Lung Cancer 155 (2021) 10–19

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Stromal FAP is an independent poor prognosis marker in non-small cell lung adenocarcinoma and associated with p53 mutation

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ARTICLE INFO

Keywords:
FAP
Fibroblast
Stroma
PDGFβR
α-SMA
Biomarkers
CD8
Non–small-cell lung cancer

ABSTRACT

Objectives: Fibroblasts regulate tumor growth and immune surveillance. Here, we study FAP, PDGFβR and α-SMA fibroblast markers in a well-annotated clinical cohort of non–small-cell lung cancer (NSCLC) for analyses of associations with immune cell infiltration, mutation status and survival.

Materials and Methods: A well-annotated NSCLC cohort was subjected to IHC analyses of stromal expression of FAP, PDGFβR and α-SMA and of stromal CD8 density. Fibroblast markers-related measurements were analyzed with regard to potential associations with CD8 density, cancer genetic driver mutations, survival and PD-L1 expression in the whole NSCLC cohort and in subsets of patients.

Results: High stromal FAP expression was identified as an independent poor prognostic marker in the whole study population (HR 1.481; 95 % CI, 1.012–2.167, $p = 0.023$) and in the histological subset of adenocarcinoma (HR 1.720; 95 % CI, 1.126–2.627, $p = 0.012$). Among patients with adenocarcinoma, a particularly strong association of FAP with poor survival was detected in patients with low stromal CD8 infiltration, and in other subpopulations identified by specific clinical characteristics; elderly patients, females, non-smokers and patients with normal ECOG performance status. α-SMA expression was negatively associated with CD8 infiltration in non-smokers, but none of the fibroblast markers expression was associated with CD8 density in the whole study population. Significant associations were detected between presence of p53 mutations and high α-SMA ($p = 0.003$) and FAP expression ($p < 0.001$).

Conclusion: The study identifies FAP intensity as a candidate independent NSCLC prognostic biomarker. The study also suggests continued analyses of the relationships between genetic driver mutations and the composition of tumor stroma, as well as continued probing of marker-defined fibroblasts as NSCLC subset-specific modifiers of immune surveillance and outcome.

1. Introduction

Experimental studies suggest that cancer-associated fibroblasts (CAFs) can affect the outcome of non-small lung cancer (NSCLC) by regulating e.g., proliferation, survival and metastatic potential [1–3] of malignant cells as well as by influencing angiogenesis [4] and immune surveillance [5].

A series of recent studies have high-lighted the existence of clinically relevant differences in the composition of the stroma compartment of common solid tumors [6]. Importantly, these differences have been found to impact both the natural course of the disease as well as response to treatment. Some of these studies, performed in NSCLC and indicate prognostic impact of certain marker-defined fibroblast subsets [7–9]

Commonly used fibroblast markers for characterization of clinical

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https://doi.org/10.1016/j.lungcan.2021.02.028
Received 18 December 2020; Received in revised form 12 February 2021; Accepted 20 February 2021
Available online 25 February 2021

samples include platelet-derived growth factor-alpha receptor (PDGF-αR), platelet-derived growth factor-beta receptor (PDGF-βR), fibroblast activation protein (FAP) and alpha-smooth muscle action (α-SMA). The PDGF/PDGFR axis is one of the best-described pathways controlling growth and migration of fibroblasts in developmental settings and in common solid tumors [10,11]. FAP is a well-studied marker of fibroblasts which is up-regulated in cancer-associated fibroblasts, and has lately been subjected to various targeting approaches [12,13].

Novel fibroblast subsets [14] have been defined by fluorescence-activated cell sorting (FACS), multi-marker immuno-histochemistry / fluorescence (mIHC) and single cell RNA seq [15–17]. Some of these studies have also contributed to functionally define these subsets, revealing the existence of novel candidate immune-regulatory subsets [5]. However, most of these analyses rely on pre-clinical models. Potential interactions and associations between fibroblast and immune cells in clinical samples, e.g. as determined e.g. by IHC analyses, therefore remain valid study topics.

The biological basis for the recognized heterogeneity in fibroblast composition remains poorly understood. Candidate factors include tumor-site properties, patients-related factors and cancer genes driver mutations, as implied by recent mouse model studies [18].

In our study, we subjected a clinically well-annotated NSCLC collection to analyses of the fibroblasts' composition using as markers PDGF-βR, FAP and α-ASMA. Associations between these markers and survival, CD8 status and driver mutations are reported.

2. Material and methods

2.1. Patients and clinical samples

Patients affected by non-small cell lung cancer (NSCLC) and undergoing surgery at Uppsala University Hospital between 2006 and 2011 were retrospectively selected [19].

Formalin-fixed and paraffin-embedded blocks from pre-chemotherapy surgeries were retrieved and representative areas were selected on a section stained with Hematoxylin and eosin (H&E) by a pathologist and the same area on the block was punched and stored in a receiving TMA block. A full description of the TMA construction method has been described before, see ref. [20].

The fourth edition of the World Health Organization Classification of Lung Tumors was used to re-evaluate the cases and exclude tissue samples with major staining artifacts or uninterpretable images by a lung pathologist [21]. Clinicopathological and survival data were obtained from the patients' records stored in the Uppsala-Örebro Regional Lung Cancer Registry and collected in a database.

This study was conducted according to the Swedish Biobank Legislation and Ethical Review Act (reference 2012/ 532, Ethical Review Board in Uppsala).

2.2. Immunohistochemistry

Immunohistochemistry (IHC) detection of FAP was performed on a Ventana autostainer (Ventana Benchmark Discovery, NexES V10.6; Tuscon, AZ, USA) using the rat antibody against human FAP (1:100 dilution, D8 Cat MABS1001, Vitatex, Stony Brook, NY) diluted in Antibody Diluent Buffer (Antibody diluent, DAKO). The staining protocol included a standard antigen retrieval step with CC1/pH9 buffer (Discovery CC1, Ventana), incubation with the primary antibody for 1 h at room temperature (RT) and incubation with the secondary antibody (UltraMab anti-rat-HRP, Ventana) for 24 min at RT. Secondary antibodies were detected by DAB.

IHC procedures combining Rabbit mAb PDGFR-β (1:100 dilution, Clone 28E1 #3169, Cell Signaling Technology, Danvers, MA) or Mouse mAb α- SMA (1: 300 dilution, Clone 1A4 M0851-, DAKO Inc, Glostrup, Denmark) with mouse mAbCD34 (Clone QBEnd 10, M7165-, DAKO Inc, Glostrup, Denmark) antibodies have been described previously [22,23]. Secondary antibodies were detected with VectraBlue (PDGFR-β and α-SMA) and VectraRed (CD34).

IHC detection of CD8 was performed manually using the mouse monoclonal antibody against human CD8A (1:250 dilution; Atlas Antibodies AMAb90883) and counterstaining with hematoxylin. A full IHC protocol can be found on the website of the human protein atlas (ref. http://www.proteinatlas.org).

IHC staining of PD-L1 was performed following the manufacturers' instruction with PD-L1 antibody from Agilent (Clone 22C3, pre-prepared dilution), including antigen retrieval at pH6. The procedure was performed on a DAKO autostainer.

2.3. Scoring of immunohistochemistry

The intensity of FAP staining was scored in each core on a 4-grade scale by two independent researchers as described in Supp. Table 2 and Supp. Fig. 1. The resulting case-based score was the mean of the core-based scoring. For dichotomization of FAP, patients-based scores <2 were considered as "Low" (25 % of the total cohort) and scores ≥ 2 were noted as "High" (75 % of the total cohort).

Data on PDGFβR and α-SMA intensity was collected and scored based on digital image analyses of samples double-stained for the fibroblast marker and CD34 as vessel marker, as previously described [24]. For dichotomization of PDGFβR- and α-SMA-scores, the cohort was divided into the lowest 25 % of marker intensity ("Low" group) and the highest 75 % of the marker intensity ("High" group) in the total population.

CD8 density was manually scored based on cell counting of the entire cores at 20x scanning magnification both in stromal and in epithelial areas. The resulting core-based scoring was the total count of the CD8 positive cell identified per single core, while the resulting case-based (patient-based) scoring was the mean of the core-based scoring. Patients-based scoring was then categorized in low and high using the median value of the cores scores as cut-off ($\tilde{x} = 15$).

The PD-L1 score in the stroma compartment was calculated by dividing the positive stroma cells by all stroma cells (immune cells, fibroblasts, endothelial cells, etc.). In the tumor compartment, the common annotation of cancer cell staining, used in clinical diagnostics (tumor proportion score) was used, which is the percentage of viable tumor cells showing a partial or complete membrane staining. Scoring increments were 0%, 1%, 5%, 10 %, 15 %, 20 %, 25 %, 30 %, 35 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, and 100 %. The overall PD-L1 score per patient was calculated as the average of the stroma and tumor compartment scores.

All scoring was done blinded to outcome data.

2.4. Statistical methods

Correlations between categorical variables were calculated using Chi- square test and correlations between continuous variables were calculated using Spearman's Ranks test. Differences in overall survival curves were analysed with Log-rank test with results reported through Kaplan- Meier graphs. Cox Regression proportional hazard models in uni- and multi-variable analyses were used to calculate Hazard ratios (HR) for clinic-pathological factors, CD8 density and fibroblast markers. *P* values were considered significant when lower than 0.05. Statistical analyses were performed at the 95 % CI, using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA).

3. Results

3.1. Inter-case-variability of fibroblast markers and associations with clinico-pathological characteristics

To characterize fibroblast marker expression in NSCLC a population-based tissue collection was employed. This collection consists of 351 cases of NSCLC diagnosed between 2006 and 2010, and has been used in

Table 2
Correlations between expressions intensity of FAP, PDGFβR and α-SMA with the top ten most frequently mutated genes (Chi-square exact test).

Top 10 mutated genes	FAP			PDGFβR			α-SMA		
	Low	High	P	Low	High	P	Low	High	P
1.TP53			0.000			0.081			0.003
Wt	51	87		19	43		37	74	
Mutated	39	160		20	86		32	145	
2.MUC16			0.325			0.126			0.040
Wt	56	139		28	73		48	118	
Mutated	37	117		14	64		25	109	
3.CSMD3			0.092			0.354			0.743
Wt	71	171		32	90		50	154	
Mutated	20	78		10	41		20	68	
4.KRAS			0.227			0.866			0.490
Wt	61	185		30	96		49	162	
Mutated	32	71		12	41		24	65	
5.LRP1B			0.077			0.529			0.716
Wt	72	178		32	98		50	162	
Mutated	18	75		9	36		21	61	
6.STK11			0.672			0.512			0.110
Wt	79	215		36	112		57	195	
Mutated	11	35		5	22		14	27	
7.EGFR			0.145			0.370			0.250
Wt	81	236		41	129		66	214	
Mutated	12	20		1	8		7	13	
7.KEAP1			0.577			0.487			0.131
Wt	81	216		34	117		58	196	
Mutated	12	39		8	20		15	30	
8.KMT2D			0.485			0.887			0.890
Wt	84	219		37	120		64	196	
Mutated	9	31		5	15		8	26	
9.PTPRD			0.075			0.852			0.068
Wt	87	221		39	126		69	196	
Mutated	6	34		3	11		4	30	
10.ATM			0.030			0.639			0.677
Wt	89	223		38	127		65	204	
Mutated	4	31		4	10		6	23	

previous biomarker studies (see e.g. [25–27]).

As shown in Supp. Table 1, the population display typical composition for such cohorts with 67 years as average age at diagnosis, 49.6 % males and 11.4 % in the never smoker group. Regarding NSCLC histology, adenocarcinoma dominated and consisted of 59.5 % of the population.

In general, large inter-case variability of fibroblast marker expression was detected. Selected cases with high and low status of the three markers are shown in Fig. 1.

Subsequent analyses explored potential associations between marker status and clinic-pathological characteristics. As shown in Table 1, none of the markers showed any associations with age, sex or smoking history. However, higher FAP and α-SMA intensity, but not PDGFβR intensity, showed a significant association with squamous cell carcinoma (SCC) histology (Table 1).

3.2. Internal associations between fibroblast markers and their relationship to CD8 infiltration and PD-L1

A further set of analyses explored the intra-case correlations of the fibroblast metrics with each other, and with stromal infiltration of CD8-positive cells.

When considering the whole population, FAP, PDGFβR and α-SMA stroma intensities indicated moderate positive association with each other (FAP / PDGFβR intensities corr. coeff. 0.426; FAP / α-SMA intensities corr. coeff. 0.475 and PDGFβR / α-SMA intensities corr. coeff. 0.478; all p-values <0.01; Fig.2).

Explorative analyses in NSCLC subsets related to clinical characteristics indicated some differences in the strengths of these associations. A particularly strong association was found in women between PDGFβR stroma intensity and α-SMA stroma intensity (corr. coeff. 0.561, $p < 0.01$; Supp. Fig. 2A). In contrast, the non-smoker subset showed largely independent expression between PDGFβR stroma intensity and α-SMA stroma intensity (corr. coeff. 0.158, $p = 0.589$; **Supp** Fig. 2B) and FAP intensity (corr. coeff. 0.149, $p = 0.612$; **Supp.** Fig. 2**B**)

Stromal CD8 density was not significantly associated with any of the fibroblast markers in analyses considering the whole population (Fig. 2). However, explorative subset-analyses identified some notable subset-specific associations between stromal CD8 status and fibroblast characteristics. A particularly strong negative association was detected within the non-smoker subset of the whole population between α-SMA stroma intensity and infiltration of stromal CD8-positive cells (corr. coeff. -0.501, $p = 0.007$; **Supp.** Fig. 2B). Even stronger negative correlation between α-SMA stroma intensity and infiltration of stromal CD8-positive cells was found in non-smoker patients with adenocarcinoma histology (corr. coeff. -0.733, $p = 0.001$; **Supp.** Fig. 2B).

PD-L1 expression, analyzed in the whole tumor or separately in the tumor stroma, did not show strong associations (corr. coeff. > 0.25 and p** (<0.01)) with any of the fibroblast markers when analyzed in the whole population (**Supp. table 3A**). Subset-specific analyses demonstrated strong positive associations among non-smokers between total and stromal PD-L1 and FAP intensity and between total PD-L1 and ASMA intensity. Furthermore, total and stromal PD-L1 was associated with FAP intensity in females (**Supp. table 3B**).

3.3. Associations between fibroblast markers and mutations in the total NSCLC population

Previous analyses of this cohort have characterized cases with regard to mutation status of 82 commonly mutated lung cancer genes [28]. This allowed us to explore the relationship between somatic cancer mutations and fibroblast-determined stroma phenotypes. For this purpose, correlation analyses were performed between the ten most commonly mutated genes and the three fibroblast markers.

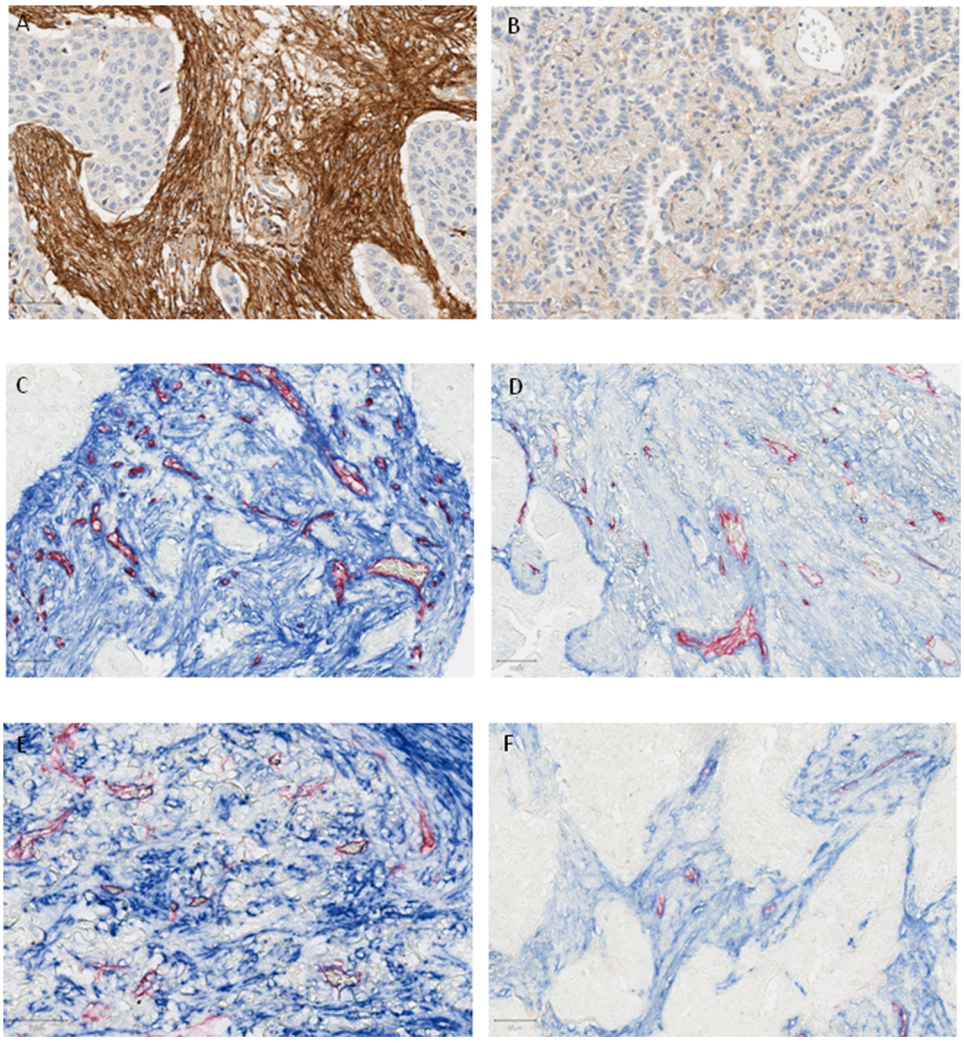

Fig. 1. Micro-photographs of TMA cores from NSCLC patients with high (left part) and low (right part) intensity expression of FAP, PDGFβR and α-SMA. A-B) High and Low FAP (FAP detected with DAB (brown) on hematoxylin stained slides); C-D) High and Low PDGFβR (PDGFβR detected with VectraBlue (blue) on slides also stained with CD34 (CD34 detected with VectraRed); E-F) High and Low α-SMA (α-SMA detected with VectraBlue (blue) on slides also stained with CD34 (CD34 detected with VectraRed). Scale bar 50 μm.

As shown in Table 2, a strong statistically significant positive correlation was detected between presence of mutations in p53 gene and high stroma intensity of FAP ($p < 0.001$) and of α-SMA ($p = 0,003$). A trend for a positive correlation between high stroma intensity of PDGFβR and presence of p53 mutations was also detected ($p = 0.081$).

Furthermore, MUC1 mutations was associated with high α-SMA intensity ($p = 0.040$), whereas ATM mutation was associated with high FAP intensity. ($p = 0.030$).

3.4. *Associations between fibroblast markers and survival in total NSCLC population and in sub-populations*

Initial analyses of potential associations between fibroblasts markers-related metrics and survival were performed on the whole study population.

According to Log-Rank tests high expression of α-SMA was significantly associated with poor survival in the whole population ($p = 0.041$). High stroma intensity of FAP and PDGFβR showed trends of being associated with shorter survival ($p = 0.073$ and $p = 0.078$, respectively), Fig. 3**; panel A–C (left)**.

These results were confirmed by uni-variable Cox-Regression analyses for high FAP (HR 1.352; 95 % CI, 0.969–1.887, $p = 0.076$; **Supp. table 4A**) PDGFβR (HR 1.525; 95 % CI, 0.947–2.456, $p = 0.083$; **Supp. table 4B**) and α-SMA (HR 1.481; 95 % CI, 1.012 – 2.167, $p = 0.043$; **Supp. table 4C**).

Multivariable analyses with adjustment for clinic-pathological characteristics, identified high FAP intensity as the only independent fibroblast marker of shorter survival (HR 1.481; 95 % CI, 1.012–2.167, $p = 0.023$; **Supp. table 4A**).

To investigate if fibroblast markers were associated with survival in sub-groups of NSCLC a set of additional analyses were performed. As shown in Forest plots of Fig. 3 **A-right)**, HRs for significant associations with poor survival were detected for high FAP stroma intensity in the sub-population of adenocarcinoma patients ($p = 0.011$ and HR 1.678; 95 % CI 1.128–2.496), of patients with normal Eastern Cooperative Oncology Group (ECOG) performance status ($p = 0.016$ and HR 1.867; 95 % CI 1.122–3.108) and CD8 stromal low infiltration cases ($p = 0.026$ and HR 1.714; 95 % CI 0.866–3.692). HRs for significant associations with poor survival were also observed for high PDGFβR stroma intensity in specific sub-groups; adenocarcinoma patients ($p = 0.042$ and HR 1.890; 95 % CI 1.024–3.487), ex-smokers ($p = 0.015$ and HR 3.209; 95 % CI 1.254–8.212) and CD8 stromal low infiltration cases ($p = 0.048$ and HR 2.003; 95 % CI 1.005–3.992); (Fig.3**B-right**). Regarding α-SMA stroma intensity, HRs were significant in the sub-populations of younger patients ($p = 0.043$ and HR 1.861; 95 % CI 1.019–3.399), and low CD8 stromal infiltration cases ($p = 0.024$ and HR 1.935; 95 % CI 1.092–3.430); **(**Fig.3**C-right).**

3.5. *Associations between fibroblast markers and survival in subsets of the adenocarcinoma population*

The Cox-regression-based poor-survival associations of high FAP and

Table 1
Correlations between fibroblast marker intensity and clinicopathological variables in NSCLC patients (Chi-square exact test).

Variables	FAP (n = 351)			PDGFβR (n = 180)			α-SMA (n = 301)		
	Low (n = 94)	High (n = 257)	P	Low (n = 43)	High (n = 137)	P	Low (n = 74)	High (n = 227)	P
Age (42−84)			0.971			0.304			0.304
<67	43	117		23	61		38	101	
>or 67	51	140		20	76		36	126	
Gender			0.177			0.454			0.575
Male	41	133		22	79		37	122	
Female	53	124		21	58		37	105	
Smoking status			0.098			0.511			0.654
Lifelong Non-Smoker	14	26		5	9		8	20	
Current Smoker	39	139		22	79		36	124	
Previous (>1 year)	41	92		16	49		30	83	
ECOG			0.752			0.248			0.829
Normal	155	54		19	75		45	129	
Slightly	99	38		24	60		28	95	
In bed<50 %	3	2		0	2		1	3	
Histology			0.000			0.219			0.004
Squamous Cell Carcinoma	11	92		10	52		11	85	
Adenocarcinoma	74	135		26	69		54	114	
Large Cell Carcinoma	8	22		6	11		7	22	
Adenosquamous	1	5		0	3		1	4	
Sarcomatoid	0	3		1	2		1	2	
pTNMStage			0.077			0.396			0.125
I A	36	106		18	48		32	82	
I B	23	53		11	24		14	50	
II A	5	35		3	22		3	33	
II B	13	21		6	15		9	21	
III A	12	37		4	24		15	33	
IV	5	5		1	4		1	8	

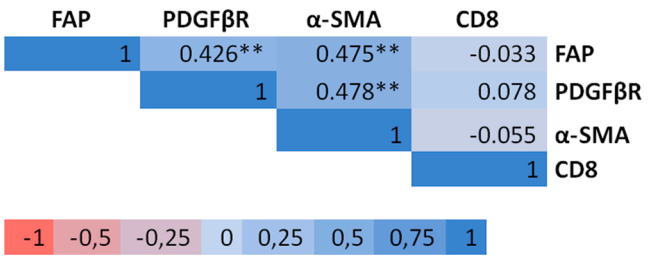

Fig. 2. Intra-case correlation between fibroblast markers and between fibroblast markers and stromal CD8 density in NSCLC patients. Figure shows results from Spearman two-tailed test and ** indicates $p < 0.01$.

PDGFβR stroma intensity in adenocarcinoma (ADC) prompted additional analyses in this subgroup. Links between fibroblast markers and survival were confirmed by Log-Rank tests (Fig. 4**-left**).

Multivariable analyses with adjustment for clinic-pathological characteristics, identified high FAP intensity as the only independent fibroblast marker of shorter survival in adenocarcinoma patients (HR 1.720; 95 % CI, 1.126–2.627, p = 0.012; **Supp. table 5A–C**).

Continued analyses were performed on subsets of the adenocarcinoma population (Fig. 4 **A–C-right**).Concerning FAP, poor prognosis associations of high FAP stroma intensity was observed in older patients (p = 0.031 and HR 1.765; 95 % CI 1.053–2.957), in women (p = 0.022 and HR 1.877; 95 % CI 1.096–2.496), in non-smoking patients (p = 0.024 and HR 5.732; 95 % CI 1.258–26.114), in patients with normal ECOG performance status (p = 0.012 and HR 2.118; 95 % CI 1.182–3.794), and in patients with CD8 low stromal infiltration (p = 0.014 and HR 2.064; 95 % CI 1.159–3.677).

All these associations of high FAP stroma intensity with poor OS remained significant in multivariable analyses (**Supp. table 6-10**).

Regarding the other markers, a significant survival association was detected for high PDGFβR stroma intensity and low OS in the subpopulation of adenocarcinoma patients with CD8 low stroma infiltration (p = 0.023 and HR 3.065; 95 % CI 1.169–8.034); (Fig. 4 **B -right**). However, this association of PDGFβR with OS was not significant at multivariable analyses (p = 0.152 and HR 1.648; 95 % CI 0.768–3.536) (**Supp. table 11**).

4. Discussion

Our study analyses the fibroblast status of surgically resected NSCLC tumor tissues, derived from a population-based collection, performed at the Uppsala University Hospital between 2006 and 2011.

Correlation analyses of FAP, PDGFβR and α-SMA staining revealed a moderately dependent expression of these three markers with each other within the whole population, which is consistent with previous findings [29]. Explorative subset analyses indicated some differences in the strength of these associations, suggesting that the mechanisms determining fibroblast composition may vary between men and women [30, 31] and between smokers and non-smokers [32].

Analyses on possible relationships between fibroblast status and $CD8^+$ cells infiltration in the whole study population failed to detect strong associations. Again, these findings are similar to an earlier study reporting Spearman correlation values below 0.2 for CD8 and the three fibroblast markers in a population of unselected stage IA-IIIA NSCLC patients [33]. Subset-analyses of the present study noted a negative association between $\alpha\text{-SMA}^+$ and $CD8^+$ cells in non-smoking patients. These findings are compatible with specific immune-cells and fibroblasts interactions in the high-mutation-load smoking population and merit further studies in additional cohorts.

While earlier studies on fibroblasts in lung cancer have not analyzed potential relationships between driver mutations and the fibroblast landscape, our study detects significant positive associations between p53 mutations and high stroma intensity of FAP and α-SMA. This finding links a well-known association of smoking and p53 mutations on the genetic level in tumor cells, to a phenotypic association between p53 mutations and a stomal fibroblast reaction. We thus hypothesize that the fibroblast composition of lung cancer specifically, and solid tumors in

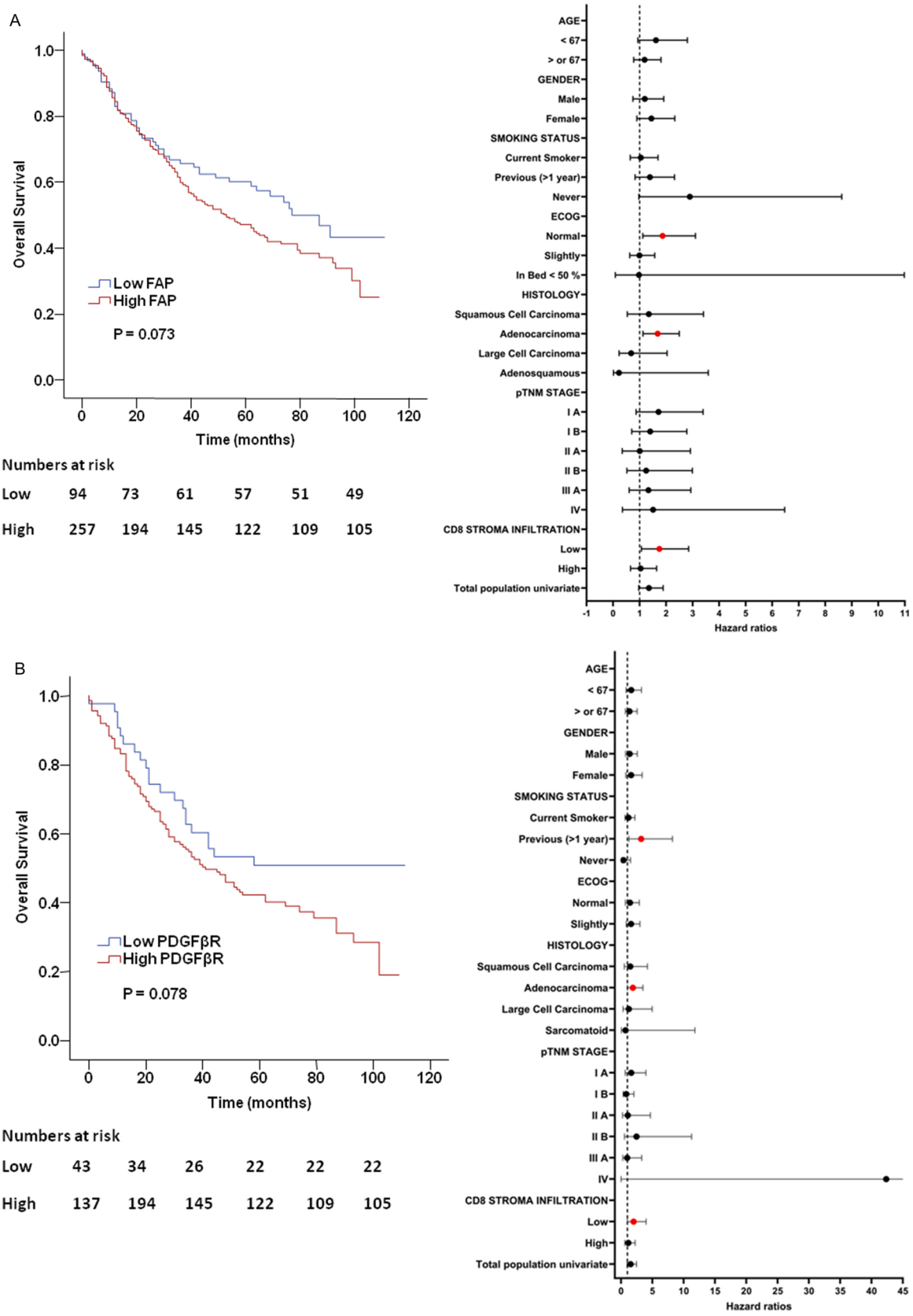

Fig. 3. Survival associations of markers in the whole NSCLC study population and in NSCLC subsets. (A—C left part) Kaplan-Meier graphs for FAP, PDGFβR and α-SMA. (A—C right part). Forest plots showing the HRs, as determine by uni-variable Cox-Regression analyses, including confidence intervals, in clinical subgroups of NSCLC.

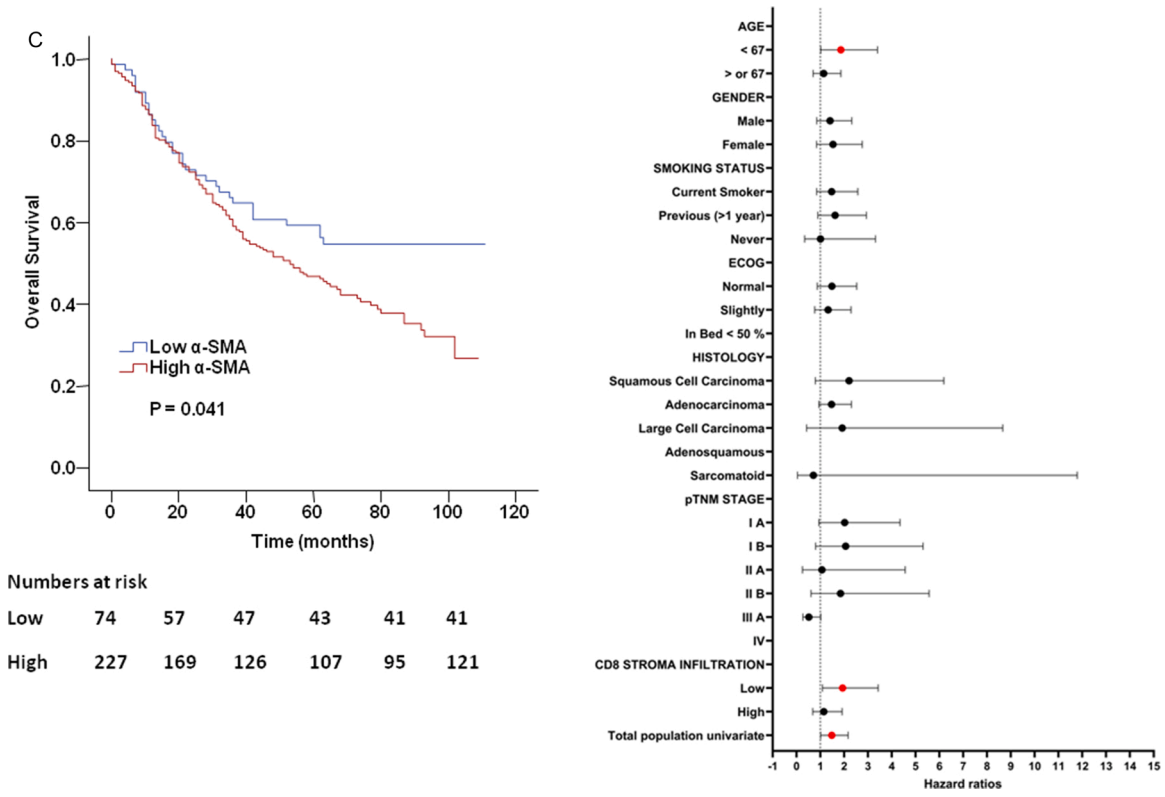

Fig. 3. (*continued*).

general, is partially determined by the genetic status of the malignant cells. Notably, emerging pre-clinical studies are also implying specific associations between genetic make-up of malignant cells and fibroblast composition of tumor stroma [18]. Future studies should investigate to what extent this finding is relevant for other tumor types as well.

High FAP intensity was identified in this study as a NSCLC stroma characteristic significantly associated with poor prognosis in multivariable analyses. Separate analyses of ADC and SCC demonstrated a similar statistically significant association between high FAP intensity and survival in ADC, but not in SCC.

FAP has been analyzed with regard to survival associations in earlier NSCLC studies [33,34] and identified as a good prognosis marker in SCC (in univariate analyses) [34] and in cases with high infiltration of CD8 cells (in multivariable analyses) [35]. Some differences exist between the present study and earlier studies, residing on the antibody used for FAP analyses, and on the scoring system, which in earlier studies referred to fraction of positive stroma cells, whereas in our study relies on FAP intensity. Collectively, the analyses regarding FAP in NSCLC still appear inconclusive and further studies are warranted.

Concerning survival associations of PDGFβR^{+} fibroblasts, this study detected a trend towards poor prognosis in the whole population, and a significant poor prognosis association in the subsets of patients with adenocarcinoma histology, in ex-smokers and in patients with low tumoral CD8 infiltration. Earlier studies on two other lung cancer cohorts with a similar focus, yielded inconclusive results but did also detect trends for poor prognosis association with PDGFβR expression in the ADC group of one of the cohorts (HR 1.45 (0.96–2.19)) [35]. Notably, this earlier study identified PDGFβR (high)/ PDGFαR (low) as a stroma characteristic associated with poor prognosis in survival analyses also considering tumor stage [35]. Possibly, a more stringent or refined method for determining PDGFβR status, will be able to identify more robust and conclusive survival associations between this marker and survival in NSCLC.

Our study represents an attempt to identify potential tumor microenvironment-derived biomarkers in lung cancer. The modest strength of the survival association signals, overall in agreement with earlier studies, suggest that more stringent high-resolution and high-content methods might be needed to uncover the biomarker potential of CAFs in lung cancer. Despite that, the present study identifies a set of subset-specific association between CAF subtypes and features such as immune cells infiltration and cancer cell driver mutations as P53, which merit further experimental exploration. Finally, the smoker-status-dependent correlation among different CAF subsets, and between CAF-subsets and CD8 status, imply that CAF composition can be traced back to known etiological mechanisms, such as cigarette smoke. Notably, this concept also suggests future experimental studies that can uncover previously unrecognized biological mechanisms.

5. Conclusion

Our analyses identified some previously unrecognized associations between cancer-associated fibroblasts markers and CD8 status in patients with NSCLC. Furthermore, novel associations were detected between p53 mutations and tumor fibroblast features, suggesting interdependencies between driver mutations and stroma features. Finally, high FAP expression was identified as an independent poor prognosis marker. In general terms, this study supports the emerging concept of clinically relevant and functionally distinct populations of fibroblasts.

Declaration of Competing Interest

Dr. Christian Klein declares employment, patents and stock ownership with Roche. All other authors declare no competing interests.

CRediT authorship contribution statement

Pablo Moreno-Ruiz: Conceptualization, Data curation,

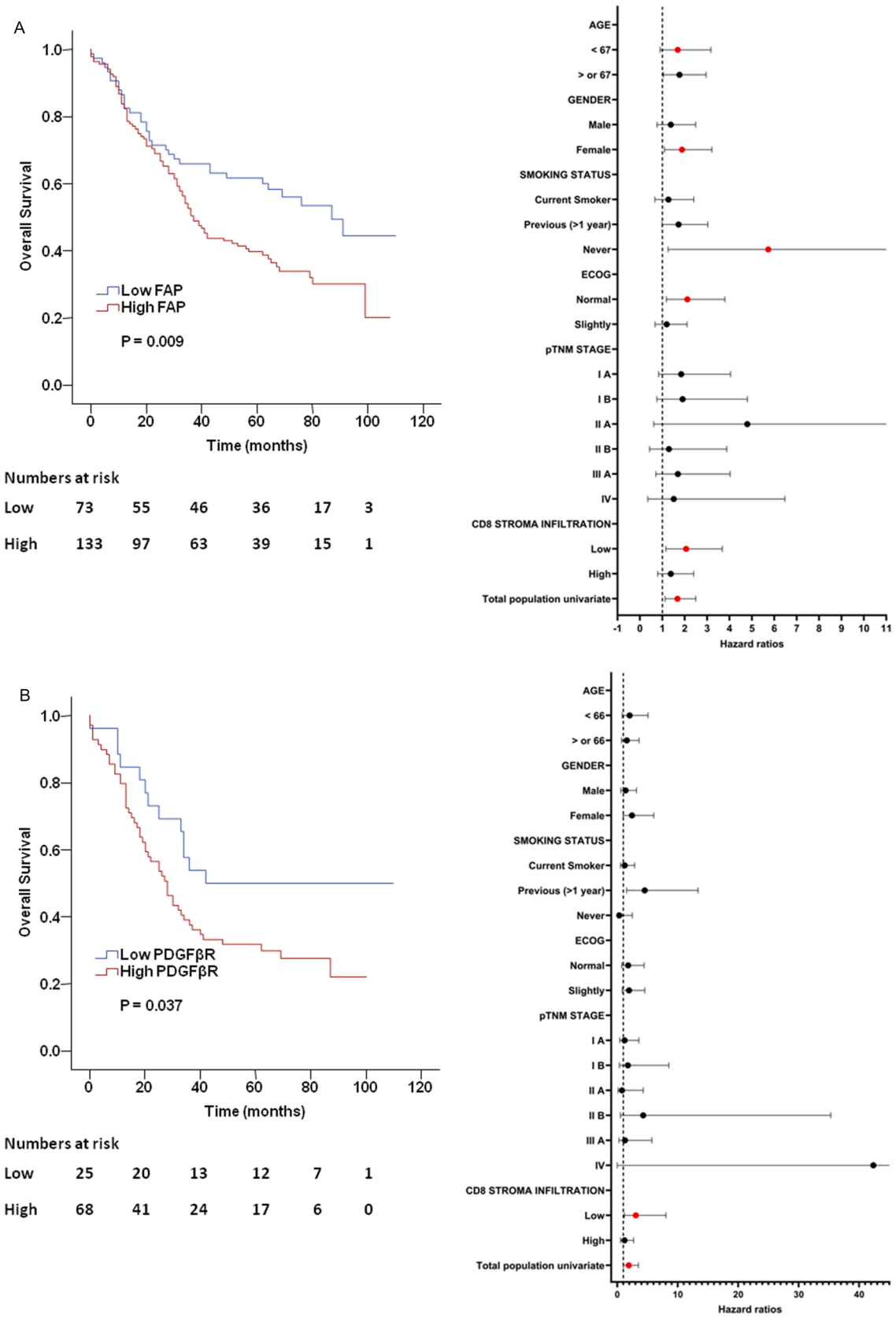

Fig. 4. Survival associations of markers in ADC and in ADC subsets. (A—C left part) Kaplan-Meier graphs for FAP, PDGFβR and α-SMA. (A—C right part) Forest plots showing the HRs, as determine by uni-variable Cox-Regression analyses, including confidence intervals, in clinical subgroups of NSCLC.

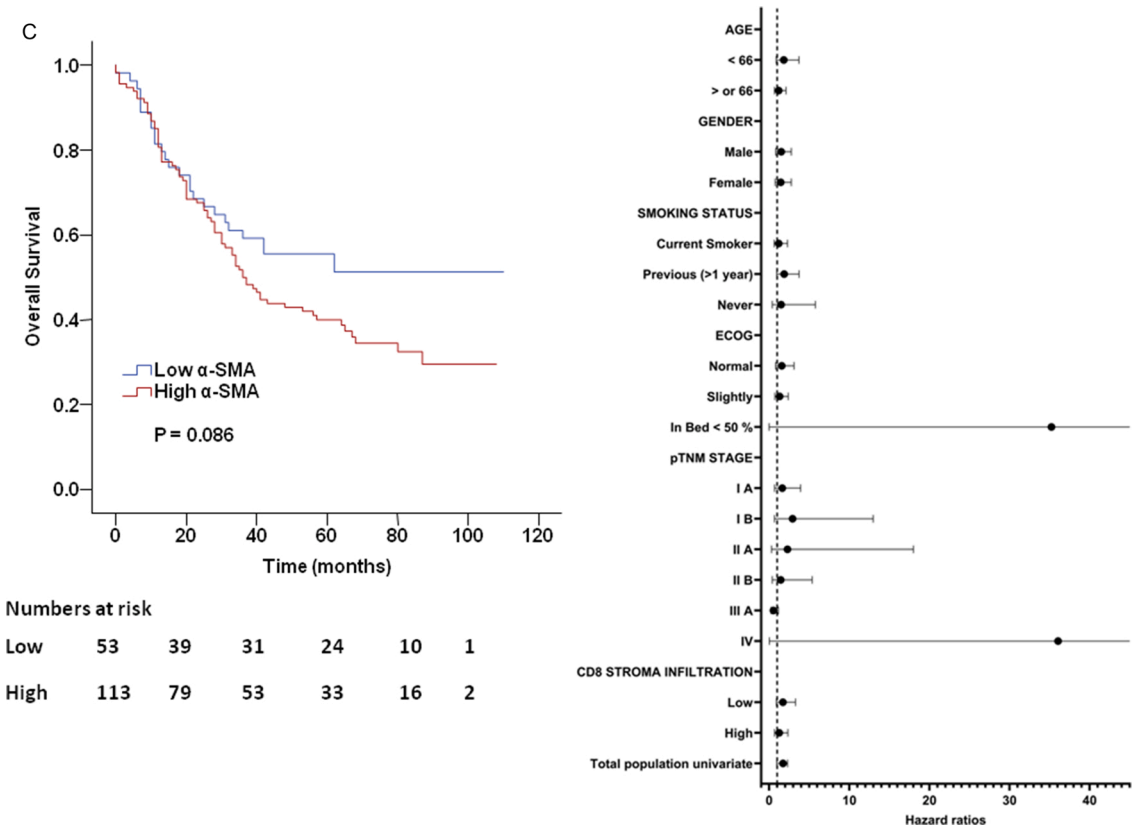

Fig. 4. (*continued*).

Methodology, Writing - original draft, Writing - review & editing. **Sara Corvigno:** Conceptualization, Data curation, Supervision, Writing - original draft, Writing - review & editing. **Nienke C. te Grootenhuis:** Data curation, Methodology, Writing - review & editing. **Linnéa La Fleur:** Data curation, Writing - review & editing. **Max Backman:** Data curation, Methodology, Writing - review & editing. **Carina Strell:** Methodology, Writing - review & editing. **Artur Mezheyeuski:** Methodology. **Gabriele Hoelzlwimmer:** Writing - review & editing. **Christian Klein:** Writing - review & editing. **Johan Botling:** Conceptualization, Supervision, Writing - review & editing. **Patrick Micke:** Conceptualization, Project administration, Supervision, Writing - review & editing. **Arne Östman:** Conceptualization, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Acknowledgements

Members of the AÖ group are acknowledged for constructive criticism throughout the project. Studies in the AÖ research group were supported by grants from Cancerfonden - contract numbers CAN 2015/707 and CAN 2018/831 (Sweden) and The Knut and Alice Wallenberg Foundation -contract number KAW 2015.0063 (Sweden).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2021.02.028.

References

[1] A.M. Richardson, L.S. Havel, A.E. Koyen, et al., Vimentin is required for lung adenocarcinoma metastasis via heterotypic tumor cell–Cancer-associated fibroblast interactions during collective invasion, Clin. Cancer Res. 24 (2018) 420–432, https://doi.org/10.1158/1078-0432.CCR-17-1776.

[2] W.J. Chen, C.C. Ho, Y.L. Chang, et al., Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling, Nat. Commun. 5 (2014), https://doi.org/10.1038/ncomms4472.

[3] T. Fang, H. Lv, G. Lv, et al., Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer, Nat. Commun. 9 (2018), https://doi.org/10.1038/s41467-017-02583-0.

[4] K. Asano, C.M. Nelson, S. Nandadasa, et al., Stromal versican regulates tumor growth by promoting angiogenesis, Sci. Rep. 7 (2017), https://doi.org/10.1038/s41598-017-17613-6.

[5] H. Xiang, C.P. Ramil, J. Hai, et al., Cancer-Associated Fibroblasts Promote Immunosuppression by Inducing ROS-Generating Monocytic MDSCs in Lung Squamous Cell Carcinoma, Cancer Immunol. Res. 8 (2020) 436–450, https://doi.org/10.1158/2326-6066.CIR-19-0507.

[6] J. Paulsson, P. Micke, Prognostic relevance of cancer-associated fibroblasts in human cancer, Semin. Cancer Biol. 25 (2014) 61–68, https://doi.org/10.1016/j.semcancer.2014.02.006.

[7] M.J. S, C.J. C, B.N. B, et al., Thy-1+ cancer-associated fibroblasts adversely impact lung Cancer prognosis, Sci. Rep. 7 (6478) (2017).

[8] J. Alcaraz, J.L. Carrasco, M. Laura, et al., MA15.10 stromal markers of activated tumor associated fibroblasts predict poor survival and are associated with necrosis in non-small cell lung Cancer, J. Thorac. Oncol. 14 (2019) S312, https://doi.org/10.1016/j.jtho.2019.08.626.

[9] K. Edlund, C. Lindskog, A. Saito, et al., CD99 is a novel prognostic stromal marker in non-small cell lung cancer, Int. J. Cancer 131 (2012) 2264–2273, https://doi.org/10.1002/ijc.27518.

[10] Paulsson J., Sjö T., Micke P., et al. Prognostic Significance of Stromal Platelet-Derived Growth Factor ␣-Receptor Expression in Human Breast Cancer.

[11] J. Paulsson, M. Ehnman, A. Östman, PDGF receptors in tumor biology: prognostic and predictive potential, Future Oncol. 10 (2014) 1695–1708.

[12] C. Kratochwil, P. Flechsig, T. Lindner, et al., 68Ga-FAPI PET/CT: tracer uptake in 28 different kinds of cancer, J. Nucl. Med. 60 (2019) 801–805, https://doi.org/10.2967/jnumed.119.227967.

[13] A. Šimková, P. Bušek, A. Šedo, J. Konvalinka, Molecular recognition of fibroblast activation protein for diagnostic and therapeutic applications, Biochim. Biophys. Acta - Proteins Proteomics 1868 (2020).

[14] E. Sahai, I. Astsaturov, E. Cukierman, et al., A framework for advancing our understanding of cancer-associated fibroblasts, Nat. Rev. Cancer 20 (2020) 174–186.

[15] S. Waise, R. Parker, M.J.J. Rose-Zerilli, et al., An optimised tissue disaggregation and data processing pipeline for characterising fibroblast phenotypes using single-cell RNA sequencing, Sci. Rep. 9 (2019), https://doi.org/10.1038/s41598-019-45842-4.

[16] M. Wang, M. Topalovski, J.E. Toombs, et al., Fibulin-5 blocks microenvironmental ROS in pancreatic Cancer, Cancer Res. 75 (2015) 5058–5069, https://doi.org/10.1158/0008-5472.CAN-15-0744.
[17] M. Yu, G. Guo, L. Huang, et al., CD73 on cancer-associated fibroblasts enhanced by the A2B-mediated feedforward circuit enforces an immune checkpoint, Nat. Commun. 11 (2020), https://doi.org/10.1038/s41467-019-14060-x.
[18] C. Vennin, P. Mélénec, R. Rouet, et al., CAF hierarchy driven by pancreatic cancer cell p53-status creates a pro-metastatic and chemoresistant environment via perlecan, Nat. Commun. 10 (2019), https://doi.org/10.1038/s41467-019-10968-6.
[19] P. Micke, J.S.M. Mattsson, D. Djureinovic, et al., The impact of the fourth edition of the WHO classification of lung tumours on histological classification of resected pulmonary NSCCs, J. Thorac. Oncol. 11 (2016) 862–872, https://doi.org/10.1016/j.jtho.2016.01.020.
[20] C. Kampf, I. Olsson, U. Ryberg, et al., Digitalization within the human protein atlas, J. Vis. Exp. 63 (2012) 3620, https://doi.org/10.3791/3620.
[21] W.D. Travis, E. Brambilla, A.G. Nicholson, et al., The 2015 world health organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification, J. Thorac. Oncol. 10 (2015) 1243–1260, https://doi.org/10.1097/JTO.0000000000000630.
[22] M. Frödin, A. Mezheyeuski, S. Corvigno, et al., Perivascular PDGFR-β is an independent marker for prognosis in renal cell carcinoma, Br. J. Cancer (2016), https://doi.org/10.1038/bjc.2016.407, 1–7.
[23] A. Mezheyeuski, M. Bradic Lindh, T. Kyrre Guren, et al., Survival-associated heterogeneity of marker-defined perivascular cells in colorectal cancer, Oncotarget 7 (2014) 41948–41958, https://doi.org/10.18632/oncotarget.9632.
[24] P. Moreno-Ruiz, L. Wik Leiss, A. Mezheyeuski, M. Ehnman, Double immunohistochemistry and digital image analysis, In: Methods in molecular biology (Clifton, N.J.). (2019) 3–11.
[25] L. La Fleur, V.F. Boura, A. Alexeyenko, et al., Expression of scavenger receptor MARCO defines a targetable tumor-associated macrophage subset in non-small cell lung cancer, Int. J. Cancer 143 (2018) 1741–1752, https://doi.org/10.1002/ijc.31545.
[26] H. Brunnström, A. Johansson, S. Westbom-Fremer, et al., PD-L1 immunohistochemistry in clinical diagnostics of lung cancer: inter-pathologist variability is higher than assay variability, Nat Publ Gr 30 (2017) 1411–1421, https://doi.org/10.1038/modpathol.2017.59.
[27] M. Grinberg, D. Djureinovic, H.R.R. Brunnström, et al., Reaching the limits of prognostication in non-small cell lung cancer: an optimized biomarker panel fails to outperform clinical parameters, Mod. Pathol. 30 (2017) 964–977, https://doi.org/10.1038/modpathol.2017.14.
[28] L. La Fleur, E. Falk-Sörqvist, P. Smeds, et al., Mutation patterns in a population-based non-small cell lung cancer cohort and prognostic impact of concomitant mutations in KRAS and TP53 or STK11, Lung Cancer 130 (2019) 50–58, https://doi.org/10.1016/j.lungcan.2019.01.003.
[29] T.K. Kilvaer, M. Rakaee, T. Hellevik, et al., Tissue analyses reveal a potential immune-adjuvant function of FAP-1 positive fibroblasts in non-small cell lung cancer, PLoS One 13 (2018), e0192157, https://doi.org/10.1371/journal.pone.0192157.
[30] C. Radkiewicz, A.L.V. Johansson, P.W. Dickman, et al., Sex differences in cancer risk and survival: a Swedish cohort study, Eur. J. Cancer 84 (2017) 130–140, https://doi.org/10.1016/J.EJCA.2017.07.013.
[31] C. Radkiewicz, P.W. Dickman, A.L.V. Johansson, et al., Sex and survival in non-small cell lung cancer: a nationwide cohort study, PLoS One 14 (2019), https://doi.org/10.1371/journal.pone.0219206.
[32] X. Li, J. Li, P. Wu, et al., Smoker and non-smoker lung adenocarcinoma is characterized by distinct tumor immune microenvironments, Oncoimmunology 7 (2018) 1–11, https://doi.org/10.1080/2162402X.2018.1494677.
[33] T.K. Kilvaer, M. Rakaee, T. Hellevik, et al., Tissue Analyses Reveal a Potential Immune- Adjuvant Function of FAP-1 Positive Fibroblasts in Non-small Cell Lung Cancer., 2018, https://doi.org/10.1371/journal.pone.0192157.
[34] T.K. Kilvaer, M.R. Khanehkenari, T. Hellevik, et al., Cancer associated fibroblasts in stage I-IIIA NSCLC: prognostic impact and their correlations with tumor molecular markers, PLoS One 10 (2015), e0134965, https://doi.org/10.1371/journal.pone.0134965.
[35] T.K. Kilvaer, M. Rakaee, T. Hellevik, et al., Differential prognostic impact of platelet-derived growth factor receptor expression in NSCLC, Sci. Rep. 9 (2019) 1–11, https://doi.org/10.1038/s41598-019-46510-3.