA1 Ab were incubated with either serially diluted standards or serum samples in duplicate, and the presence of SCCA1-IgM complex was revealed by the addition of enzyme-conjugated anti-human IgM. The plates were then washed, and the substrate solution was incubated for 10-15 minutes. Subsequently, the plates were read on a microtiter plate

	Low SCCA1 (<10 ng/mL)	High SCCA1 (>10 ng/mL)	Whole group
No. of patients	55	20	75
Age, years	62 (53-70)	62.5 (56-73.5)	62 (54-70)
Male sex	49	18	67 (89.3)
Tumor site			
Lower esophagus	5	1	6 (8.0)
Esophagogastric junction	50	19	69 (92.0)
Neoadjuvant therapy	44	12	56 (74.7)
Response after neoadjuvant therapy			
Mandard 1	8	2	10 (17.9)
Mandard 2-3	13	5	18 (32.1)
Mandard 4-5	23	5	28 (50.0)
Tumor stage			
0	8	2	10 (13.3)
I	11	5	16 (21.3)
II	13	5	18 (24.0)
III	23	8	31 (41.4)
Nodal metastasis	32	7	39 (52.0)
Vascular invasion	26	9	35 (46.6)
Perineural invasion	26	9	35 (46.6)
Lymphomononuclear tumor infiltration	39	16	55 (73.3)

TABLE 1 Epidemiological and clinical characteristics of the patients included in the study, grouped according to level of squamous cell carcinoma antigen-1 (SCCA1)

Dichotomous data are expressed as n (%) and continuous data as median (interquartile range). No statistically significant difference was observed between the 2 groups.

reader at 450 nm. The amount of immune complex was expressed in Arbitrary Units/mL (AU/mL). The cut-off value of 156 AU/mL was calculated as the 95th percentile on the distribution curve of the assay in healthy subjects.²⁶ Forty-four healthy volunteers, used as controls, had their free SCCA1 and SCCA1-IgM serum levels quantified and compared to the corresponding EAC patients' serum levels.

2.5 | Flow cytometry analysis of esophageal mucosa

Tumor and corresponding normal mucosa tissue samples were mechanically dissected and passed through a sterile nylon filter (BD Falcon, Heidelberg, Germany). The single cell suspension was pelleted, suspended in FACS buffer (PBS/2% FCS/0.02% sodium azide) and stained with fluorochrome-conjugated Abs (Table S1). Staining was undertaken in flow cytometry buffer for 30 minutes at 4°C after 20 minutes of incubation with human Fc receptor binding inhibitor (eBioscience, San Diego, CA, USA). After two washes, samples results were acquired on a FACSCalibur based on CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). At least 10 000 cells were analyzed for each sample.

2.6 | RNA isolation and real-time quantitative PCR

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's

instructions. Complementary DNA synthesis was carried out using the Applied Biosystems (Foster City, CA, USA) cDNA synthesis kit according to the manufacturer's directions. Specific mRNA transcripts of stem cells markers (CD90, CD34, OCT-4, and NSTM), paracrine adipose tissue mediators (adiponectin and leptin), costimulatory molecules (CD80 and CD86), innate immunity markers (Toll-like receptor 4 [TLR4] and MyD88) as well as lymphocyte activation markers (CD38 and CD69) were quantified with SYBR Green PCR Master Mix in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the primers listed in Table S2. The expression of the target molecule was normalized to the expression of *Actb* housekeeping gene.

2.7 | Immunohistochemistry

The immunohistochemical (IHC) expression of SCCA1 (polyclonal, rabbit HEPA-Ab; Xeptagen) was carried out on the automated Leica Microsystems Bondmax (Leica, Wetzlar, Germany). Immunostaining was scored jointly by 2 pathologists (MF and MR). Both cytoplasmic and nuclear staining was retained for scoring. Immunostaining was semiquantified using 3-tier scoring based on intensity of staining (0, negative; 1, weak/moderate; and 2, strong). Antigen-presenting cells, cytotoxic lymphocytes, and checkpoint gene expression were quantified and localized by immunohistochemistry. Moreover, angiogenesis and lymphangiogenesis markers (CD31 and podoplanin)

within the peritumoral adipose tissue were also evaluated by IHC. The primary Abs, murine IgG1, specific for the CD80, CD4, CD8, programmed death ligand-1 (PD-L1), PD-L2, and CD107 (Table S1) were added and incubated for 30 minutes at room temperature. After several washes, the secondary Ab (horse Ig conjugated with biotin, directed against murine Igs) was added and incubated for further 30 minutes. The slides were washed in PBS with a final wash of 30 minutes with the avidin-biotin-peroxidase complex. The peroxidase of the detecting system reacted with 3'3'-diaminobenzidine that was added to the slides for 5 minutes. In order to quantify the number of positive cells, the percentage of cells stained by the avidin-biotin complex (ABC) system was counted in 10 random fields at $\times 60$ magnification.

2.8 | Effect of SCCA1 on immune checkpoint gene expression

Recombinant LPS-free SCCA1 was obtained in our laboratory as previously described²⁷ and used at 200 ng/mL on isolated human primary monocytes (peripheral mononuclear cells [PMCs]). Unless otherwise indicated, all other agents were of analytical grade and were purchased from Sigma (Saint Louis, MI, USA).

2.8.1 | Isolation and culture of human PMCs

Peripheral mononuclear cells from healthy donors were isolated by centrifugation on Ficoll-Paque solution at 500 rcf for 30 minutes. Mononuclear cells were harvested, resuspended in RPMI-1640 medium with 10% FCS and seeded (2×10^6 per well) with or without recombinant SCCA1-1 (200 nmol/L) in 12-well plates in RPMI-1640-10% FCS for 24 hours, then harvested for analysis.

2.8.2 | Flow cytometry

Primary PMCs, stimulated or not with recombinant SCCA1 (200 nmol/L), were harvested and stained with fluorochrome-conjugated Abs (Table S1) and isotype-matched, fluorochrome-labeled Abs as controls. Staining was carried out in PBS/2% FCS/0.02% sodium azide for 30 minutes at 4°C after 20 minutes of incubation with human Fc receptor binding inhibitor (eBioscience). After 2 washes, sample results were acquired on a FACSCalibur based on CellQuest software (Becton Dickinson). Monocytes were identified by CD14⁺ staining. At least 10 000 cells were analyzed for each sample. Results were expressed as mean of fluorescence intensities (MFI).

2.8.3 | External validation series

The validation series consisted of gene expression data from 75 samples from the University of Texas accessed from the Gene Expression Omnibus (GEO) databank (dataset ID: GSE13898).²⁸ According to the GEO entries, 75 frozen biopsy specimens of tumors and 28 paired surrounding nontumor esophageal tissues

endoscopically obtained before treatment from 64 EAC patients were selected from fresh-frozen tissue stored at the University of Texas for microarray experiments, and were hybridized by Illumina microarrays (San Diego, CA, USA). Our selected gene panel was tested on the downloaded dataset, and the correlation between SCCA1 and PD-L1 was tested with the nonparametric Spearman correlation test.

2.9 | Real-time proliferation and cytotoxicity assay of esophageal cell lines

Two different esophageal cell lines were used, preliminarily selected on the basis of the extent of SCCA1 expression. The cell line OE19 (kindly provided by Dr. S. Realdon) showed low SCCA1 expression at both transcription and protein levels. The cell line OE33 (kindly provided by Dr. S. Realdon) showed high levels of SCCA1 expression (Figure S1). In these cell lines, PD-L1 expression was measured as MFI by flow cytometry analysis. Moreover, OE19 cells were also transiently transfected with a plasmid vector (pcDNA3.1) carrying the gene for SCCA1 or the plasmid vector alone, as control, to have a cell line able to produce very high levels of SCCA1. The transfections were carried out with Lipofectamine 3000 (Life Technologies Europe, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Real-time cell proliferation was carried out on the xCELLigence DP instrument (ACEA, San Diego, CA, USA) as described in the supplier's instruction manual. Briefly, 30 000 cells/well were seeded on E-plates, then 16 and 24 hours after seeding the cells were exposed to cisplatin, 5-fluorouracil (5-FU), epirubicin, and docetaxel at 1 mmol/L concentration, or to medium alone. Proliferation of the cells was monitored every 15 minutes at 37°C in 5% CO₂ atmosphere up to 24 hours from chemotherapy treatment, using the incorporated sensor electrode arrays of the E-plates. The electrical impedance of each well was measured by the RTCA-integrated software (version 1.2; ACEA) of the xCELLigence system as a dimensionless parameter termed cell index (a quantitative measure of the cell number present in a well) to evaluate real-time proliferation and the cytotoxic effect of the 4 tested chemotherapies.

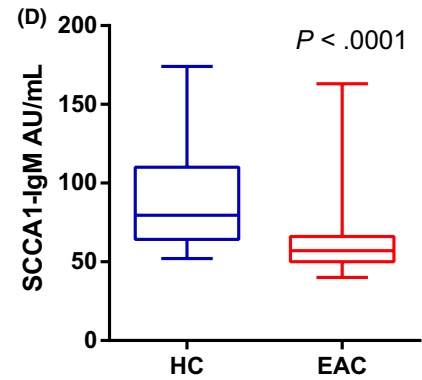
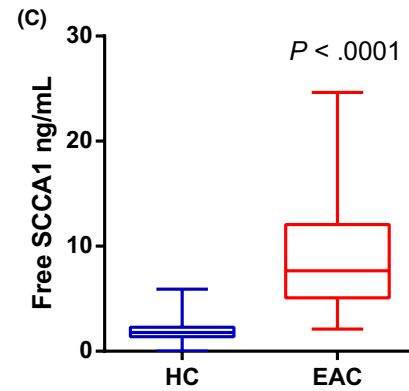
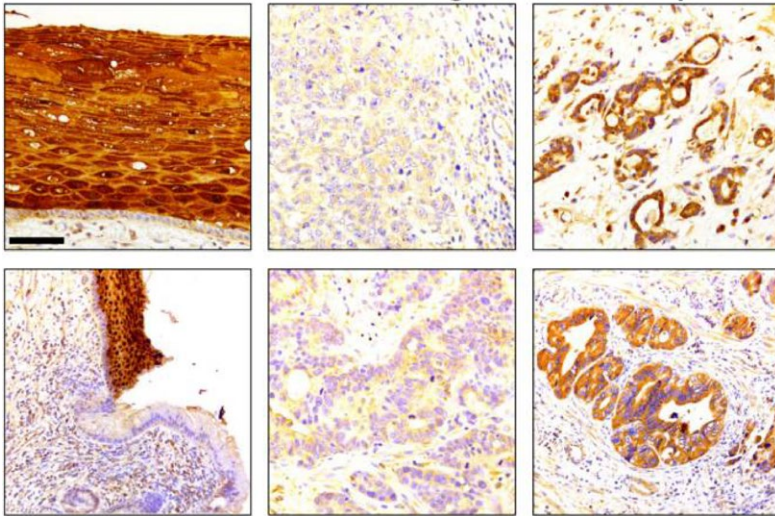
2.10 | Statistics

Data are presented as median with interquartile range or number of patients with percentage, where appropriate. The association between SCCA1 and dichotomous variables was evaluated using the Mann-Whitney U test. The association between SCCA1 and continuous variables was evaluated using Spearman's rank correlation. A Cox regression model was estimated to evaluate the effect of SCCA1 on overall survival and disease-free survival, adjusting for age, neoadjuvant therapy, and tumor stage. In the cytotoxicity analysis, differences between groups were evaluated using the paired t test, Mann-Whitney U test, and modified Kruskal-Wallis nonparametric test for trend, as appropriate. All calculations were obtained using the RTCA-integrated software of the xCELLigence system.

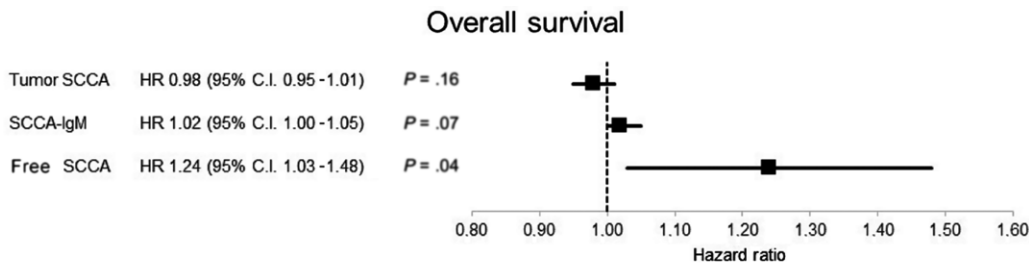
(A)

	Tumor SCCA (mRNA)	SCCA-IgM (AU/ml)	Free SCCA
n of patients	46	44	44
Average (St Dev)	26.6 (43.9)	62.3 (22.1)	9.1 (4.7)
Median (IQR)	4.4 (0.7-28.2)	56.8 (45.1-64.2)	7.6 (5.6-12.3)
Min-Max	0.002-150.7	39.5-163	3.6-24.6

(B) Normal SCCA neg SCCA pos



(E)



(F)

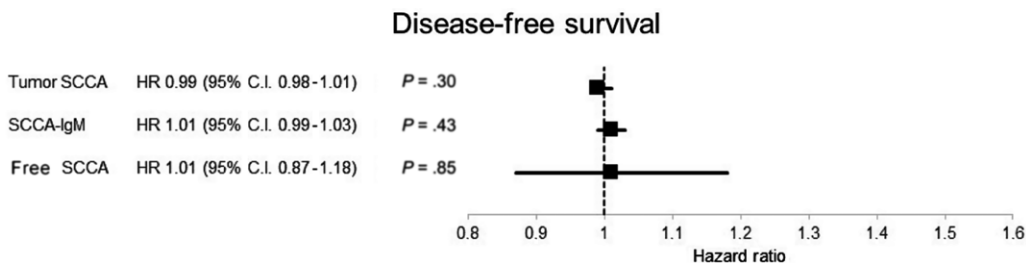


FIGURE 1 Squamous cell carcinoma antigen-1 (SCCA1) serum levels are associated with a worse overall survival after esophageal adenocarcinoma (EAC) surgery. A, Levels of tumor SCCA1, SCCA1-IgM, and free SCCA1 in EAC patients. B, Representative immunohistochemical stains showing moderate to strong cytoplasmic and nuclear SCCA1 expression of normal esophageal squamous mucosa, negative (neg) SCCA1 EAC, and positive (pos) SCCA1 EAC (original magnifications, $\times 10$ and $\times 40$). C,D, Levels of SCCA1-IgM and free SCCA1 in healthy controls (HC) and EAC patients. E,F, Forest plots showing a Cox regression analysis including tumor SCCA1, SCCA1-IgM, and free SCCA1 as possible predictors of overall (E) and disease-free (F) survival

The RTCA software undertook a curve-fitting of selected sigmoidal dose-response equations to the experimental data points and calculated the half maximum inhibitory concentration IC_{50} values at

a given time point. Data analysis was undertaken using R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria)²⁹ and Stata Statistical Software: Release 12 (StataCorp LP, College Station, TX,

USA). In the cytotoxicity analysis, differences between groups were tested by applying the (unpaired) *t* test, using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). *P* values < .05 were considered significant for all the above-mentioned analysis.

3 | RESULTS

3.1 | Levels of SCCA1 and patients' survival after EAC surgery

The extent of expression of SCCA1 within the tumor at transcription level is shown in Figure 1A. Figure 1B illustrates SCCA1 protein expression within tumors in a positive and a negative case. Free SCCA1 serum levels were significantly higher in EAC patients than in healthy controls (5.9 [0-2.11] ng/mL vs 7.67 [1.8-24.65] ng/mL, *P* < .0001) (Figure 1C). In contrast, SCCA1 IgM-conjugated form serum levels were significantly higher in healthy controls than in EAC patients (79.5 [52-174] AU/mL vs 57 [40-163] AU/mL, *P* < .0001) (Figure 1D). In the multivariable analysis, SCCA1 levels within the tumor and the form conjugated with IgM in serum were not associated with overall survival, although SCCA1-IgM serum levels tended to be associated to worse overall survival (hazard ratio = 1.02 [95% confidence interval (CI), 1.00-1.05], *P* = 0.07). In contrast, free SCCA1 serum levels were associated with a statistically significant worse overall survival (HR = 1.24 [95% CI, 1.03-1.48], *P* = .04) (Figure 1E). None of these parameters were correlated with disease-free survival (Figure 1F).

3.2 | Clinical parameters, SCCA1, and neoadjuvant therapy

As SCCA1 levels were inversely associated with patients' survival, we tested the possible associations between SCCA1 and age, neoadjuvant therapy, tumor stage, and N stage (Table S3). Age was negatively associated with SCCA1-IgM ($\rho = -0.29$, *P* = .05) and positively associated with free SCCA1 ($\rho = 0.28$, *P* = 0.06) levels (Figure 2A). Patients who had previously received neoadjuvant therapy had lower serum SCCA1 levels (*P* = .01) and slightly higher tumor SCCA1 levels (*P* = .07) (Figure 2B). Among the patients receiving neoadjuvant therapy, the most frequent components of the chemotherapy scheme were 5-FU (33 patients), cisplatin (30 patients), docetaxel (12 patients), and oxaliplatin (6 patients). Tumor SCCA1 was higher in patients receiving docetaxel (*P* = .003) (Figure 3A), whereas SCCA1-IgM and free SCCA1 levels in serum were not significantly different in patients receiving docetaxel (Table S3).

To investigate the possible modulation of cell viability by SCCA1 in the presence of different chemotherapeutic drugs, we compared the cell rate viability at 24 hours after treatment with cisplatin, 5-FU, epirubicin, and docetaxel in 2 cell lines with different SCCA1 expression, namely the OE33 cell line, with high SCCA1 expression, and the OE19 cell line, in which SCCA1 is barely expressed. The OE33 cells, overexpressing SCCA1, were significantly more resistant to cell death than the control OE19 cells after treatment with both epirubicin and docetaxel, whereas cell viability was not affected by treatment with 5-FU (Figure 3B). Analyzing the chemoresistance to docetaxel treatment of EAC cell lines with different SCCA1 expression levels, we found a

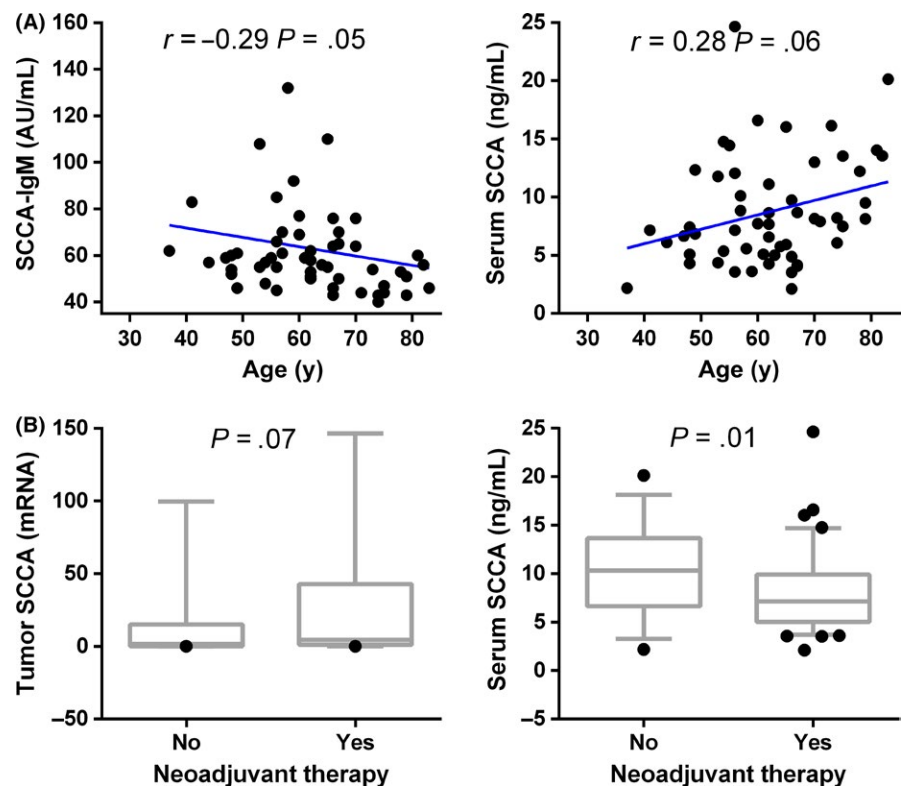


FIGURE 2 Squamous cell carcinoma antigen-1 (SCCA1), clinical parameters, and neoadjuvant therapy in patients with esophageal adenocarcinoma. A, Spearman rank correlation analysis of SCCA1-IgM and free SCCA1 with age. B, Association of tumor SCCA1 or free SCCA1 in serum with neoadjuvant therapy was evaluated by the Mann-Whitney U test

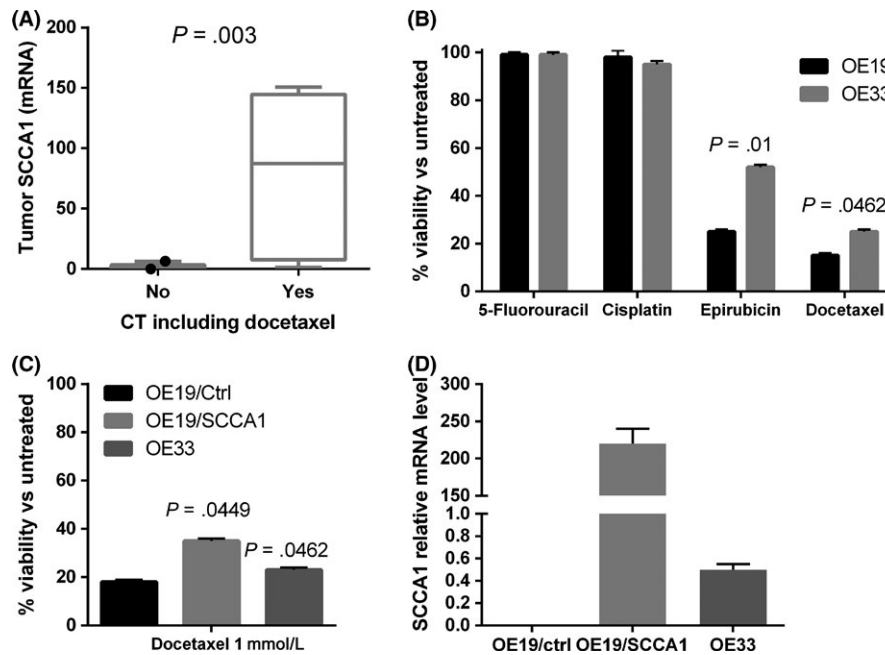


FIGURE 3 Squamous cell carcinoma antigen-1 (SCCA1) confers resistance to chemotherapy drugs in patients with esophageal adenocarcinoma. A, Association of tumor SCCA1 with neoadjuvant therapy including docetaxel was evaluated by Mann-Whitney U test. B, Real-time cell proliferation was undertaken on OE19 (low SCCA1 expression) and OE33 (high SCCA1 expression) cells exposed to 5-fluorouracil, cisplatin, epirubicin, or docetaxel at 1 mmol/L, or medium alone. Proliferation was monitored every 15 min at 37°C in 5% CO₂ atmosphere up to 24 h from chemotherapy treatment. Data shown are representative results of at least 3 independent experiments. All graphs depict mean \pm SEM. C, Real-time cell proliferation of OE19 transfected with the plasmid vector alone (OE19/Ctrl), OE19 transfected to overexpress SerpinB3 (OE19/SCCA1), and of OE33 exposed to docetaxel treatment at 1 mmol/L for 24 h. The resistance of the cells overexpressing SCCA1 to chemotherapy treatments was evaluated with unpaired t test (statistical significance calculated vs control cells). D, Basal levels of SCCA1 mRNA in the corresponding cell lines

significant association between chemoresistance and SCCA1 expression levels. Indeed, the OE19 cells, not expressing SCCA1, acquired the highest viability after docetaxel treatment when transfected to overexpress SCCA1 and they became even more resistant than OE33 cells (Figure 3C,D).

3.3 | Squamous cell carcinoma antigen-1 and immune microenvironment within the tumor

To verify the hypothesis of a direct role of SCCA1 in EAC patients' survival, we have investigated the possible cross-talk between SCCA1 and the tumor immune microenvironment. To address this goal, we have evaluated the following parameters: (i) the activity of epithelial cells as antigen-presenting cells (cytokeratin⁺/HLA-ABC⁺ and cytokeratin⁺/CD80⁺ cell rate within the tumor); (ii) the antigen-presenting cell activity (CD80 and CD86 mRNA expression within the tumor, CD80⁺ cell rate within the tumor at IHC); (iii) the innate immunity (TLR4 and MyD88 mRNA expression within the tumor); (iv) the activity of cytotoxic lymphocytes (CD8⁺ and CD107⁺ cell rate in the peritumoral lamina propria and intraepithelial and CD69 and CD38 mRNA within the tumor); and (v) the gatekeeper expression (PD-L1 and PD-L2 cell rate within the tumor) and in peritumoral lymphocyte infiltration (CD45⁺ cell rate within the adipose tissue surrounding EAC) (Table S4).

Tumor SCCA1 mRNA expression was inversely correlated to the MFI of CD80 expression on epithelial cells ($\rho = -0.68$, $P = .03$) (Figure 4A). Both SCCA1-IgM and free SCCA1 levels in serum were inversely correlated with immune activation markers. In particular, SCCA1-IgM serum levels were inversely correlated with CD80, CD86, and CD38 mRNA levels within the tumor (Figure 4B). In addition, SCCA1-IgM was significantly higher in patients with low peritumoral intraepithelial expression of CD107 (Figure 4C,D). Free SCCA1 serum levels were inversely correlated to TLR4 and CD38 mRNA levels within the tumor (Figure 4C) and they were significantly higher in patients with a low intraepithelial CD8⁺ lymphocyte rate (Figure 4C,D).

3.4 | Squamous cell carcinoma antigen-1 enhances the immune checkpoint genes expression

To verify the hypothesis of a possible direct role of SCCA1 in the depression of the tumor immune response, human recombinant SCCA1 was added to the medium of cultured mononuclear cells and immune subpopulations were analyzed for the expression of immune checkpoint molecules (PD-L1, PD-L2, CD80, and CD86 on monocytes, PD-1 on lymphocytes). A significant increase of the inhibitory molecule PD-L1, but not of PD-L2, was observed in CD14⁺ monocytes (Figure 5A), whereas the expression of costimulatory molecules

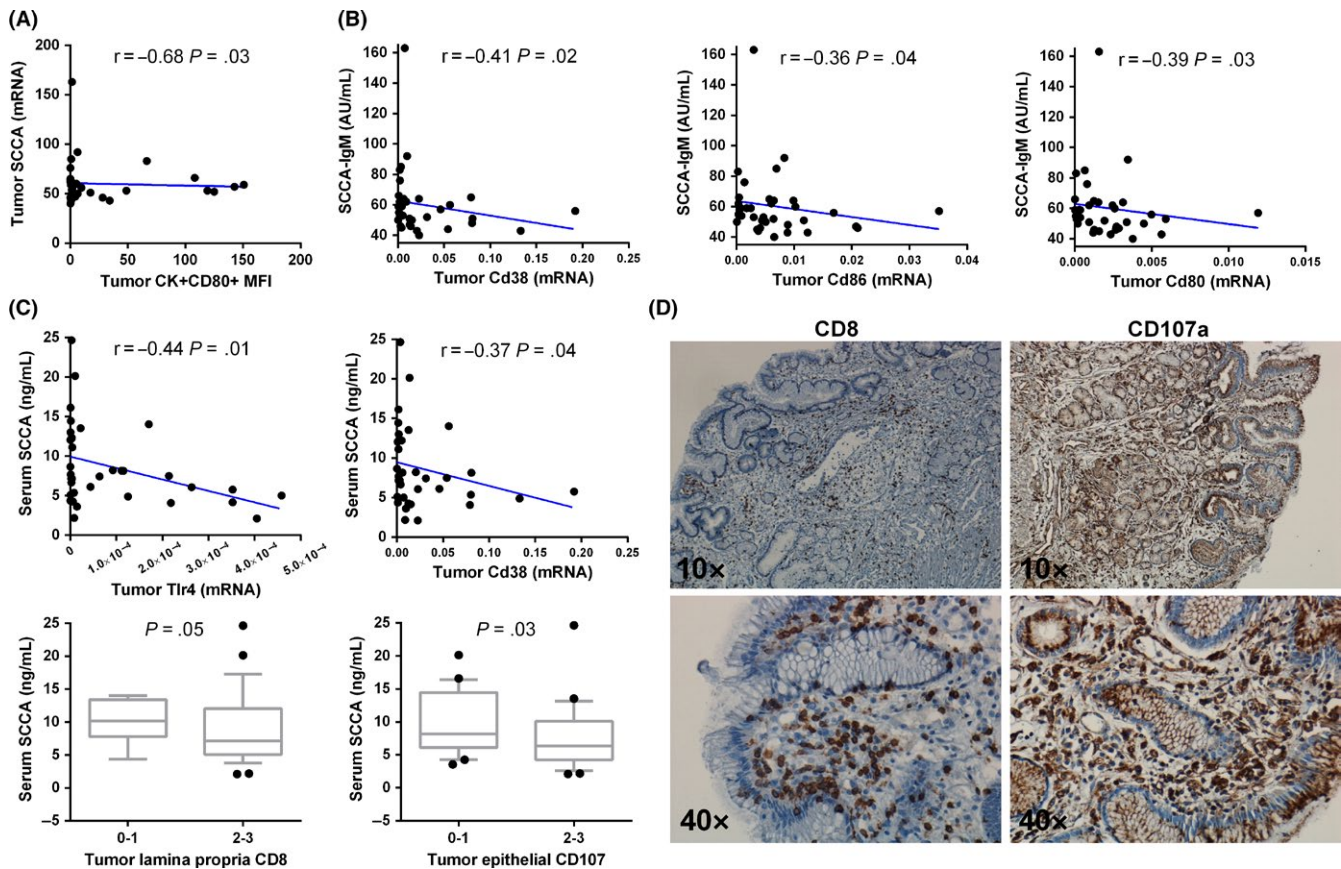


FIGURE 4 Squamous cell carcinoma antigen-1 (SCCA1) and immune microenvironment parameters in patients with esophageal adenocarcinoma. A, Spearman rank correlation analysis of tumor SCCA1 mRNA and epithelial CD80 mean fluorescence intensity. B, Spearman rank correlation analysis of tumor SCCA1-IgM and tumor Cd38, Cd86, or Cd80 mRNA levels. C, Association of free SCCA1 in serum with tumor Tlr4 or Cd38 mRNA levels was evaluated by Spearman rank correlation and with tumor lamina propria CD8⁺ cells or tumor CD107⁺ epithelial cells was evaluated by Mann-Whitney U test. D, Representative IHC stains of CD8 and CD107a on peritumoral esophageal mucosa (original magnifications, $\times 10$ and $\times 40$)

such as CD80 and CD86 was unaffected by the addition of SCCA1 (Figure 5B). No increase of PD-1 expression was observed in B or T cell subsets (Figure S2). The induction of PD-L1 by SCCA1 was corroborated by the direct correlation of the two molecules observed in serial paraffin sections of EAC obtained from resected patients, as shown in the examples of Figure 5C. Moreover, data from 2 cell lines, OE19 and OE33 expressing low and high SCCA1 levels, respectively, showed that the last ones significantly expressed more PD-L1 (Figure 5D). Finally, in the external validation cohort GSE13898, a significant direct correlation was observed between SCCA1 and PD-L1 mRNA levels within the tumor tissue (Figure 5E).

3.5 | Squamous cell carcinoma antigen-1 and peritumoral microenvironment

To investigate the association between SCCA1 and peritumoral microenvironment, we evaluated mRNA expression of stem cell markers (CD90, CD34, OCT-4, and NSTM) and paracrine adipose tissue mediators (adiponectin and leptin) in peritumoral adipose tissue. Moreover, angiogenesis and lymphangiogenesis markers (CD31 and podoplanin) within the peritumoral adipose tissue were also

evaluated by IHC. In peritumoral adipose tissue, SCCA1 was detectable in only 9 (20.9%) of the patients. It is interesting to note that the extent of SCCA1 expression within the tumor was directly associated with the levels of leptin in peritumoral adipose tissue (Figure 6A). In contrast, SCCA1 mRNA expression within the tumor was inversely correlated with CD34 mRNA levels both within the tumor and in the peritumoral adipose tissue (Figure 6B). The other variables analyzed were not associated with SCCA1 expression in any site (Table S5).

4 | DISCUSSION

Recent findings indicate that SCCA1 is upregulated during Barrett's carcinogenesis and predicts EAC resistance to neoadjuvant chemotherapy.⁸ In fact, several studies have highlighted a significant association between SCCA1 overexpression and poor cancer prognosis, also associated with chemoresistance in different tumor types.^{9,15-17} Squamous cell carcinoma antigen-1 confers to cancer cells resistance inhibiting apoptosis and reactive oxygen species generation and cell death.^{9,15} Moreover, cancer immune surveillance mechanisms might significantly influence the prognosis of EAC and in this cancer setting SCCA1

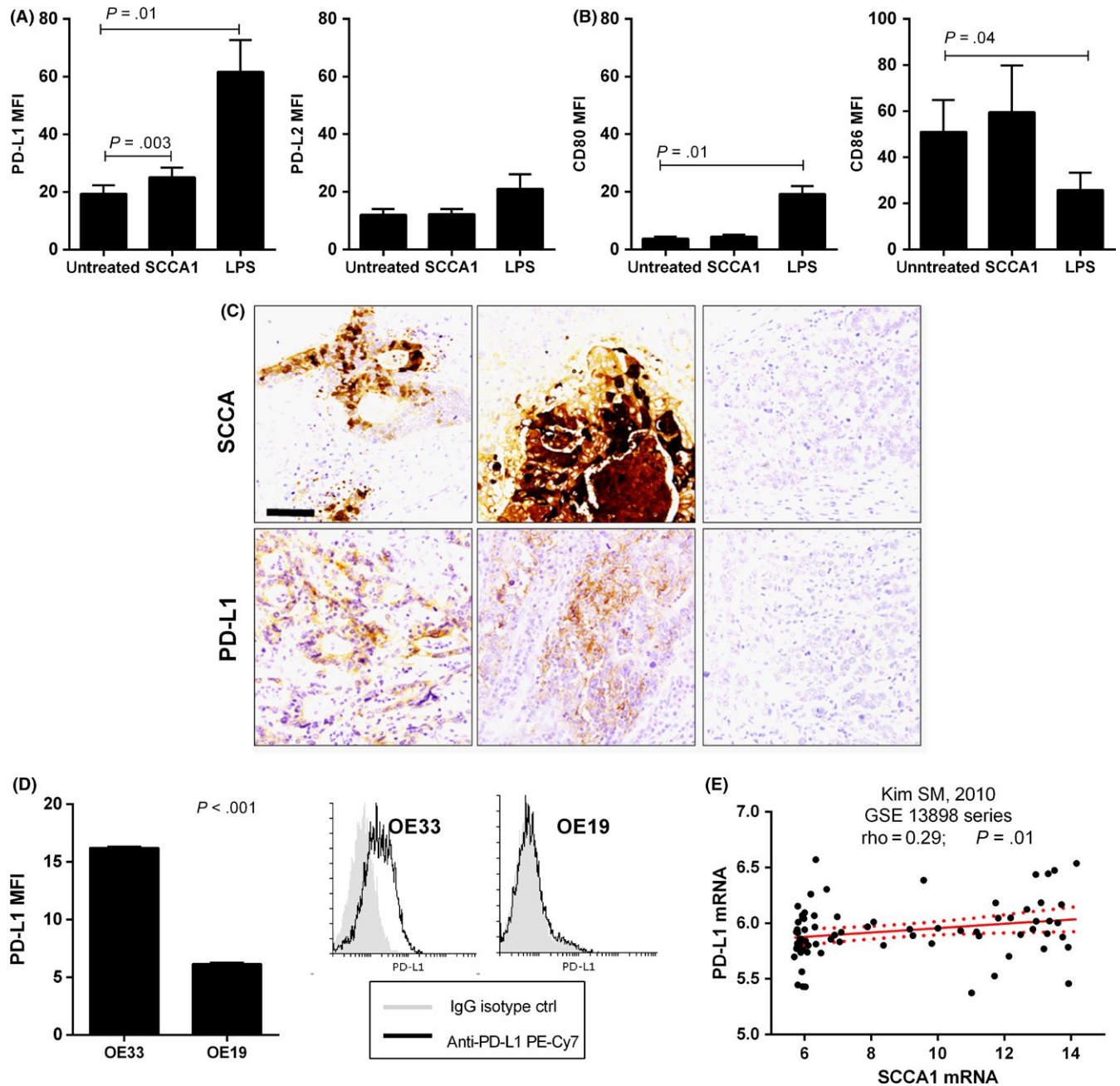


FIGURE 5 Squamous cell carcinoma antigen-1 (SCCA1) induces programmed cell death ligand-1 (PD-L1) expression in human monocytes. A,B, Expression of PD-L1 and PD-L2 suppressive molecules (A) and CD80 and CD86 costimulatory molecules (B) was analyzed by flow cytometry in monocytes, defined as CD14⁺ cells. Mean fluorescence intensities (MFI) were compared between monocytes treated or not with SCCA1 for 24 h. C, Representative immunohistochemical stains of SCCA1 and PD-L1 on consecutive slides of 3 different esophageal adenocarcinomas (original magnification, $\times 10$). D, Expression of PD-L1 was analyzed by flow cytometry and MFI was compared between OE33 and OE19 cells. Data shown are representative results of at least 3 independent experiments. All graphs depict mean \pm SEM. E, An independent study in the NCBI GEO database showed a significant correlation between SCCA1 and PD-L1 mRNA levels in esophageal adenocarcinoma tissues

expression has never been investigated in relation to immune surveillance profile and to the peritumoral adipose tissue microenvironment.

In our series of EAC patients, free SCCA1 serum levels were significantly higher and SCCA1-IgM serum levels were significantly lower than in healthy controls. Moreover, in EAC patients, SCCA1 high serum levels of free SCCA1 were associated with significantly

worse overall survival, and SCCA1-IgM serum levels showed a trend to a worse overall survival. These findings might reflect a decline of the natural immune surveillance, as IgM bound to SCCA1 is likely an expression of immune control, in line with the recent theory of cancer immunoediting.³⁰ The impairment of immunosurveillance, which can physiologically slightly increase with age,³¹ might

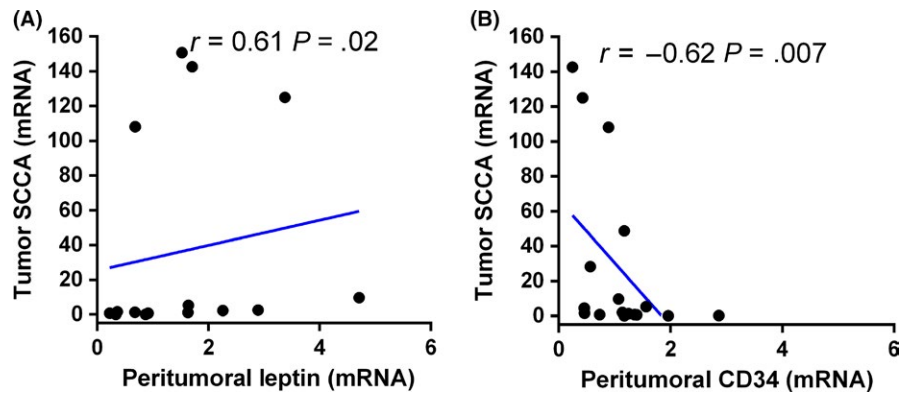


FIGURE 6 Squamous cell carcinoma antigen-1 (SCCA1) and peritumoral microenvironment parameters in patients with esophageal adenocarcinoma. A, Spearman rank correlation analysis of tumor SCCA1 mRNA and peritumoral Leptin mRNA levels. B, Spearman rank correlation analysis of tumor SCCA1 mRNA and peritumoral CD34 mRNA levels

explain the higher levels of free SCCA1 detected in serum of the patients with worse survival. In line with our results, positivity for SCCA1 identified a subgroup of patients with non-small-cell lung carcinoma who had poor survival after chemotherapy.³²

In our series, patients who had previously received neoadjuvant therapy had slightly higher tumor SCCA1 levels; this finding might somehow reflect the selection criteria for the patients undergoing anticancer treatment, as this approach was reserved for patients with more aggressive features (i.e. nodal metastasis). In a previous study, we had observed that SCCA1 overexpression was associated with chemoresistance in patients with EAC.⁸ Furthermore, SCCA1 overexpression has also been associated with chemoresistance in several different other tumor types.^{16,17} Moreover, as we observed that tumor SCCA1 mRNA expression was higher in patients receiving docetaxel, we focused on the possible modulation of cell viability by SCCA1 in the presence of different chemotherapeutic drugs. We observed that OE33 cells, overexpressing SCCA1, were more resistant to cell death than the control OE19 cells after treatment with epirubicin, docetaxel, and cisplatin, whereas cell viability was not affected by treatment with and 5-FU. Moreover, OE19 cells transfected to overexpress SCCA1 were more resistant to docetaxel treatment than OE33 expressing SCCA1 levels approximately 200 times less. This result is consistent with the higher SCCA1 mRNA expression in those who had received docetaxel because in these patients the cells surviving this type of chemotherapy are probably expressing SCCA1. Moreover, our research group has observed that SCCA1 shields cells from the toxicity of drugs with a pro-oxidant action, such as doxorubicin and cisplatin.¹⁵ It is worth noting that these drugs are among the most commonly used in neoadjuvant therapy for EAC. Thus, a reduced chemosensitivity in patients with high levels of SCCA1 could explain, at least in part, the poor prognosis of these patients.

To complete the possible cause of worse survival in patients with high SCCA1 levels, we verified the hypothesis of possible cross-talk between SCCA1 and the tumor immune microenvironment. In fact, we observed that tumor SCCA1 mRNA expression was inversely correlated to the MFI of CD80 expression on epithelial cells. CD80 is a co-stimulatory molecule necessary to the proper activation of T

cells during antigen presentation.³³ Its expression on epithelial cells was reported to be the main driver of immune surveillance mechanisms in inflammatory colorectal carcinogenesis.¹⁸ Ipilimumab, an anti-CTLA-4 Ab (CTLA-4 is the physiological inhibitor of CD80 and CD86) was indeed declared molecule of the year in 2013 at the beginning of the immunotherapy revolution.³⁴ The inverse correlation of SCCA1 with CD80 expression on the epithelial cell surface might suggest an important clue on the immune inhibitory role of SCCA1. In fact, total bulk of SCCA1, including IgM-bound and free forms, was inversely correlated with immune activation markers such as CD80, CD86, TLR4, and CD38 mRNA levels within the tumor and as peritumoral intraepithelial expression of CD8⁺ and CD107⁺ cells. All these data indicate the presence of a direct or an indirect effect of high levels of SCCA1 expression on the immune surveillance mechanisms, resulting in intratumoral immunity impairment that might concur with the worse prognosis of patients with high SCCA1 expression.

To verify the hypothesis of a possible direct role of SCCA1 in the depression of the tumor immune response, human recombinant SCCA1 was added to different immune cell subsets. In the CD14⁺ monocyte cell subset a significant increase of PD-L1, but not PD-L2, was observed and these findings were confirmed by IHC in tumor specimens. Moreover, PD-L1 levels were higher in OE33 cells, expressing SCCA1, than in OE19 cells, not expressing SCCA1. It is worth noting that in the external validation cohort GSE13898,²⁸ a significant direct correlation between SCCA1 and PD-L1 mRNA levels within the tumor tissue was also detected. Taken together, all these data provide different and consistent *in vivo* and *in vitro* proof that these 2 molecules are expressed at the same time in EAC, although the precise pathogenetic mechanism of their association still remains to be elucidated. In addition, these results agree with the recent report of a direct association between SCCA1 and the expression of immune checkpoint regulators PD-L1/PD-L2 in head and neck squamous cell carcinoma.³⁵ Interestingly, in a very recent multicohort, phase IB study, pembrolizumab, an anti-PD-L1 Ab, showed manageable toxicity and durable antitumor activity in patients with heavily pretreated, PD-L1-positive advanced esophageal carcinoma.³⁶ The increased levels of PD-L1 expression in EAC, induced by SCCA1, might contribute to the observed

intratumoral immune impairment. These findings suggest that SCCA1 might be used as a predictive marker of response to immunotherapy and might support the use of different strategies to enhance the response to immunotherapy in future studies.

Finally, to investigate the association between SCCA1 and the peritumoral microenvironment, we evaluated SCCA1 expression, stem cell markers, and paracrine adipose tissue mediators in peritumoral adipose tissue that had been proven to play a significant role in the nodal metastatic process and in chemoresistance.^{21,36} In our series, SCCA1 expression within the tumor was inversely correlated with CD34 levels, both within the tumor and in the peritumoral adipose tissue. It is likely that hypoxia, more evident in tumors with low angiogenesis and therefore low levels of the endothelial marker CD34, can contribute to SCCA1 induction through a hypoxia-inducible factor-2 α mediated mechanism.³⁷ Moreover, we observed that the extent of SCCA1 expression within the tumor was directly correlated with the levels of leptin in peritumoral adipose tissue. As we failed to determine a direct effect of SCCA1 in leptin secretion in vitro (data not shown), this direct correlation might be due to the independent activity or dependence of other factor(s) of both molecules, leading to poor response to chemotherapy and poor prognosis.²¹

In conclusion, SCCA1 is associated with poor prognosis in esophageal cancer patients. This finding might be due to the following identified mechanisms induced, at least in part, by SCCA1: (i) reduced tumor chemosensitivity; or (ii) peritumoral immunity impairment, possibly mediated by the induction of the PD-L1 immune checkpoint regulator.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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