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ORIGINAL ARTICLE

Immunohistochemical analysis of the expression of cancer-associated fibroblast markers in esophageal cancer with and without neoadjuvant therapy

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Abstract

Esophageal carcinoma (EC) is one of the most aggressive human malignancies with high rates of resistance to conventional anticancer treatment. Cancer-associated fibroblasts (CAFs) are an important part of the tumor microenvironment and associated with tumor progression. COL11A1, SPARC, and CD90 have been identified as rather specific CAF markers, with COL11A1 expression particularly shown to influence response to chemotherapy. We investigated the impact of CAFs in esophageal cancer with a special focus on response to neoadjuvant treatment (nTX). Two collections of esophageal carcinomas were investigated: 164 cases treated with primary resection and 256 cases receiving nTX before resection. The expression of CAF markers was determined using next-generation tissue microarray (ngTMA®) technology and immunohistochemistry. The presence of COL11A1 and SPARC in fibroblasts within both primary resected cases and nTX-treated cases was associated with unfavorable clinicopathological variables such as higher (y)pT category and lymphatic invasion ($p<0.001$ each). The presence of COL11A1-positive CAFs was associated with worse overall survival in primary resected cases (HR: 2.162, $p = 0.004$, CI 95% 1.275–3.686). While in tumors showing regression after nTX, COL11A1-positive CAFs were detected less frequently, SPARC-positive CAFs were enriched after nTX, in both responding and non-responding patients ($p < 0.001$). Our results support the concept of CAFs as an important factor of tumor promotion and maintenance in EC. The population of CAFs increases with tumor progression and decreases, partly depending on the subtype, after regression following nTX. CAFs may serve as potential target for future therapeutic approaches for these highly aggressive tumors.

Keywords COL11A1 · SPARC · CD90 · Cancer-associated fibroblasts · Esophagus · Chemoresistance

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Introduction

Esophageal carcinoma (EC) is one of the most aggressive cancers and the seventh most common cause of cancer-related deaths worldwide. Overall, the 5-year survival rate remains only around 20%. Esophageal squamous cell carcinoma (ESCC) is the more common histological subtype worldwide, whereas adenocarcinoma (EAC) is the most commonly seen in Western countries with increasing incidence [1].

Multimodal treatment concepts with a combination of neoadjuvant therapy (nTX) followed by surgery have been widely implemented in the therapy of locally advanced EC, aiming to improve the prognosis for these patients. However, the effects of nTX differ between patients, and the survival outcome is still unsatisfactory for non-

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responders [2]. It is therefore important to enhance the knowledge about resistance to chemotherapy (CTX), or radiochemotherapy (RCTX), but also to identify markers or conditions that predict the responsiveness before initiating neoadjuvant treatment.

The tumor microenvironment (TME) has been identified as a regulator of cancer progression, and cancer-associated fibroblasts (CAFs) represent the major cellular component of the tumor stroma [3, 4]. Several studies have shown that TME plays an important role for resistance to anticancer treatment. In particular, CAFs have been shown to promote progression and chemoresistance of malignant cells. Moreover, they share more characteristics with tumor stem cells compared to normal fibroblasts [5, 6]. These observations have also been corroborated in previous EC studies [7, 8]. Although this fact makes CAFs a potential target for anticancer therapy [9, 10], it is still not well elucidated as to how CAFs are involved in resistance to nCTX or nRCTX in EC.

Several markers have been used to identify CAFs like alpha-smooth muscle actin (a-SMA) or fibroblast activation protein (FAP). However, these markers are frequently also expressed in other cells and fibroblasts of other non-cancer-related processes (tissue repair, development, inflammatory diseases) [11]. This lack of specificity hampers the detection of CAFs in tissue specimens and causes problems in therapeutic targeting. In order to find specific markers to distinguish CAFs from other types of cells, studies based on gene microarray technology have shown a relevant function for collagens in neoplastic transformation. Among the upregulated genes, collagen type XI alpha I (COL11A1), secreted protein acidic and rich in cysteine (SPARC), and CD90 (Thy-1) were the most highly elevated [12–14].

Using immunohistochemistry for COL11A1, SPARC, and CD90 for the characterization of CAFs, we investigated the impact of this particular cellular component of the tumor microenvironment in EC, including both EAC and ESCC tumor types with a special focus on tumors treated with nTX.

Material and methods

Case collections

This work was performed on cases collected from the Institutes of Pathology of the University of Bern (Switzerland) and the Technische Universität München (Germany). Out of these collectives, two groups were built: cohort 1 ($N = 164$) comprised 113 cases of EAC and 51 cases of ESCC, which did not receive nTX and had undergone primary resection; cohort 2 ($N = 256$) comprised 198 EAC patients and 58 ESCC patients treated with nTX followed by surgical resection: for EAC, 125 cases were treated only with platinum/5-fluorouracil based (nCTX), and 73 cases were treated with nCTX and 45Gy + platinum/5-fluorouracil based (nRCTX), whereas for ESCC patients, 10 cases were treated only with nCTX, and 48 cases were treated with nRCTX. The following clinicopathological variables were analyzed: age, (y)pTNM categories, differentiation (grading), and tumor regression grade (TRG) according to Becker [15] for cases treated with nTX. According to the prognostic impact demonstrated before [16], patients with TRG 1a–1b were classified as responders and TRG 2–3 as non-responders. The overall survival was calculated from the day of surgery. The clinicopathological variables are summarized in Supplementary Table 1.

ngTMA® construction

All FFPE blocks were cut at 2.5 μm and stained with hematoxylin and eosin (HE). Tissue microarray construction was performed using a next-generation tissue microarray (ngTMA®) approach (www.ngtma.com) [17]. HE slides were scanned using a Pannoramic P250 scanner (3DHistech, Hungary). Next, digital slides were annotated using a TMA tool (Pannoramic Viewer, 3DHistech, Hungary). The digital slides of each surgical resection were annotated using a 0.6-mm tool as follows: six cores per tumor of the tumor center or the tumor bed including scar tissue in nTX cases, three cores of normal epithelium, and three cores of non-neoplastic stroma, usually obtained from the resection margins. Next, these annotated core regions were punched out using an automated tissue microarrayer, TMA Grandmaster (3DHistech, Hungary).

Immunohistochemistry

Immunohistochemical (IHC) staining was performed using an automated system (BOND RX, Leica Biosystems, UK). All whole sections and TMA sections were cut at 2.5 μm, deparaffinized, and rehydrated in dewax solution (Leica Biosystems). Endogenous peroxidase activity was blocked with H_2O_2 solution for 4 min. All samples were incubated with the following primary antibodies for 30 min at room temperature: COL11A1, clone 1E8.33 (Oncomatrix, Spain); CD90, clone EPR3132 (Abcam, ab92574, UK), and SPARC (Santa Cruz, sc398419). Tris buffer (pH 9) at 95°C for 30 min and citrate buffer (pH 6.5) at 100°C for 30 min were used for antigen retrieval. Antibody detection was done with the Bond Polymer Refine Detection kit (Leica Biosystems, DS9800) following the manufacturer's instructions.

Immunostaining validation and assessment

Interpretation of staining results was in accordance with the "reporting recommendations for tumor marker prognostic studies" (REMARK) guidelines [18]. Readout of stainings were performed by JW and JAG, and discrepancies were

discussed with RL on a multi-header microscope to gain a final consensus. For scoring, the cytoplasmic staining of COL11A1, SPARC, and CD90 in the fibroblasts was taken into account. The immunostaining was recorded as follows: 0, no signal; 1, detectable in <1% of cells; 2, readily detectable in 1%–5% of cells; 3, readily detectable in 6%–10% of cells; and 4, signals in >10% of cells (adapted from Galván et al.) [19].

In addition, for the assessment of potential bias due to intratumoral heterogeneity and to demonstrate the validity of the IHC stainings applied on the TMAs, 40 tumor blocks (20 primary resected cases and 20 treated cases) were cut, and the full slides were stained with COL11A1, SPARC, and CD90 in parallel with the TMA cuts to check the reliability of TMA for the marker detection.

Statistical analysis

Associations between the expression of the markers and categorical clinicopathological features were performed using the chi-square test. For the agreement between full slide sections and TMA punches, Kappa statistic was used. Univariate analyses for overall survival and disease-free survival were performed using the Kaplan–Meier method and log-rank tests. Statistically significant variables from the univariate analysis and those that met the proportional hazard assumptions were further analyzed by multivariate Cox regression analysis. *P* values <0.05 were considered statistically significant. All analyses were carried out using IBM SPSS v24 (IBM, NY, US).

Results

Previous considerations

A preliminary survival analysis in primary resected tumors (n=164), using the scoring in five categories, was performed. While the absence of CAFs was significantly associated with the best outcome ($p = 0.011$), there was no difference between the categories <1% and 1%–5% and 6%–10% and > 10% ($p = 0.1$, Supplementary Fig. 1). Another aspect was the disproportion of cases among the different categories: 0%, 86 cases; <1%, 14 cases; 1%–5%, 24 cases; 6%–10%, 13 cases; and > 10%, 25 cases. We therefore considered it appropriate to use the dichotomization into absent and present (86 cases vs. 76 cases) for further analysis and proceeded with the other markers likewise.

Comparison of COL11A1, SPARC, and CD90 stainings in full slide sections with tissue microarrays

The comparison between the whole sections and the staining results obtained from the TMAs (dichotomized categorization) revealed the following: Regarding COL11A1 stainings, the percentage in agreement was 80%, Kappa value 0.583 (CI 95% 0.318–0.838), $p < 0.001$. Similar results were found for SPARC and CD90 stainings, the percentage in agreement was 87.5%; Kappa 0.541 (CI 95% 0.095–0.844), $p < 0.001$ and 87.5%; and Kappa 0.398 (CI 95% 0.197–0.599) $p = 0.002$, respectively.

Presence of COL11A1, SPARC, and CD90 in normal tissue and esophageal cancers

The presence of these markers was only present in neoplastic tissue. The immunosignal with granular pattern was located within the cytoplasm of fibroblasts. CD90 is also expressed by endothelial cells; however, this was not included in the evaluation. Figure 1 shows a representative case with COL11A1-, SPARC-, and CD90-positive CAFs (40× magnification).

In non-neoplastic tissue, no CAFs were detected (0/68 cases with normal tissue from primary resected cases and 0/82 cases from nTX cases) (Fig. 2). In primary resected cases, COL11A1 was present in 76/163 (46%) of cases ($p < 0.001$), while SPARC-positive CAFs were present in 141/162 cases (87%) $p = 0.85$ and CD90 present in 150/162 total (92.6%) $p = 0.015$. Similar results were found in the nTx cases, with COL11A1 presence found in 69/256 (26.9%) $p > 0.001$; the presence of SPARC and CD90 was found in 227/256 (88.6%).

By tumor subtype, COL11A1 was present in 78/310 EAC cases (25.2%) and 67/109 ESCC cases (61.5%), $p > 0.001$. In contrast, SPARC was present in 279/309 EAC (90.3%) and 89/109 ESCC (81.7%) $p = 0.017$ and CD90 in 271/309 EAC (87.7%) and 106/109 ESCC (97.2%) $p = 0.004$.

Staining features and clinicopathological variables

The presence of COL11A1 and SPARC in primary resected cases was associated with parameters indicating aggressive tumor behavior, in particular higher pT category ($p < 0.001$ and $p = 0.034$, respectively) and the presence of lymphatic invasion ($p < 0.001$ and $p = 0.033$, respectively). CD90 expression was associated with more clinicopathological variables such as higher pT category ($p < 0.001$), the presence of lymph node metastasis ($p = 0.015$), worse tumor differentiation ($p = 0.006$), the presence of lymphatic invasion ($p < 0.001$), venous invasion ($p = 0.017$), and perineural invasion ($p = 0.008$). (Table 1)

In contrast, in the nTX cases, COL11A1 and SPARC expressions were associated with higher ypT category, $p < 0.001$), the presence of lymph node metastasis, ($p < 0.001$), higher tumor grade ($p = 0.053$), the presence of lymphatic invasion ($p = 0.002$), and perineural invasion ($p < 0.001$) (Table 2).

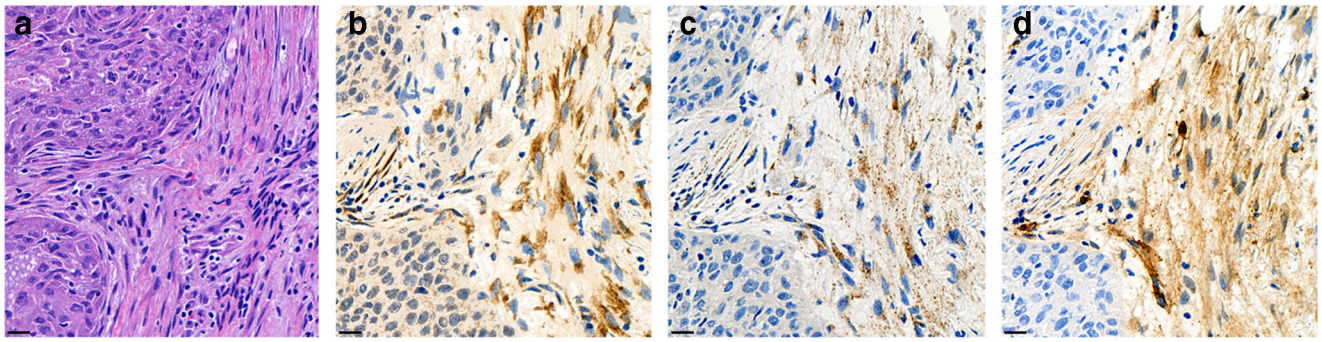

Fig. 1 CAFs markers in detail: H&E (**a**), COL11A1 (**b**), SPARC (**c**), and CD90 (**d**) immunostaining in one representative case at 40× magnification. Scale bar 20 μm

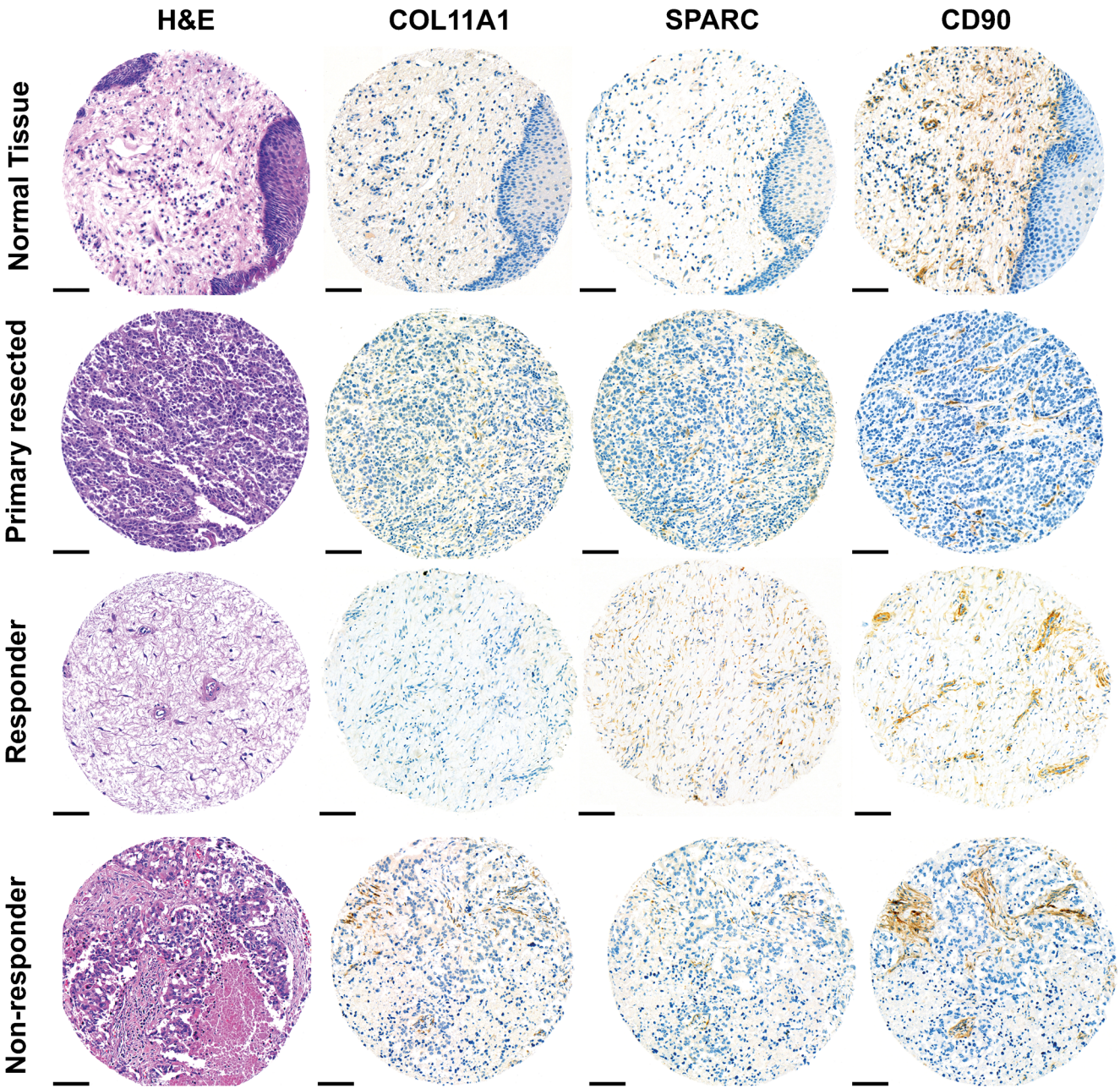

Fig. 2 CAFs markers (COL11A1, SPARC and CD90) immunostaining in normal tissue, primary resected cases and nTX cases (responder and non-responder) in Esophagus Adenocarcinoma cases. Scale bar 100 μm, 20× magnification

Table 1 Statistical associations between clinicopathological features and CAF markers in primary resected cases

		Esophageal Cancer Cohort - primary resection								
		COL11A1 expression			SPARC expression			CD90 expression		
		Absent	Present	*p*	Absent	Present	*p*	Absent	Present	*p*
Gender	Male	74	58	0.156	16	115	0.559	10	121	0.821
	Female	13	18		5	26		2	29	
Age	<65 y	46	38	0.714	10	73	0.722	74	58	0.156
	>65 y	41	38		11	68		13	18	
Histology	ESCC	10	41	0.001	7	44	0.845	0	51	0.015
	EAC	77	35		14	97		12	99	
pT category	T0-T2	43	17	0.001	12	47	0.034	10	50	0.001
	T3-T4	44	59		9	94		2	100	
Lymph node metastases	Absent	50	30	0.022	13	66	0.197	10	70	0.015
	Present	37	46		8	75		2	80	
Distant metastases	Absent	85	70	0.099	21	133	0.263	12	142	0.412
	Present	2	6		0	8		0	8	
Tumor grade	G1	13	7	0.345	5	15	0.228	5	15	0.006
	G2	36	39		8	66		4	70	
	G3	38	30		8	60		3	65	
Resection margins	Negative	76	59	0.067	17	117	0.765	12	123	0.114
	Positive	10	17		4	23		0	26	
Lymphatic invasion	Absent	34	12	0.001	10	35	0.033	10	36	0.001
	Present	52	63		11	104		2	112	
Venous invasion	Absent	65	48	0.086	15	97	0.842	12	100	0.017
	Present	21	28		6	43		0	49	
Perineural invasion	Absent	51	38	0.203	14	74	0.149	11	77	0.008
	Present	34	38		6	66		1	71	
Lauren classification	Intestinal	30	18	0.131	7	40	0.64	8	39	0.003
	Non-intestinal	57	58		14	101		4	111	

CAFs and neoadjuvant therapy

COL11A1-positive CAFs were present in 33/121 cases (27.3%) which were treated with nRCTX, and 36/135 (26.7%) were treated only with nCTX. This was significantly lower compared to primary resected tumors 76/163 (46.6%; $p = 0.001$). The presence of SPARC, 102/121 (84.3%) and 125/135 (92.6%), and the presence of CD90, 116/121 (95.9%) and 111/135 (82.2%), were similar in the cases treated with nRTCX as well as in cases treated with CTX, respectively.

In general, the presence of COL11A, SPARC, and CD90 was associated with tumor regression grade although its interpretation is different. In tumors which responded to nTX, COL11A1-positive cases were observed only very infrequently (18/122 cases, 14.8%; $p < 0.001$). In contrast, tumors of non-responders frequently showed the presence of SPARC (127/132 cases, 96.2%; $p < 0.001$). 20/29 (69%) of patients who developed metastasis showed CD90-positive CAFs ($p < 0.04$).

According to therapy response and tumor type, EAC cases which did not respond to the therapy showed higher levels of COL11A1 38/113 (33.62%) versus patients who responded to the therapy 5/83 (6%) ($p < 0.0001$) (Fig. 2). However, SPARC presence in both non-responders (109/113, 96.5%) and responders (71/83, 85.5%) ($p = 0.029$) was higher (Fig. 2). For ESCC cases, 13/19 (68%) cases were positive for COL11A1 staining in non-responders versus 13/39 (33%) cases in therapy responders ($p = 0.012$). Similarly, the presence of SPARC in non-responders was higher (18/19, 94.7%) versus responders (27/39, 69.2%) ($p = 0.029$). Comparable observations were found for CD90 fibroblasts (Supplementary Fig. 2).

Survival analysis

A negative impact on survival was found for the following clinicopathological variables in the primary resected cohort: higher pT category, the presence of lymph nodes metastasis, higher tumor grade ,and the presence of lymphatic invasion (*p*

Table 2 Statistical association between clinicopathological features and CAFs markers in nTX cases

		Esophageal cancer cohort – nTX								
		COL11A1 expression			SPARC expression			CD90 expression		
		Absent	Present	*p*	Absent	Present	*p*	Absent	Present	*p*
Gender	Male	156	59	0.687	19	196	0.004	24	191	0.848
	Female	31	10		10	31		5	36	
Age	<65	60	22	0.811	15	67	0.239	4	78	0.235
	>65	50	20		8	62		1	69	
Histology	ESCC	32	26	0.001	13	45	0.002	3	55	0.093
	EAC	155	43		16	182		26	172	
ypT category	yT0-yT2	119	24	0.001	24	119	0.002	18	125	0.475
	yT3-yT4	68	45		5	108		11	102	
Lymph node metastases	Absent	109	22	0.001	23	108	0.001	17	114	0.394
	Present	78	47		6	119		12	113	
Distant metastases	Absent	168	59	0.332	27	200	0.424	20	207	0.04
	Present	19	10		2	27		9	20	
Tumor grade	G1	23	2	0.053	6	19	0.128	1	24	0.408
	G2	57	26		10	73		11	72	
	G3	61	28		8	81		12	77	
Resection margins	Negative	161	57	0.144	28	190	0.118	26	192	0.715
	Positive	19	12		1	30		3	28	
Lymphatic invasion	Absent	72	22	0.003	20	74	0.005	5	89	0.132
	Present	21	20		1	40		0	41	
Venous invasion	Absent	82	34	0.609	21	95	0.142	5	111	0.344
	Present	13	7		1	19		0	20	
Perineural invasion	Absent	59	23	0.03	19	63	0.015	4	78	0.218
	Present	15	15		1	29		0	30	
Lauren classification	Intestinal	27	8	0.557	4	31	0.984	0	35	0.023
	Non-intestinal	160	61		25	196		29	192	
Becker TRG	Responder	104	18	0.001	24	98	0.001	16	106	0.413
	Non-responder	81	51		5	127		13	119	

< 0.001 for all) (Supplementary Table 2). Among CAF markers, the only marker with a negative impact on survival was the presence of COL11A1 (HR: 2.162, $p = 0.004$, CI 95% 1.275–3.686) (Fig. 3). In multivariate regression analysis, we included the following variables, which were significant in univariate analysis: COL11A1 expression, pT category, pN category, pM category, tumor grade, and lymphatic invasion. Here, only pT category showed an independent prognostic value (HR: 3.36, $p < 0.001$, CI 95% 1.789–6.31) (Supplementary Table 3).

Different results with regard to CAFs were found among clinicopathological variables in the nTX cohort. Here, the following parameters were associated with worse outcome: higher ypT category, the presence of lymph metastasis, the presence of metastasis, higher tumor grade, the presence of lymphatic invasion, the presence of venous invasion, and positive resection margins ($p < 0.05$ for all) (Supplementary Table 4). Among these, after multivariate regression analysis Cox, the presence of metastasis and positive resection margins were the two variables with an independent prognostic (HR: 3.84, $p = 0.016$, CI 95% 1.28–11.4) and (HR: 3.57, $p = 0.021$, CI 95% 1.2–10.5), respectively (Supplementary Table 3).

Discussion

EC is a highly aggressive disease, and only a subset of patients can be cured by surgery alone. Resistance to nCTX and nRCTX is a major problem in EC disease management, comprising the two major histologic subtypes, EAC and ESCC, and both in the setting of multimodal treatment (i.e., nTX followed by surgery) and metastatic or recurrent disease which would require systemic and/or local anticancer treatment.

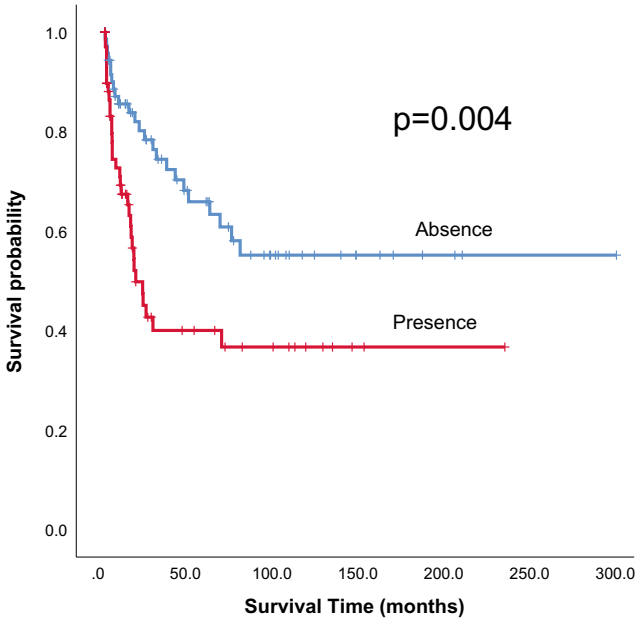

Fig. 3 Kaplan–Meier curves of overall survival after resection in patients with COL11A1-presence versus COL11A1-absence tumors

CAFs play an important role in the mechanism of drug resistance of tumors and are also reported to be the main cause of relapse in many patients [20]. The investigation of CAFs may therefore provide important information about these specialized cells that may also be target for a specific anticancer treatment. In rectal cancer, nTX increases the number of CAFs favoring tumor progression [4].

In the present study, we investigated the role of CAFs in EC using IHC for three CAF markers, COL11A1, SPARC, and CD90, in both types of esophageal cancer specimens (EAC and ESCC), in two distinct cohorts, i.e., primary resected tumors and tumors treated with nTX. We have demonstrated that, depending of the expression of certain markers, the presence of CAFs is not only associated with tumor progression and poor outcome but also with therapy response.

The first of these markers, COL11A1, has been proposed to act as a potential invasion-associated gene in multiple epithelial cancer types [21], with negative impact on the survival rates [22, 23]. These statements have been confirmed in our study on EC patient-derived tissues. COL11A1 has also been shown to be involved in chemoresistance in lung [24] and ovarian cancer [25]. However, only He et al. identified by bioinformatics analysis the upregulation of *COL11A1* in ESCC cases [26]. Zhang H et al. found that CAFs conferred chemoresistance of ESCC cells via secretion of transforming growth factor-β1 (TGF-β1) [7] . It is well established that CAFs secrete a number of growth factors, including TGF-β1. TGF-β1 is not only an extracellular signal triggering tumor promotion, epithelial-mesenchymal transition process, and chemoresistance [27–29] but also a factor that maintains the CAF phenotype by an autocrine mechanism [30]. Both mechanisms are related with previous findings by which COL11A1 expression is directly linked to TGF-β1 signaling [19, 31]. Most data, however, generate from in vitro and animal studies, while an in situ tissue-based analysis of secreted factors such as TGF-β in the context of CAFs is challenging. Given the potential high impact of the role of the tumor microenvironment on several aspects of tumor biology, in particular resistance to antitumoral treatment; furthermore, ex vivo studies addressing to the complex interaction between CAFs, cancer cells, and other cells of the tumor microenvironment, in particular using the TGFβ signaling, are highly demanded.

SPARC was originally identified as a collagen-binding glycoprotein and interacts with matrix metalloproteinases (MMPs) and several growth factors, such as transforming growth factor-beta (TGFβ). SPARC is involved in numerous mechanisms in cancer, such as tumor progression, migration, and metastasis. SPARC mRNA and protein are highly expressed in ESCC and negatively correlated with lymph node metastasis and poor prognosis which was not associated with postoperative survival of ESCC patients [32–34].

CD90 (Thy-1), a protein present in a subtype of CAFs, has been shown to mediate cell–cell interactions by binding to integrins and facilitates the attachment of tumor cells to endothelial cells during the metastasis process [35]. Until now, CD90 expression has only been described in ESCC to be associated with lymph node metastasis [36].

Our work highlights the involvement of CAFs in the tumor progression in ESCC cases and EAC cases. Our results show that the presence of COL11A1, SPARC, and CD90 is similar in both tumor types and associated with most of unfavorable clinicopathological variables, providing further evidence on the role of these markers in the tumor invasiveness. Of note, we could confirmthese associations in both cohorts: higher pT category, the presence of lymph nodes metastasis, and lymphatic invasion - observations supporting those recently published [24, 37].

However, in tumors treated with nTX, the presence of CAFs was different depending on the marker analyzed. After therapeutic treatment, the number of COL11A1(+) fibroblasts decreases with regression. This is in line with the previous evidence associating increased chemoresistance with COL11A1 presence in other tumors, such as lung cancer [24]. In contrast, SPARC(+) and CD90(+) fibroblasts are enriched after therapy. Similar results have been found in other tumors of gastrointestinal origin such as the colon [38] and rectum [3, 4] irrespective of clinical response classification. These findings suggest different CAF subtypes with different tumor biologies, different susceptibilities to the treatment, and distinct properties and levels of activation. This fact has also been recently showed in breast cancer by Costa et al. [39].

So far, one of the biggest limitations to identify CAFs has been the absence of specific markers. The most widely used

markers have been α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), vimentin, desmin, platelet-derived growth factor receptor-α and β (PDGFR α and β), and fibroblast-specific protein-1 (FSP-1). However, they can also be detected in other cells, like normal stromal cells or even epithelial malignant cells [40]. In the present study, we have demonstrated that COL11A1, SPARC, and CD90 could be used as CAF markers, since they are not expressed in healthy tissue or scars but associated with the presence of vital tumor and its progression, similar to the findings of previous studies in other types of tumors [22, 23].

Limitations in this work are the absence of experimental analysis (e.g. with CAFs isolated from esophageal specimens or deeper functional study at the gene expression level). Further studies to isolate CAFs from EC, for example, using a reliable method previously published by Underwood et al. [8] would be required to understand the functional significance of these markers. Yet, their presence already anticipates that drug resistance in EC might be affected in part by the participation of CAFs.

Moreover, subsets of CAFs can be specifically identified through the three markers used in this study, and their presence is associated with the existence of tumor cells. In addition, the study of CAFs could be carried out with ngTMA® technology. This possibility allows the digital selection of histological areas with high precision. With the construction of a TMA which includes hundreds of cases, many samples can be analyzed at the same time.

The focal staining of COL11A1, shown in previous studies [19, 22, 23], has required a comparison between cases using entire sections with tissue punches represented in the TMA. We found a highly significant correlation between the results obtained from full slide sections to those from TMAs in a selected case series. We therefore are confident that the TMA approach with its technical advantages is suitable to study CAFs in EC.

In summary, this work has shown that CAFs increase with tumor progression in primary resected cases. Depending on the marker, CAF subtypes increase or decrease after neoadjuvant therapy. Further specific studies are necessary to define the role of these markers before and after therapy. The immunohistochemical detection of CAFs can be a valuable tool for cancer research but also for clinical diagnostics.

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Author contributions JAG and RL conceived and planned the study. JSH, MF, KO, DK, and CAS provided the human tissue samples. JW and RL selected the human tissue samples and performed the annotations of the TMA cores. JAG constructed the ngTMAs and carried out the immunostainings. JW and JAG carried out the scoring and supervised by RL. JAG carried out the statistical analysis. JAG and RL contributed to the interpretation of the results. JAG wrote the manuscript with the support of RL. All authors provided critical feedback and helped shape the manuscript.

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Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of local ethics commission (Kantonale Ethikkommission Bern, Switzerland, 200/14 and Medizinische Fakultät of the Technische Universität München, 2056/08) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflicts of interest.

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