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Original Research

FAPα and αSMA mark different subsets of fibroblasts in normal kidney and conventional renal cell carcinoma

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Abstract

Several studies suggested a correlation between cancer associated fibroblasts (CAF) and cancer progression, but data on conventional renal cell carcinoma (cRCC) is still lacking. We aimed to analyse the impact of αSMA positive myo-CAF and FAPα expressing i-CAF on postoperative relapse of cRCC. We applied immunohistochemistry on tissue-multiarray (TMA) containing 736 consecutively operated cRCC without metastasis at the time of diagnosis. We analysed the correlation between the amount and pattern of αSMA and FAPα expressing CAFs and tumour cells and postoperative tumour relapse. Stromal fibroblasts of each cRCC displayed αSMA immunreaction but only 142 of the 736 tumours showed positive FAPα staining. There was no correlation between the amount of αSMA and or FAPα positive CAFs and tumour progression. However, tumours with large tourtous vessels with strong αSMA positive immunreaction have more then two times higher risk of postoperative tumour relapse (RR=2.198, $p = 0.005$). Patients with cRCC (57) showing cytoplasmic αSMA staining of tumour cells had a nearly two times higher risk for postoperative progression (RR=1.776, $p = 0.014$).
There is no significant correlation between the density of αSMA or FAPα positive CAFs and postoperative relapse of cRCCs, therefore CAFs in cRCC are not suitable targets for therapy. Further limitation of anti-CAF therapy of cRCC that stromal cells of normal kidney are positive with αSMA antibody.

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Keywords: αSMA, FAPα, CAF, immunohistochemistry, conventional renal cell carcinoma

Introduction

In addition to malignant biological behavior of cancer cells, the inflammatory tumour microenvironment (TME) containing cancer associated fibroblasts (CAF) have been implicated in progression of several types of cancer [1]. CAFs have a central role in producing and remodeling of extracellular matrix (ECM) and in changing the TME to pro-metastatogenic [2]. Recently, different functions have been attributed to CAFs, which are executed by at least two different subtypes. CAFs exhibiting contractile phenotype are termed as myo-CAFs, whereas CAFs regulating tumour associated inflammation are termed as inflammatory CAF, i-CAF [3]. Alpha smooth muscle antigen (αSMA) and fibroblast associated protein alpha (FAPα) are used to identify the two types of fibroblasts with overlapping function [4]. CAFs and tumour cells steadily communicate via growth factors and inflammatory cytokines [5–8]. Each type of tumour exerts specific secretome that activates their own fibroblasts through paracrine effect [4].

Recent years several reports have been published on the correlation between αSMA and FAPα expressing CAFs and cancer progresson [9–18]. The impact of αSMA and FAPα positive CAFs on biological behaviour of conventional renal cell carcinoma (cRCC) is not yet known. One previous report suggested the association between FAPα positive CAFs and progression of cRCC [14]. The aim of our study was to analyse the role of αSMA positive myo-CAF and FAPα expressing i-CAF in progression of cRCC. We applied immunohistochemistry on tissue-multiarray (TMA) of tumours without metastatic growth at the time of diagnosis. We analysed the

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correlation between clinical and pathological parameters and postoperative tumour relapse and the pattern of αSMA and FAPα expressing CAFs and tumour cells.

Material and methods

Patients

We have selected 736 cRCC patients operated between 2000 and 2015 without clinically detectable metastasis at the time of operation. Patients with metastatic tumor at the time of first observation (75) or died due to other diseases or those without follow-up data were available (34) were excluded from the study. Follow-up was defined as a time interval between the operation and last recorded control or tumor specific death. The data were obtained from Tumor registry of Department of Urology. Progression free survival was defined as the time between the diagnosis and the clinically detected relapse.

Of the 736 patients, 426 (58%) were males and 310 (42%) females, the mean age of the cohort was 60.9±11.2 years (range 23–88 years). The average tumour size was 49.5 ±25.3 mm. During the median follow-up of 66±29 months, tumour relapse was observed in 119 patients (16%). The vast majority of 736 tumours (78%) were classified as pT1, as G1 (69%) and stage I and II (91%).

Tumour samples and immunohistochemistry

The histology of original tumor samples was evaluated according to the Heidelberg Classification and a 1-3 tiered grading system [19]. We restrained to the Heidelberg Classification because it is based on robust tumor specific genetic alterations. According to this classification approximately 70-80% of conventional RCCs are composed of "clear" cells and the rest of "eosinophilic", (earlier "granular") cells or showing rhabdoid or sarcomatous histology. For tumor staging we used the 2016 TNM systems [20]. Adult kidney samples from radical tumor nephrectomy as well as fetal kidneys obtained from 10, 12, 15 and 17 weeks of gestation were included in this study. After marking representative tumour areas in hematoxylin and eosin stained slides, TMA was constructed by taking three to four core biopsies with a diameter of 0.6 mm from each tumour and placed in recipient blocks using a Manual Tissue Arrayer (MTA1, Beecher Instruments, Inc., Sun Prairie, USA).

The paraffin was removed by xylol from the 4 μm thick sections and after rehydration the heat-induced epitope retrieval was carried out in citrate buffer (pH 6.0) five minutes at 121 Celsius in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase was blocked with Envision FLEX Peroxydase Blocking Reagent (DAKO, Glostrup, Denmark) for 10 min at room temperature. The slides were covered with anti-αSMA antibody (ab124964, Abcam, Cambridge, UK) at the dilution of 1:1000 and rabbit anti-FAPα antibody (ab207178, Abcam, Cambridge, UK) at the dilution of 1:200 and incubated at room temperature for one hour. After application of EnVision FLEX horse-radish-peroxydase conjugated secondary antibody (DAKO) for 30 minutes at room temperature the immunoreaction was developed with DAB substrate (DAKO). The slides were then counterstained with Mayer's haematoxylin (Lillie's modification, DAKO) and after bluing in ammoniumhydroxid solution were mounted with Glycergel (DAKO). For positive control we used fetal and adult kidneys and for negative control we omitted the primary antibody.The expression pattern of αSMA and FAPα in stromal fibroblast and myoendothelial cells was evaluated by a board certified pathologist blinded to clinical data. Tumour cells were scored as positive or negative.

Statistical analysis

The objective of this study was to evaluate the correlation between postoperative tumour relapse and pattern of FAPα and αSMA positive cells in cRCC. We used Fisher's exact test to estimate the correlations between categorical variables. We applied the Kaplan-Meier method to estimate the cumulative survival and the long-rank test for calculation the differences between groups. We used the univariate and multivariate Cox proportional hazard regression to evaluate the significance of clinical and pathological variables. We performed the analyses by the IBM SPSS Statistics v.27 for windows (Chicago IL, USA). *p-value* <0.05 was considered the limit of statistical significance.

Results

Expression of αSMA and FAPα in foetal and adult kidneys

In foetal kidneys of 10, 12, 15 and 17 weeks of gestation no immunostaining was seen with αSMA antibody (Fig. 1a). In the same series of foetal kidneys a strong FAPα positivity was seen in loosely associated cell population around cap blastemal cells and in stromal mesenchyme (SM) (Fig. 1b). In the cortical areas SM cells showed elongated fibroblast like shape and several of these tiny bands displayed a lumen resembling blood vessels. In the differentiated medullary part of foetal kidneys stromal fibroblasts and blood vessels were negative with FAPα staining. In normal adult kidneys strong αSMA staining was detected in stromal cells between kidney tubules (Fig. 1c), but no FAPα staining was seen in adult kidneys (Fig. 1d).

Pattern of aSMA and FAPa positive CAFs in cRCC

All the 736 cRCC samples displayed αSMA positive CAFs although with different histological apperance. Based on vascular architecture, amount of fibrotic stroma and growth pattern we have separated three groups of tumours. The most frequently observed growth pattern showing trabecular or alveolar histology and regular vascular network of thin-walled blood vessels was found in 428 (58%) tumours, which were designed to group A (Fig. 2a). Group B consisted of 168 (23%) cRCCs showing a high number of proliferating αSMA positive CAFs leading to increased intratumoral fibrosis (Fig. 2c). In some of these tumours, broad fibrotic areas of αSMA positive CAFs occupied more than half of tumour biopsy. We have selected 140 (19%) cRCCs displaying 1-4 large dilated and tortuously growing vessels embedded in sheets of solid growing of tumour. The vessels showed strong αSMA positive staining of myofibroblasts and myoendothelial cells (Fig. 2e). Tumours with this type of growth pattern and vasculature were designed as group C. More than half (53%) of high grade (G3) and 56% of high stage (stage III) tumours occurred group C. Clinical-pathological parameters of the 736 cRCCs and the distribution of the three patterns of αSMA expressing CAFs is shown in Table 1.

Focal or diffuse FAPα expression was detected in cells of the fine capillary meshwork in 142 (19%) of cRCCs (Fig. 2b) or rarely in fibrous stroma. In 594 cRCC no FAPα expression was seen either in fibrotic stroma or in the wall of large tortuous vessels (Fig. 2d, f).

Pattern of αSMA positive CAFs and postoperative relapse

Kaplan-Meier cox regression analysis identified significant differences in the survival of patients with tumours belonging to the three groups (Fig. 3). The 5-year overall survival rate for cRCC with the histological structure of αSMA positive CAFs "A", "B" and "C" were 96.1%, 85.3% and 68.4% respectively. The estimated mean survival for patients with "A" was 176 (157-194) ± 10, with "B" 134 (122-146) ± 6 and with "C" only 94 (77-110) ±8 months, by overall survival of 154 (143-164) ± 5 months.

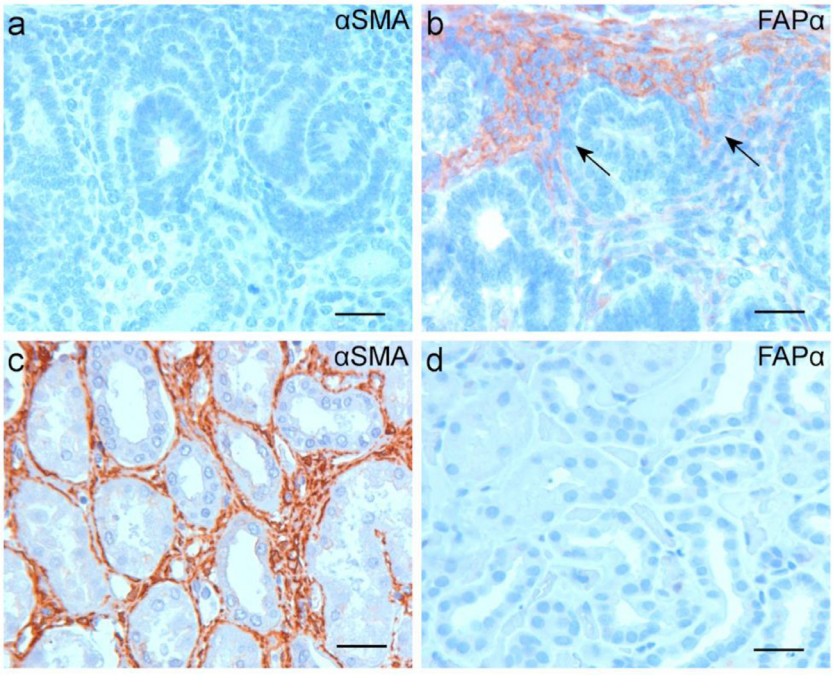

Fig. 1. Expression of αSMA and FAPα in foetal and adult kidneys. a, No expression of αSMA in cortical area of a 12 weeks old foetal kidney. b, FAPα immunoreaction in the emerging fibroblasts and endothelial cells in the nephrogenic zone of a 12 weeks old foetal kidney (arrows). c, Strong αSMA staining of the fibroblasts and endothelial cells in stroma of adult kidney. d, No FAPα expression can be seen in adult kidney. Scale bar: 30 μm. (1.5 column).

Table 1

Clinical-pathological parameters of the 736 and pattern of αSMA expressing CAFs.

		Nr of cases (736)	aSMA positive CAF			*p*-value
			A (428)	B (168)	C (140)	
Gender						<0.001
	male	426	223	102	101	
	female	310	205	66	39	
Status						<0.001
	AWD	617	403	139	75	
	PTR	119	25	27	65	
Size						<0.001
	< 4 cm	301	217	58	26	
	4 - 7 cm	286	155	70	61	
	> 7 cm	149	56	40	53	
T Stadium						<0.001
	pT1	574	376	125	73	
	pT2	99	44	24	31	
	pT3	63	8	19	36	
Grade						<0.001
	G1	510	362	96	52	
	G2	177	60	55	62	
	G3	49	6	17	26	
Stage						<0.001
	I	570	375	123	72	
	II	98	43	25	30	
	III	68	10	20	38	

AWD – alive without disease; PTR - postoperative tumour relapse; A - capillary network; B - fibrosis; C - large vessels.

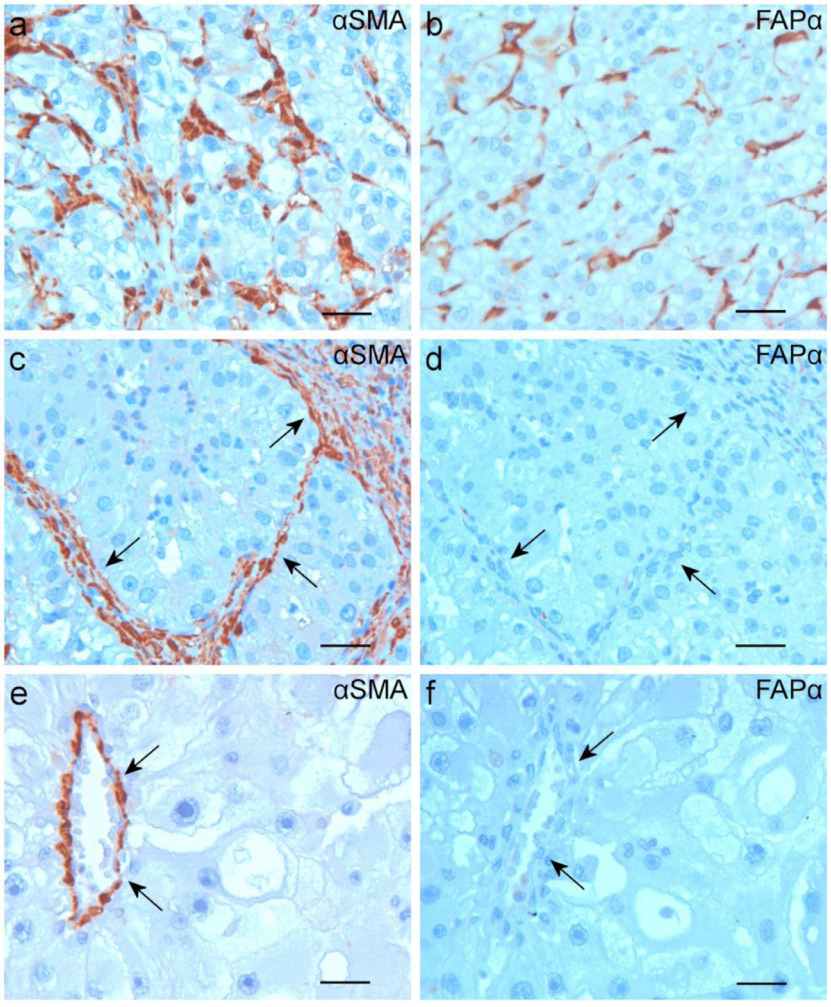

Fig. 2. Expression of αSMA and FAPα in cRCC. a, Endothelial/myoendothelial cells of capillary network in a cRCC with classical alveolar histology (group A) display a strong αSMA positive staining. b, FAPα positive endothelial cells in core biopsy from the same cRCC. c. Solid growing tumour areas embedded in αSMA expressing fibrotic stroma (arrows) in a cRCC (group B). d, Lack of FAPα positive straining in the same tumour area (Fig. 1 d). Arrows show the same areas as in Fig 1 c. e, Large dilated vessel in solid growing tumour (group C) shows strong αSMA positive immunreaction (arrows). f, The same tumour area without FAPα staining of fibroblasts and myoendothelial cells (arrows). Scale bar: 30 μm. (1.5 column).

In univariate analysis all clinical and pathological parameters as well as the distinct patterns of αSMA positivity were significantly associated with the tumour relapse (all $p < 0.001$). However, in multivariate analysis only tumour grade, stage, and pattern of αSMA positive large vessels (group C) remained independent prognostic factor (Table 2). Patients with tumours belonging to group C have more then two times risk of postoperative tumour relapse (RR=2.198, $p = 0.005$). Increased amount of αSMA positive CAF in tumour stroma (group B) was not and independent risk factor.

Expression of aSMA in tumour cells predicts postoperative tumour relapse

Positive αSMA staining of tumour cells has been seen in 57 (8%) cRCC, including those with epithelial characteristics or sarcomatoid transformation (Fig. 4a, c). A positive αSMA staining of tumour cells occurred in group A (2%) less frequently as in group B (13%) and group C (20%). Altogether, postoperative tumour relapse occurred in 12% of αSMA negative cases whereas 60% of the αSMA positive tumours showed progression. Cytoplasmic FAPα positivity in tumour cells occurred only in 21 cRCC. Eleven tumours showed cytoplasmic expression of FAPα and αSMA as well (Fig. 4c, d).

Kaplan-Meier estimates for disease free survival of 736 patients without metastatic disease at the time of operation showed that expression of αSMA protein in tumour cells has prognostic value (Fig. 5). The 5-year overall survival rate for patients with αSMA positive and negative tumours were 54.2% and 91.2%, respectively. The mean survival for patients with αSMA positive tumours was 76 (58-94) ± 10 and with negative staining 160 (149-172) ± 6 months by overall survival of 154 (143-164) ± 5 months.

In univariate analysis all clinical pathological parameters as well as αSMA positivity were significantly associated with the tumour relapse (all <0.001). However, in multivariate analysis only tumour grade, stage and αSMA positivity in tumour cells remained as independent prognostic factor. Patients with an αSMA positive tumours has nearly two times higher risk to develop a postoperative relapse. (RR=1.776, $p = 0.014$).

Discussion

Correlation between αSMA positive CAF and tumour progression has been described in several types of cancer with exception of cRCC [9–13].

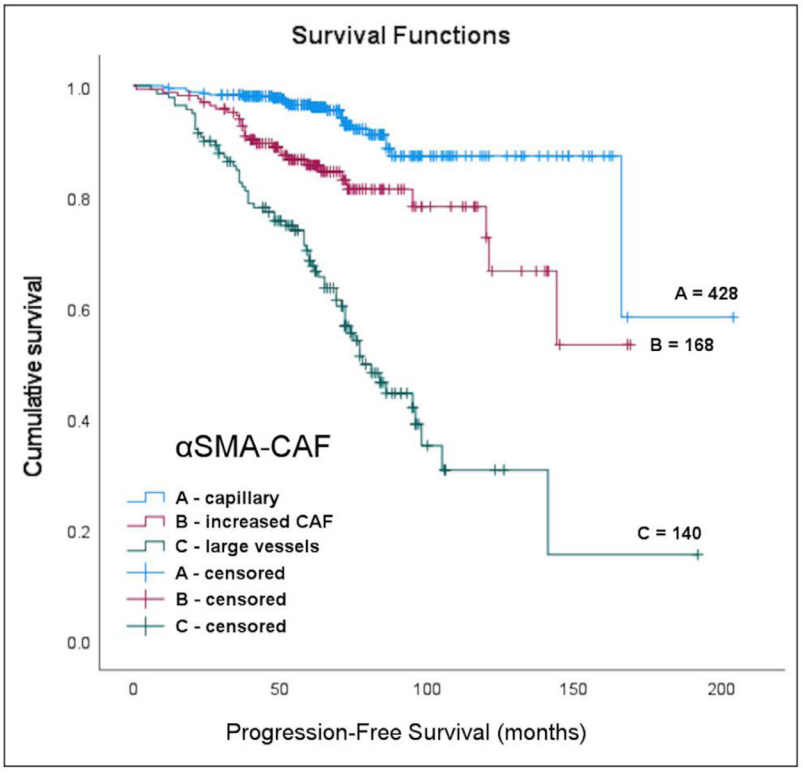

Fig. 3. Kaplan-Meier cox regression analysis shows distinct progression free survival for tumour groups of A, B and C ($p < 0.001$).(1.5 column).

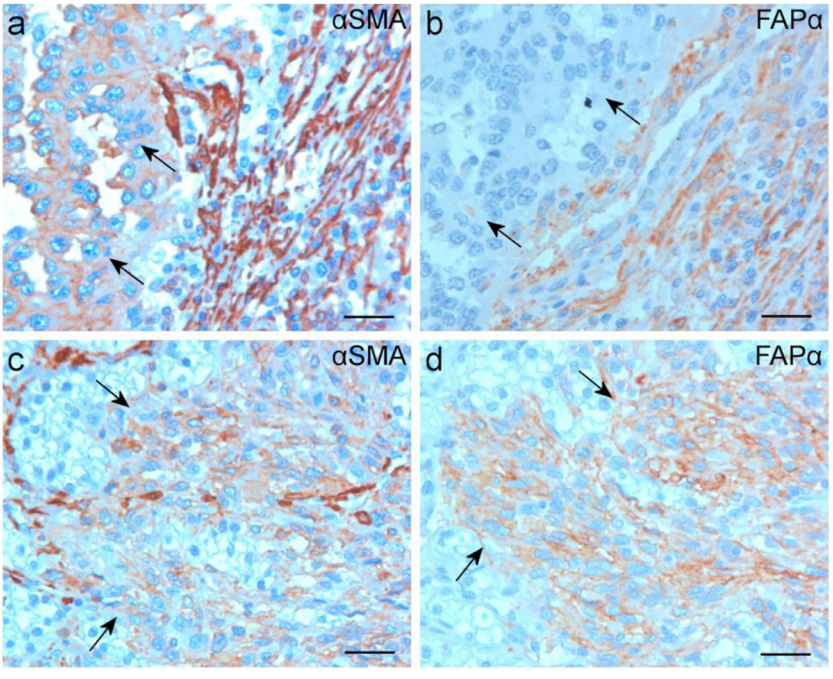

Fig. 4. Expression of αSMA and FAPα in cRCC. a, Positive αSMA staining in tumour cells (arrows) and in myofibroblasts. b, The same tumour area shows FAPα positive CAFs in fibrotic stroma, whereas tumour cells remain negative (arrows). c, Sarcomatous changes in cRCC displaying αSMA positive tumour cells (arrows) and also strong immunostaining of CAFs. d, The same tumour area shows FAPα positive tumour cells (arrows), but the CAFs remain negative. Scale bar: 30 μm. (1.5 column).

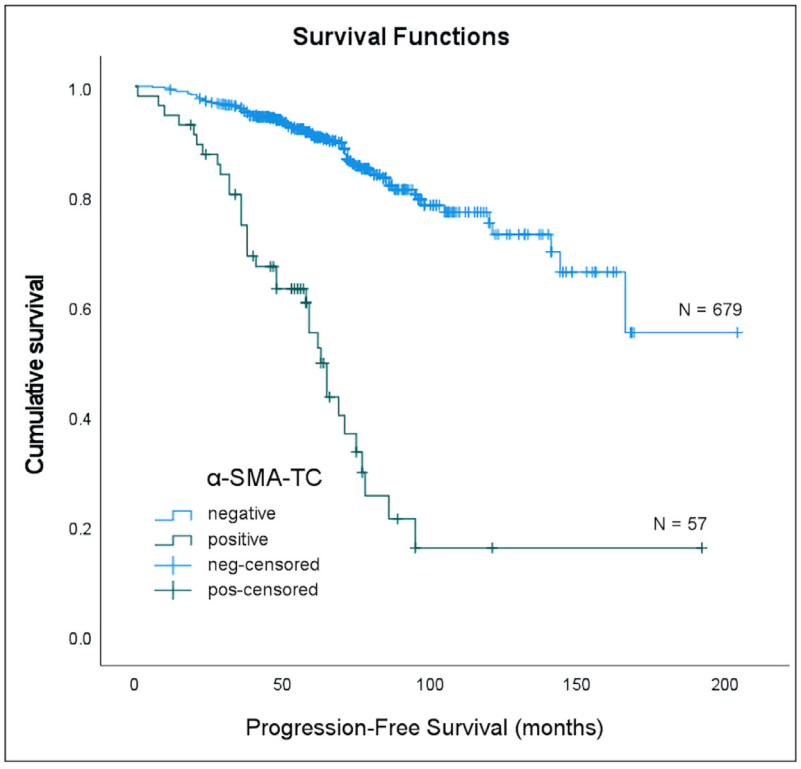

Fig. 5. Kaplan Meier plot showing the significant progression free survival between two groups of tumour depending on αSMA straining of tumour cytoplasma ($p < 0.001$). (αSMA-TC = αSMA positive or negative tumour cells). (1.5 column).

Table 2

Multivariate analysis of clinical pathological parameters and histological pattern of αSMA positive CAF in cRCC.

	RR	95.0% CI		*p*-value
		Lower	Upper	
Size				
≤4 cm				0.048
4<x≤7 cm	1.513	0.781	2.932	0.219
>7 cm	0.805	0.346	1.874	0.615
T1				0.452
T2	0.753	0.114	4.974	0.768
T3	1.638	0.370	7.251	0.516
G1				0.006
G2	1.923	1.186	3.117	0.008
G3	2.598	1.416	4.770	0.002
Stage I				0.026
Stage II	5.975	0.932	38.295	0.059
Stage III	7.999	1.774	36.061	0.007
CAF A				0.010
CAF B	1.332	0.746	2.376	0.332
CAF C	2.198	1.263	3.826	0.005

Although, we found a strong αSMA expression in CAF of cRCC, Kaplan-Meier spot and multivariate analysis did not show a significant correlation between αSMA positive CAFs and tumour progression. This finding is contrary to those obtained in other types of cancers. However, we found a significant association between vascular architecture marked by αSMA positive myo-fibroblasts and myo-endothelial cells and postoperative cRCC relapse (RR= 2.198, $p = 0.005$). Interestingly, only 6% of the 428 tumours showing regular vascular network (group A), whereas 46% of the 140 tumours displaying αSMA positive large tortous vessels (group C) progressed during the the median follow-up of 66±29 months. This can be explained by the fact that rapid growing tumours of group C have a substantially decreased blood vessel density and blood supply in relation to the number of cancer cells and consequently a hypoxemic condition triggering their growth and metastatic capacity [21].

Naïve fibroblasts maintain the normal tissue architecture and inhibit cancer development [22,23]. Transiently activated FAPα positive mesenchymal cells/fibroblasts play a role in embryonal development of kidney stroma as it was shown in this study. During tissue repair in wound healing local naïve fibroblasts are transiently activated until the end of healing process. [24]. However, CAFs remain perpetually activated in the inflammatory TME of malignant tumours [23]. The switch of tissue-resident normal fibroblasts into CAFs is suggested to be one of the important steps in progression of cancer [2]. In inflammatory TME endothelial cells can also undergo endothelial to mesenchymal transition to become CAF [25]. In addition to supporting tumour growth and progression, several other functions have been attributed to CAFs. CAFs have a central role in deposition of fibrillary proteins into ECM, produce matrix-degrading proteases and they play an important role in remodeling the ECM and in changing the TME to pro-metastogenic [26]. CAF communicate steadily with cancer cells via growth factors and inflammatory cytokines such as IL-6, TGFβ, FGF, HGF [6–8]. Cancer cells can also activate secretion of proinflammatory citokines and chemokines in CAF by paracrine mechanism [27]. CAF can trigger the production of MMPs with ECM remodeling capacities and can also promote epithelia to mesenchymal conversion of epithelial cells [28].

Recent investigations indicate that functions attributed to CAF might be executed by different subtypes such as myo-CAF and iCAF [3]. FAPα and αSMA antibodies can identify subsets of CAFs with different gene signature [4]. It was shown that FAPα expressing fibroblasts predominantly synthetize

ECM components such as COL1, COL3, EDA-FN and can secrete ECM degrading enzymes such as MMPs as well [4]. This indicates the possible role of FAPα positive fibroblasts in disruption of desmoplastic tumour stroma and in tumour progression. On contrary, αSMA expressing fibroblasts mediate contraction and stiffeness of tumour stroma by crosslinking the ECM collagens [29,30]. A subset of fibroblasts can also co-express αSMA and FAPα [31].

There are controversal data on tumour promoting or suppressing function of CAFs. CAF with high αSMA and/or high FAPα expression might have an opposite affect on tumour progression. There are conflicting reports on the αSMA positive CAFs which can act as pro- and anti-tumourigenic factor [32]. Depletion of αSMA expressing fibroblasts in transgenic mice resulted in increased aggressiveness of pancreatic adenocarcinoma [33]. The stromal elements such as CAFs rather restrained than supported progression of pancreas carcinoma [34]. Restoring the normal homeostasis between tumour cells and fibroblasts can lead to regulatory control and proliferation of cancer cells can be restrained or reversed [35]. The reciprocity between activated (CAF) and wound healing-like fibroblasts is regulated by their self-generated ECM which may promote or sustain tumour supportive programms [36].

The different expression of αSMA and FAPα in foetal and adult kidneys and in cRCCs indicates the presence of two types of activated fibroblast with distinct functions. FAPα positive stromal mesenchymal cells in the nephrogenic zone are progenitors for interstitium, smooth muscle and endothelial cells and are only transiently activated until the kidney stroma is developed. As FAPα did not expressed in adult kidneys, its expression in CAFs and myo-endothelial cells in cRCC can be evaluated as de novo expression during tumorigenesis. On the other hands, we found a strong αSMA expression in stromal fibroblasts of normal kidney and in cRCC but not in foetal kidneys. Therefore, a question arises, what is the difference in function between αSMA positive stromal fibroblasts in adult kidney and those located in tumour stroma [35].

In previous studies of distinct types of cancer both FAPα and αSMA expression has been detected exclusively in CAF, whereas tumour cells remained unstained [9–18]. In our study cytoplasmic αSMA expression was seen in tumour cells, and the positive cytoplasmic staining was significantly corrrelated with the postoperative tumour relapse (RR=1.776, $p = 0.014$). Out of 679 αSMA negative tumours only 12% showed a postoperative tumour relapse, whereas 60% of the αSMA positive tumours developed metastasis during the median follow-up of 66±29 months. Expression of αSMA and FAPα in cytoplasma of cRCC cells is a unique finding among distinct types of carcinoma.

This seemingly contradictory result between our study and published by others may be explained by the fact, that proximal tubules of kidney, the cRCC originates from, are developing from metanephric mesenchyme via mesenchymal to epithelial transition (MET) [37]. The stromal fibroblasts and endothelial cells of the kidney vasculature also develops from nephrogenic blastema [37]. Therefore, the cRCC should be regarded as a "carcinoma" of mesenchymal origin. The vast majority of cRCCs retain the polarized epithelial characteristics of proximal tubules, but some of them may undergo an epithelium to mesenchyme transition (EMT) by gradually loosing epithelial characteristics [38]. This transition may explain the strong positive cytoplasmic reaction of tumour cells with αSMA and also FAPα. Of interest, each of 44 gastric glomus tumours analysed previously by immunohistochemistry displayed strong positive αSMA staining in tumour cells [39–41]. The common characteristics of glomus tumours and cRCCs is their mesodermal origin.

Closing remarks

In the last years, efforts has been made to target activated fibroblast, but until now there is no breakthrough [42]. In cRCC only the vascular architecture, marked by αSMA positive fibroblasts and myoendothelial cells showed significant correlation with postoperative tumour relapse. This correlation rely on the vascular structure rather than αSMA positive cells. Our study showed that αSMA positive CAFs have no influence on cRCC progression. A small group of αSMA positive tumours indicates a risk of postoperative tumour relapse, and these cases might be taken into consideretion for anti-CAF therapy. However, we have to aware that a succesful threatment will target αSMA expressing normal kidney stroma and harm normal kidney structures. Our study indicates that cRCC, a "carcinoma" of mesodermal origin, substantially differs in several aspects from true carcinomas of ectodermal and endodermal origin.

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Institutional review board statement

The collection and use of all tissue samples for this study were approved by the Ethics Committee of the University Pecs, Hungary (No. 8466.PTE 2020).

Informed consent statement

Informed consent was obtained from all patients involved in this study.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Lehel Peterfi: Investigation, Data curation, Writing – original draft. **Maria V. Yusenko:** Formal analysis. **Gyula Kovacs:** Conceptualization, Supervision, Writing – review & editing. **Tamas Beothe:** Visualization, Writing – original draft.

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