

Identification of key molecules in micropapillary progression of lung adenocarcinoma: A comprehensive gene expression analysis study using the spatial gene expression solution methodology

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Abstract. The micropapillary histological subtype is a high-grade element and a poor prognostic marker in lung adenocarcinoma (LUAD). This subtype develops through the lepidic-filigree micropapillary (filigree)-conventional/overt micropapillary (mPAP) pathway. The present study aimed to identify key molecules that promote this progression. To this end, gene expression profiles specific to lepidic, filigree and mPAP elements were investigated in histological sections obtained from 4 different LUAD cases. The 10x Genomics Visium Spatial Gene Expression Solution was used due to its superior resolution compared with conventional microdissection techniques. Cellular retinoic acid binding protein 2 (CRABP2), carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) and mucin 21 (MUC21) were

identified as common molecules with significantly elevated levels along the lepidic-filigree-mPAP pathway. Furthermore, the present findings indicated that CRABP2 may serve an important role in the early stage of this process, as its level significantly increases during the transition from the lepidic to the filigree substage. Immunohistochemical analysis of the expression of CRABP2, CEACAM5 and MUC21 proteins in 207 surgically resected LUAD samples (expanded sample size) was performed. The present study revealed an increase in the expression levels of CRABP2 between the lepidic and filigree elements, and between filigree and mPAP for CEACAM5 and MUC21. Thus, these three proteins were demonstrated to serve roles in the lepidic-filigree-mPAP pathway at different stages. Notably, these molecules were associated with poor prognosis, characterized by an elevated recurrence rate and poor survival rate. In conclusion, crucial molecules that promote the lepidic-filigree-mPAP pathway, and exhibit potential clinical utility as prognostic markers and molecular therapeutic targets, were identified.

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Abbreviations: CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; FFPE, formalin-fixed paraffin-embedded; LUAD, lung adenocarcinoma; mPAP, conventional/overt micropapillary; MUC21, mucin 21; OS, overall survival; RFS, recurrence-free survival

Key words: lung adenocarcinoma, micropapillary histology, filigree, progression, prognosis

Introduction

Lung cancer is the leading cause of cancer-related deaths in the developed world, with lung adenocarcinoma (LUAD) being the most prevalent histological type. Various grading methods based on a histological perspective have been proposed to predict prognosis. The micropapillary subtype is a high-grade histological element that serves as a marker of poor prognosis (1,2), particularly among patients with *EGFR*-mutated LUAD (3). We previously suggested that the micropapillary element may develop from a pure lepidic type *via* intermediate forms exhibiting a low papillary structure (4). The 5th edition

of the World Health Organization (WHO) classification defines this low papillary structure as a filigree pattern, consisting of tumor cells growing in delicate, lace-like, narrow stacks of three cells without fibrovascular cores (5). As the filigree and micropapillary patterns exhibit comparable malignant potential, the filigree type should be classified as a variant of the micropapillary element (6). This supported our theory and has prompted us to investigate the molecular basis of the lepidic-filigree micropapillary (filigree)-conventional/overt micropapillary (mPAP) progression pathway.

The Visium Spatial Gene Expression Profiling System (10x Genomics, Pleasanton, CA, USA) has facilitated comparisons of mRNA expression among various histologically distinct elements from a single tissue section (7). Conventional methods compare mRNA levels between tissue elements within a single tumor by separately isolating RNA from each region using microdissection. However, LUAD is histologically heterogeneous within a single tumor, limiting the effectiveness of conventional methods for analyzing spatial gene expression. Notably, the Visium Spatial Gene Expression Profiling System compares different histological elements while preserving morphological information within a single tissue section (8). Recently, detailed analysis using the Visium spatial transcriptomics platform has revealed the mechanism by which lung tumors develop through interactions with their surrounding micro-environment (9).

In the present study, we aimed to identify key molecules promoting LUAD progression using the Visium Spatial Gene Expression Solution, which offers better resolution than microdissection. We examined gene expression profiles specific to lepidic, filigree, and mPAP elements in identical histological sections to reveal key molecules that promote the lepidic-filigree-mPAP pathway.

Materials and methods

Patients. All LUAD tissues analyzed in this study were surgically resected at the Kanagawa Prefectural Cardiovascular and Respiratory Center between January 1997 and December 2022. The proportion of smokers in lung adenocarcinoma in our study [50.5% (104/207)] was comparable with the previous study investigating Japanese cohort (56.2–61.9%) (10–12). For the prognostic analysis, patients with lung cancer who underwent surgery between 1997 and 2014 were included. All patients provided written informed consent for the use of these samples for research purposes. This study was approved by the ethics committee at the Kanagawa Prefectural Cardiovascular and Respiratory Center [Approval ID: KCRC-17-0016; October 1, 2018] and the materials (patient tissues and data) were used based on a mixed retrospective and prospective study design.

Histopathological analyses. Formalin-fixed paraffin-embedded (FFPE) tissue sections were sliced and stained with hematoxylin and eosin (HE). The proportions of the histological elements (lepidic, acinar, papillary, mPAP, and solid elements) were recorded in 5% increments according to the WHO classification (5). The filigree pattern was classified as part of the micropapillary subtype (6,13).

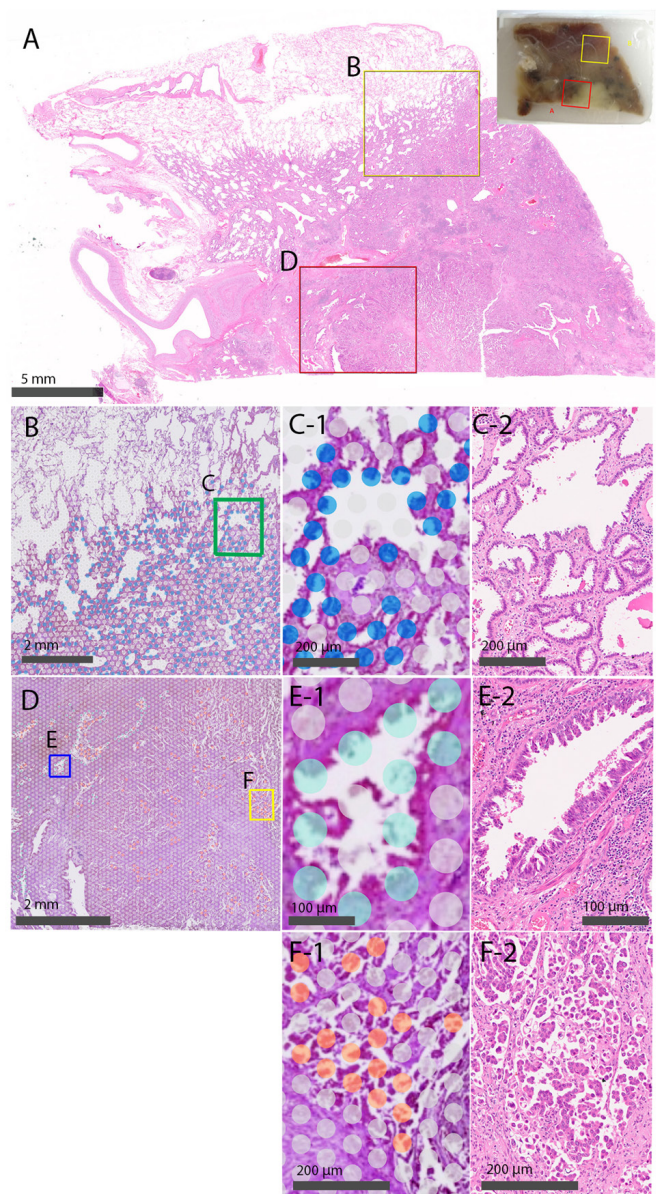


Figure 1. Representative H&E staining images of patient 1 acquired using Visium profiling. (A) Loupe slide of surgically resected lung adenocarcinoma. Tissue sections *in situ* and invaded areas were analyzed using Visium profiling. Scale bar, 5 mm. (B) Tumor cells consisted of lepidic elements *in situ*. Scale bar, 2 mm. (C) High magnification view of the green square in (B). The lepidic element was characterized by neoplastic cell extension along the alveolar wall surface. (C-1) Blue spots. (C-2) Serial section. Scale bar, 200 μ m. (D) Tumor cells in the invaded area comprised both filigree and mPAP elements. Scale bar, 2 mm. (E) High magnification view of the blue square in (D). Filigree element consisted of stacked tumor cells extending from alveolar walls towards airspace without fibrovascular cores. (E-1) Light green spots. (E-2) Serial section. Scale bar, 100 μ m. (F) High magnification view of the yellow square in (D). mPAP elements were characterized by the formation of tufted papillary structures with a central fibrovascular core and floated in the alveolar space. (F-1) Orange spots. (F-2) Serial section. Scale bar, 200 μ m. mPAP, conventional/overt micropapillary.

Spatial gene expression profiling. Four LUAD samples were analyzed using the Visium Spatial Gene Expression Profiling System (10x Genomics, Pleasanton, CA, USA). Briefly, the quality of RNA was analyzed using FFPE slides, and all samples that satisfied the DV200 requirement as a standard of RNA integrity were then subjected to the adhesion test. Eight

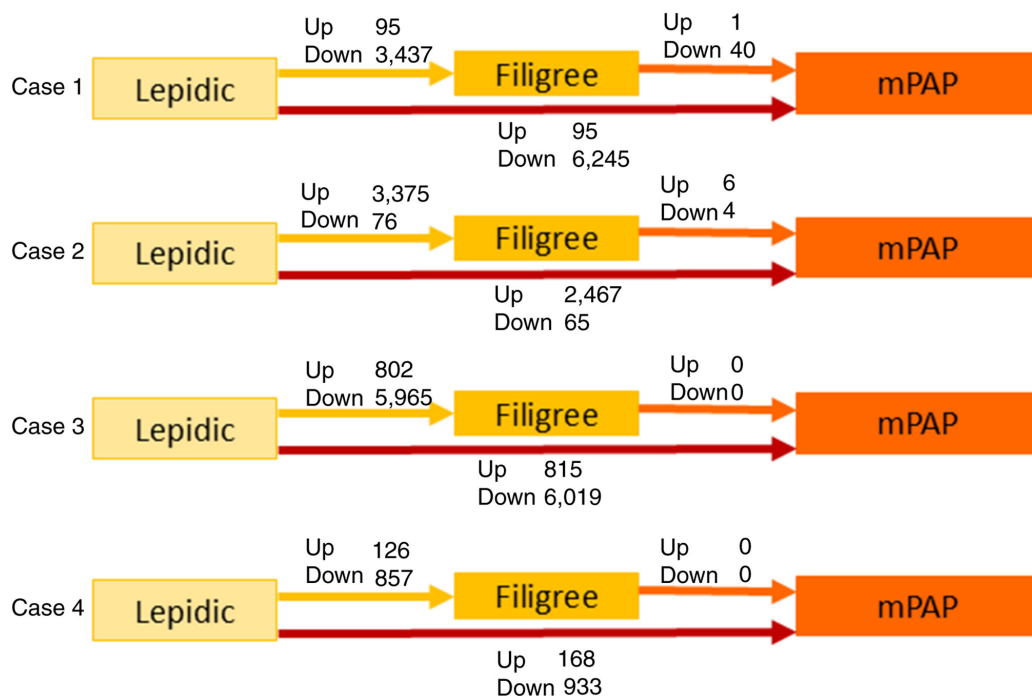


Figure 2. Number of significantly differentially expressed genes among histological elements in 4 cases. The numbers of changes in gene expression between lepidic and mPAP elements were a few dozen to several thousand. Most of the changes in gene expression during the transition from lepidic to mPAP elements occurred between the lepidic and filigree elements (approximately one hundred to several thousand). By contrast, relatively few changes were observed between filigree and mPAP elements (<50). mPAP, conventional/overt micropapillary.

tissue sections (n=2 each from the four LUAD samples) that included lepidic, filigree, and mPAP elements were mounted onto Visium Spatial Gene Expression Slides in oligo-barcoded capture areas (6.5x6.5 mm). Sequencing libraries were generated using Visium Spatial Gene Expression kits, followed by spatial transcript analysis using Visium Spatial Gene Expression Solution (10x Genomics) (Fig. 1). The Visium Spatial Gene Expression Profiling System contains approximately 5,000 circular spots with a diameter of 55 μ m within a 6.5x6.5 mm² analysis area on an FFPE slide, with each spot containing 3-5 cells. RNA transcriptome data were obtained from individual spots corresponding to each histological element (Fig. 1C-1: lepidic element, D-1: filigree element, E-1: mPAP element). Quantitative gene expression data were normalized and mapped onto tissue sections using a Bio Turing Lens (Bio Turing, San Diego, CA, USA). The mRNA levels in the lepidic, filigree, and mPAP components were then compared and analyzed. Differentially expressed genes among the three elements were identified using t-tests with significance criteria set at fold change (FC) >1.5 and a false discovery rate (FDR) <0.05.

Immunohistochemical analyses. Immunohistochemical analysis was performed on 207 surgically resected LUAD samples. FFPE tissue sections were incubated with primary antibodies against cellular retinoic acid binding protein 2 (CRABP2; polyclonal; Sigma-Aldrich, St. Louis, MO, USA), carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5; clone CB30; Lifespan Biosciences, Washington, DC, USA), and mucin 21 (MUC21; polyclonal; Novus Biologicals, Littleton, CO, USA). The slides were autoclaved to retrieve antigens, and immunoreactivity was then visualized using

Simple Stain MAX-PO (MULTI; Nichirei, Tokyo, Japan). The reactions were further developed using 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. The intensity of CRABP2, CEACAM5, and MUC21 staining in neoplastic cells was judged as negative (0), weak (1), or strong (2) and scored as: 1x (proportion of area with weak intensity) + 2x (proportion of area with strong intensity (%), in 5% increments).

Statistical analysis. Categorical and continuous variables were respectively analyzed using Pearson chi-square tests or Fisher exact tests, the Kruskal-Wallis test with Dunn's multiple comparison test and the Cox proportional hazards model. Receiver operating characteristic (ROC) curves of recurrence were generated to establish a cut-off to distinguish 'high' from 'low' immunohistological scores. Kaplan-Meier curves of recurrence and survival were plotted, and differences in recurrence-free survival (RFS) and overall survival (OS) rates were analyzed using log-rank tests. Values with P<0.05 were considered significant. All data were statistically analyzed using JMP 9.0.2 (SAS Institute, Cary, NC, USA).

Results

Changes in gene expression in each LUAD subtype. The number of differentially expressed genes was considerably higher during the transition from lepidic to filigree than from filigree to mPAP among all four cases (Fig. 2). This result suggests that the gene expression profile primarily changes during the early stages of the progression.

Differentially expressed genes during the lepidic-filigree-mPAP progression. Among the four cases, three genes

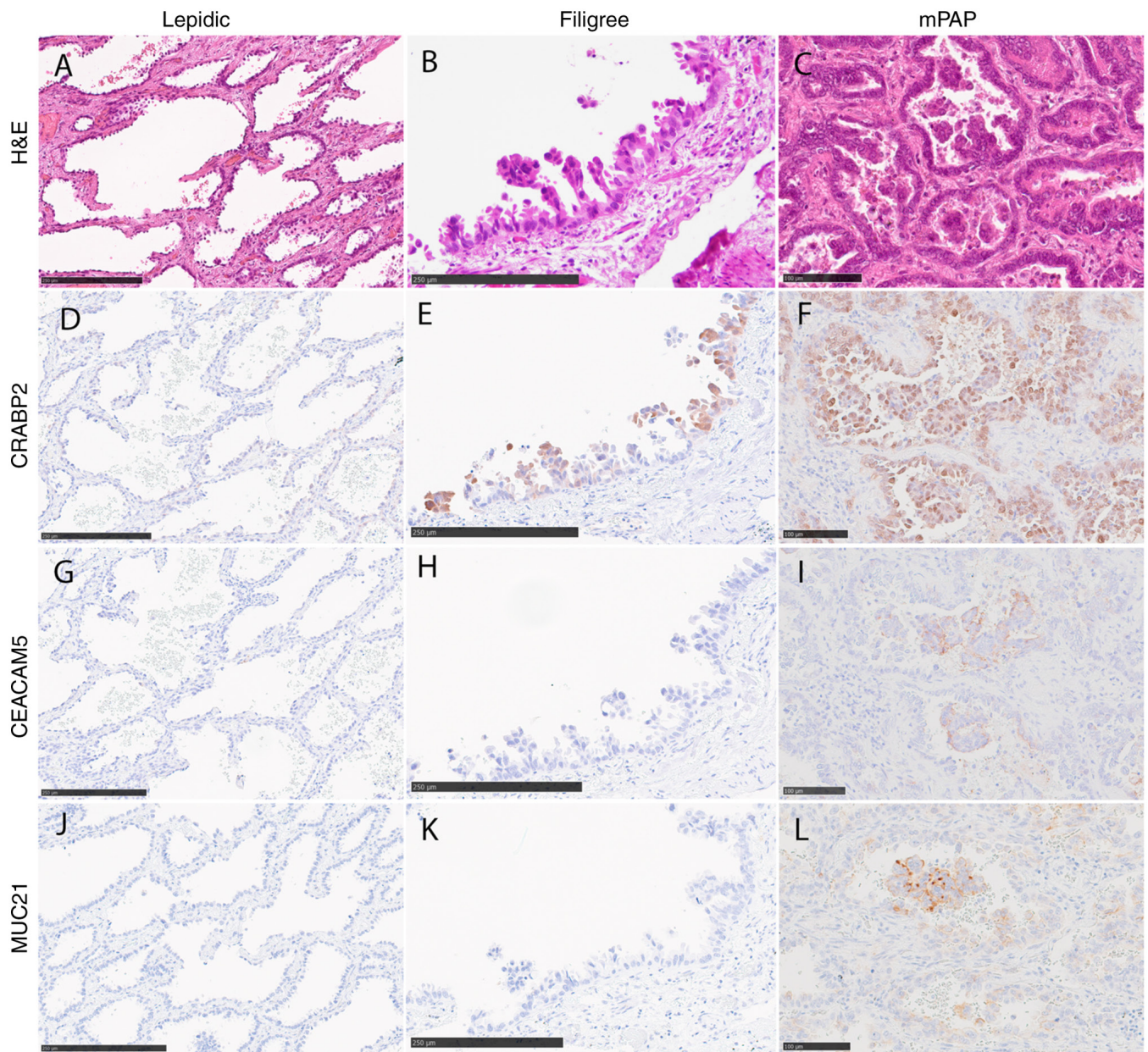


Figure 3. Representative images of CRABP2, CEACAM5 and MUC21 immunohistochemical staining. (A) Lepidic, (B) filigree and (C) mPAP elements stained with H&E. (D) CRABP2, (G) CEACAM5 and (J) MUC21 were rarely expressed in lepidic elements. CRABP2 was expressed in (E) filigree and (F) mPAP elements. (I) CEACAM5 and (L) MUC21 were expressed only in mPAP elements. (H) CEACAM5 and (K) MUC21 were rarely expressed in filigree elements. Scale bar, 250 μ m (A, B, D, E, G, H, J and K) or 100 μ m (C, F, I and L). CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; mPAP, conventional/overt micropapillary; MUC21, mucin 21.

(*CRABP2*, *CEACAM5*, and *MUC21*) were commonly upregulated during the transition from lepidic to mPAP elements (Tables SI–SIII). Notably, *CRABP2* was upregulated in the early stage (from lepidic to filigree elements). Conversely, 14 genes were commonly downregulated during the transition from lepidic to mPAP elements, whereas 6 genes were downregulated during the transition from lepidic to filigree elements (Tables SIV–SVI).

Immunohistochemical expression of *CRABP2*, *CEACAM5*, and *MUC21* in different histological elements. Among the identified genes, we focused on three—*CRABP2*, *CEACAM5*, and *MUC21*—and examined their protein expression in a larger cohort of LUAD cases using immunohistochemical analysis

(Fig. 3). *CRABP2* was preferentially expressed both in filigree and mPAP elements (Fig. 3E and F). Conversely, *CEACAM5* and *MUC21* expression was specific to mPAP elements, with positive rates were 50.6% (39/77) and 66.2% (51/77), respectively (Fig. 3I and L). Positive immunohistochemical signals for *CEACAM5* and *MUC21* revealed predominantly negative staining in the filigree element, with positive rates of 38.7% (60/155) and 21.3% (33/155), respectively (Fig. 3H and K). To confirm this observation, we semi-quantified the immunohistochemical expression levels in the different histological elements, including lepidic, filigree, acinar, papillary, mPAP, and solid elements, using a scoring system (as described in the Materials and Methods). Representative images of tumors with varying immunohistochemical scores are presented in Fig. 4.

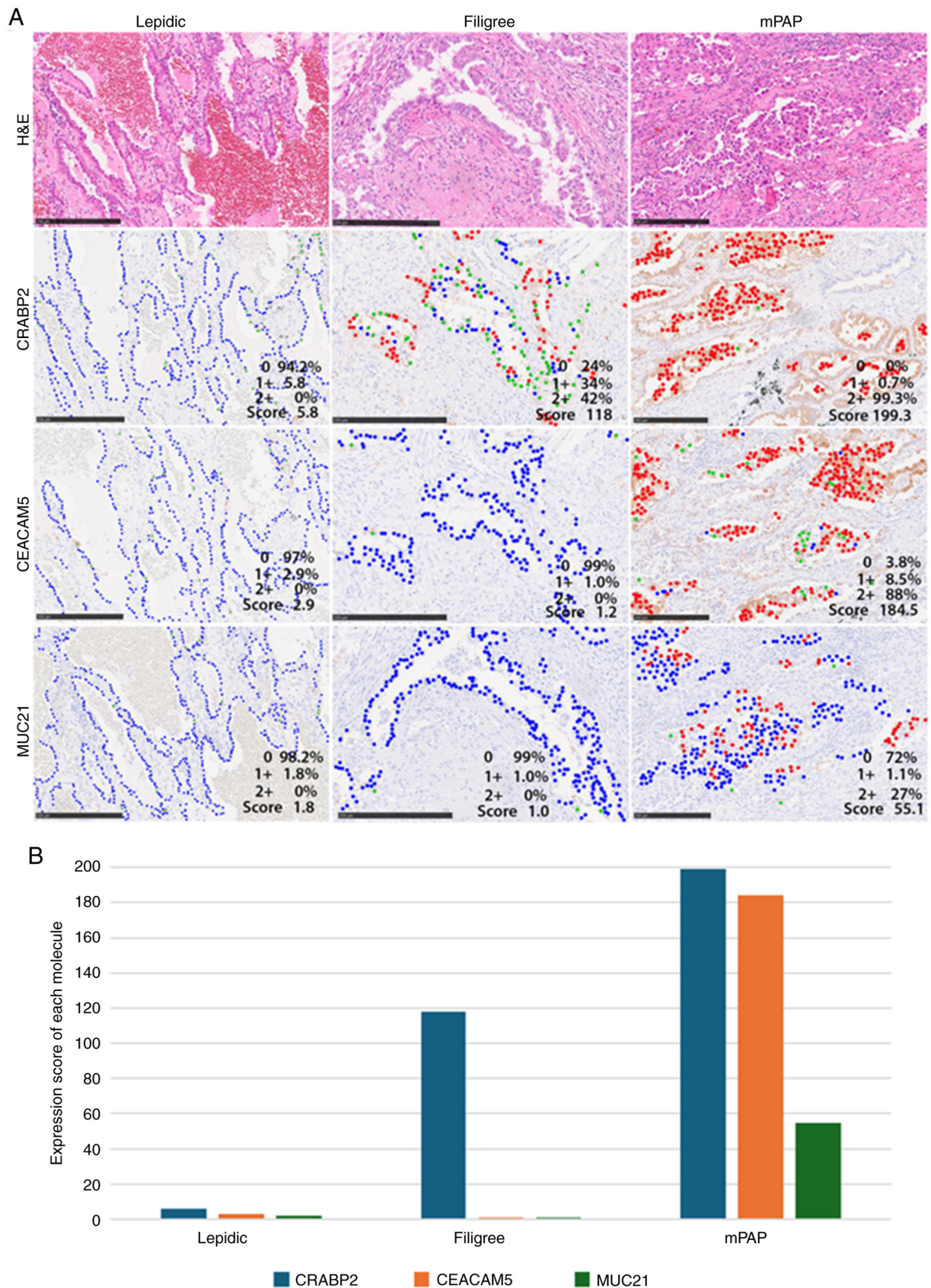


Figure 4. Representative images of tumors with various immunohistochemical scores. Red, green and blue dots indicate tumor cells with strong, weak and no signals (intensity, 2, 1 and 0, respectively) (A) Lepidic elements (left) rarely exhibited positive signals for each antibody (CRABP2, CEACAM5 and MUC21 scores: 5.8, 2.9 and 1.8). Filigree elements (center) showed some strong and weak CRABP2 signals in most neoplastic cells (score, 118=1x34+2x42). Signals for CEACAM5 and MUC21 were rarely positive (scores, 1.2 and 1.0, respectively). mPAP elements (right) exhibited stronger signals in most neoplastic cells for CRABP2, CEACAM5 and MUC21 than the other elements (lepidic and filigree) (scores, 199.3=1x0.7+2x99.3, 184.5=1x8.5+2x88 and 55.1=1x1.1+2x27, respectively). Scale bar, 250 μ m. (B) All scores were low in lepidic elements. Only the expression score for CRABP2 was high in the filigree element, whereas the scores for all three proteins were high in mPAP elements. CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; mPAP, conventional/overt micropapillary; MUC21, mucin 21.

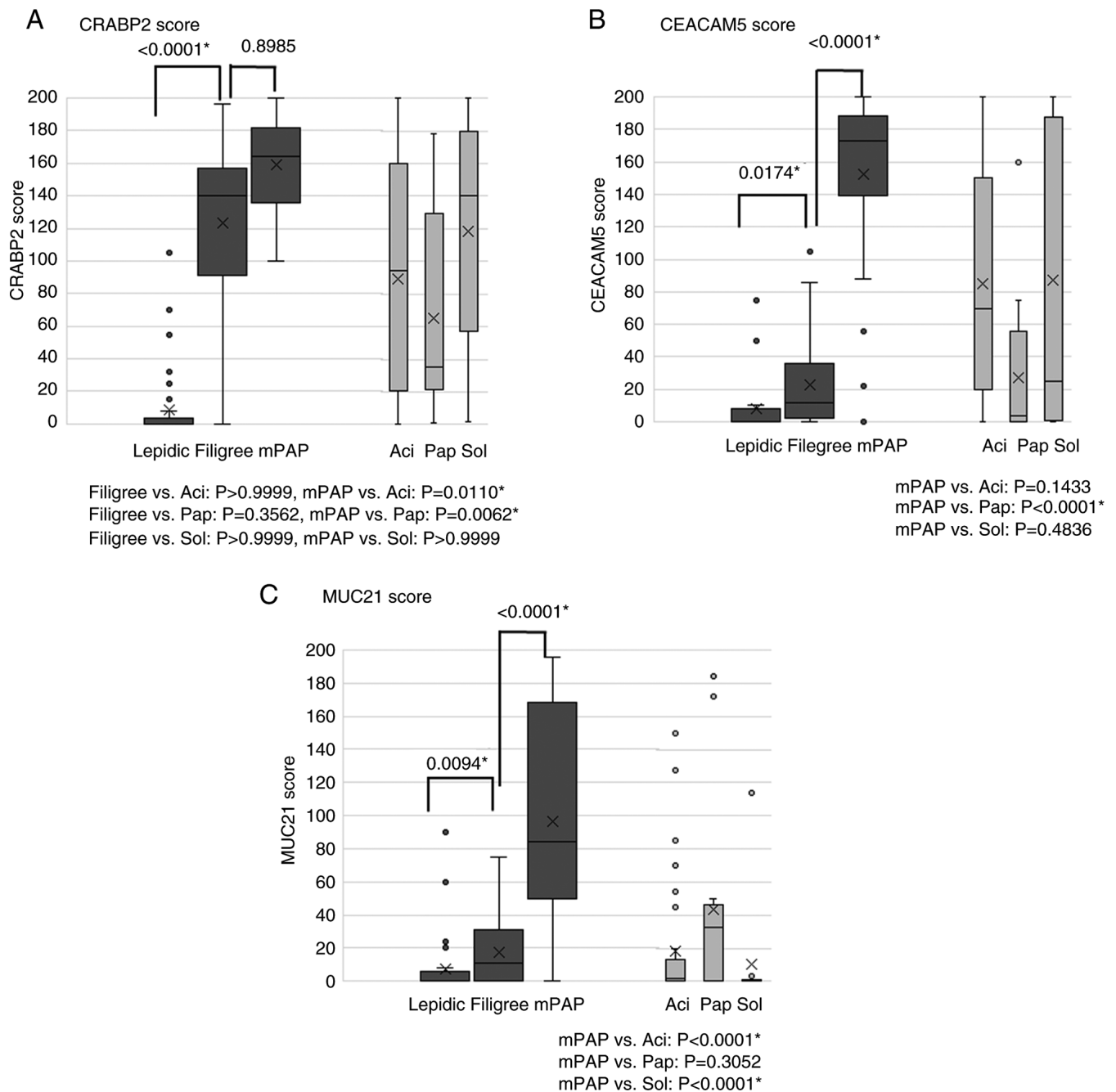


Figure 5. Association between histological elements and CRABP2, CEACAM5 and MUC21 immunohistochemical scores. (A) The CRABP2 score was significantly higher in filigree elements than in lepidic elements ($P<0.0001$) but no significant difference was observed between mPAP elements and filigree elements ($P=0.8985$). (B) The CEACAM5 score was significantly higher in filigree elements than in lepidic elements ($P=0.0174$) and significantly higher in mPAP elements than in filigree elements ($P<0.0001$). This difference was more significant between filigree and mPAP elements than between filigree and lepidic elements. (C) The MUC21 score was significantly higher in filigree elements than in lepidic elements ($P=0.0094$) and significantly higher in mPAP elements than in filigree elements ($P<0.0001$). This difference was more significant between filigree and mPAP elements than between filigree and lepidic elements. Box-and-whisker plots show immunohistochemical scores for 50 surgically resected lung adenocarcinoma cases (median, thick line; 25th-75th percentiles, box; 10th-90th percentiles, whiskers; outliers, dots). $P<0.05$ (Kruskal-Wallis test with Dunn's multiple comparison test). *Statistically significant. Aci, acinar; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; mPAP, conventional/overt micropapillary; MUC21, mucin 21; Pap, papillary; Sol, solid.

Consistent with our initial observation, the scores of CRABP2 were significantly higher in filigree than lepidic elements; however, no significant difference was observed between mPAP and filigree elements (Fig. 5A). The scores of CEACAM5 and MUC21 were significantly higher in mPAP than in filigree elements, and the scores of these were significantly higher in filigree than in lepidic elements (Fig. 5B and C). The increase

in the CRABP2 score between lepidic and filigree elements was considerably larger than that in CEACAM5 or MUC21 expression scores (Fig. 5). This result suggests that CRABP2 may play a role in the earlier stages of the lepidic-filigree-mPAP progression. High scores of CRABP2 and CEACAM5 were also observed in solid elements, another high-grade element, as well as mPAP elements (Fig. 5A and B). In some

Table I. Relationships between CRABP2, CEACAM5 and MUC21 expression and clinicopathological factors in lung adenocarcinoma.

Clinicopathological factors	CRABP2			CEACAM5			MUC21		
	Low expression (N=90)	High expression (N=117)	P-value	Low expression (N=93)	High expression (N=114)	P-value	Low expression (N=169)	High expression (N=38)	P-value
Age			0.7698			0.6693			0.4535
Young (≤65 years), % (n)	36.7 (33)	34.2 (40)		37.6 (35)	33.3 (38)		36.7 (62)	28.9 (11)	
Older (>65 years), % (n)	63.3 (57)	65.8 (77)		62.4 (58)	66.7 (76)		63.3 (107)	71.1 (27)	
Median, years (range)	69 (47-84)	69 (36-86)		67 (38-85)	69 (36-86)		68 (36-86)	72 (47-83)	
Sex, % (n)			0.3247			0.5758			0.1502
Female	58.9 (53)	51.3 (60)		57.0 (53)	52.6 (60)		52.1 (88)	65.8 (25)	
Male	41.1 (37)	48.7 (57)		43.0 (40)	47.4 (54)		47.9 (81)	34.2 (13)	
Smoking status, % (n)			0.7799			0.2645			0.0122 ^a
Never smoked	51.1 (46)	48.7 (57)		45.2 (42)	53.5 (61)		45.6 (77)	68.4 (26)	
Smoking	48.9 (44)	51.3 (60)		54.8 (51)	46.5 (53)		54.4 (92)	31.6 (12)	
Tumor size, % (n)			0.3961			0.1194			>0.9999
≤20 mm	45.6 (41)	39.3 (46)		48.4 (45)	36.8 (42)		42.0 (71)	42.1 (16)	
>20 mm	54.4 (49)	60.7 (71)		51.6 (48)	63.2 (72)		58.0 (98)	57.9 (22)	
Lymphatic invasion, % (n)			<0.0001 ^a			0.0171 ^a			0.0112 ^a
Present	38.9 (35)	66.7 (78)		45.2 (42)	62.3 (71)		50.3 (85)	73.7 (28)	
Absent	61.1 (55)	33.3 (39)		54.8 (51)	37.7 (43)		49.7 (84)	26.3 (10)	
Vascular invasion, % (n)			0.0007 ^a			0.0334 ^a			0.5646
Present	17.8 (16)	40.2 (47)		22.6 (21)	36.8 (42)		29.6 (50)	34.2 (13)	
Absent	82.2 (74)	59.8 (70)		77.4 (72)	63.2 (72)		70.4 (119)	65.8 (25)	
pStage, % (n)			0.0020 ^a			0.0117 ^a			0.0343 ^a
IA, IB	91.1 (82)	74.4 (87)		89.2 (83)	75.4 (86)		84.6 (143)	68.4 (26)	
IIA, IIB, IIIA, IIIB	8.9 (8)	25.6 (30)		10.8 (10)	24.6 (28)		15.4 (26)	31.6 (12)	
Histological subtype, % (n)			0.0050 ^a			0.0011 ^a			0.1858
Lepidic ^b	63.3 (57)	45.3 (53)		65.6 (61)	43.0 (49)		55.0 (93)	44.7 (17)	
Acinar	21.1 (19)	31.6 (37)		16.1 (15)	36.0 (41)		26.6 (45)	28.9 (11)	
Papillary	3.3 (3)	11.1 (13)		5.4 (5)	9.6 (11)		5.9 (10)	15.8 (6)	
Micropapillary ^c	0.0 (0)	1.7 (2)		0.0 (0)	1.8 (2)		0.6 (1)	2.6 (1)	
Solid	5.6 (5)	9.4 (11)		6.5 (6)	8.8 (10)		7.7 (13)	7.9 (3)	
Mucinous	6.7 (6)	0.9 (1)		6.5 (6)	0.9 (1)		4.1 (7)	0.0 (0)	
Cytological subtype, % (n)			0.0117 ^a			0.1114			0.1987
TRU	87.8 (79)	91.5 (107)		88.2 (82)	91.2 (104)		88.2 (149)	97.4 (37)	
Non-TRU/BSE	10.0 (9)	1.7 (2)		8.6 (8)	2.6 (3)		6.5 (11)	0.0 (0)	
Unclassifiable	2.2 (2)	6.8 (8)		3.2 (3)	6.1 (7)		5.3 (9)	2.6 (1)	
EGFR, % (n)			0.0377 ^a			0.0498 ^a			0.0188 ^a
Mutated	45.6 (41)	59.0 (69)		45.2 (42)	59.6 (68)		49.1 (83)	71.1 (27)	
Wild-type	54.4 (49)	41.0 (48)		54.8 (51)	40.4 (46)		50.9 (86)	28.9 (11)	

^aP<0.05 (statically significant). ^bLepidic histological subtypes in this analysis included adenocarcinoma *in situ* and minimally invasive adenocarcinoma. ^cMicropapillary histological subtypes in this analysis included filigree pattern. BSE, bronchial surface epithelium; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; MUC21, mucin 21; N, number; TRU, terminal respiratory unit.

cases, CRABP2 and CEACAM5 expression was observed in the acinar and papillary elements. However, the expression scores in these elements were lower than those in filigree

and/or mPAP elements (Fig. 5A and B). The expression score of MUC21 was consistently lower in the papillary, acinar, and solid elements than in the filigree and mPAP components

Table II. Multivariate analysis performed using the Cox proportional hazards model.

Variable	Recurrence-free survival			Overall survival		
	HR	95% CI	P-value	HR	95% CI	P-value
CRABP2						
Expression score (≤ 100 vs. >100)	1.894	1.05-3.59	0.0325 ^a	1.804	0.93-3.75	0.0842
Sex (female vs. male)	1.100	0.61-2.01	0.7547	1.373	0.70-2.73	0.3588
Smoking status (never smoked vs. smoking)	1.077	0.59-1.98	0.8107	1.116	0.56-2.25	0.7555
Tumor size (≤ 20 vs. >20 mm)	1.193	0.66-2.13	0.5520	1.095	0.58-2.05	0.7769
Vascular invasion (absent vs. present)	1.385	0.74-2.64	0.3149	1.222	0.58-2.62	0.5989
Lymphatic invasion (absent vs. present)	1.128	0.57-2.23	0.7269	0.691	0.32-1.45	0.3315
pStage (I vs. II, III)	4.859	2.65-8.88	0.0090 ^a	5.778	3.02-11.1	$<0.0001^a$
CEACAM5						
Expression score (≤ 50 vs. >50)	3.045	1.67-5.95	0.0002 ^a	1.547	0.82-3.08	0.1849
Sex (female vs. male)	1.023	0.56-1.87	0.9398	1.346	0.68-2.69	0.3928
Smoking status (never smoked vs. smoking)	1.256	0.69-2.30	0.4578	1.191	0.60-2.42	0.6228
Tumor size (≤ 20 vs. >20 mm)	1.308	0.72-2.35	0.3736	1.103	0.58-2.06	0.7605
Vascular invasion (absent vs. present)	1.344	0.71-2.60	0.3697	1.192	0.56-2.58	0.8480
Lymphatic invasion (absent vs. present)	1.191	0.60-2.35	0.6118	0.759	0.36-1.58	0.4643
pStage (I vs. II, III)	4.720	2.55-8.70	$<0.0001^a$	5.795	2.99-11.3	$<0.0001^a$
MUC21						
Expression score (≤ 80 vs. >80)	1.135	0.59-2.09	0.6971	1.100	0.52-2.22	0.7995
Sex (Female vs. Male)	1.127	0.62-2.07	0.6974	1.383	0.70-2.77	0.3526
Smoking status (never smoked vs. smoking)	1.163	0.62-2.19	0.6355	1.165	0.57-2.45	0.6809
Tumor size (≤ 20 vs. >20 mm)	1.169	0.64-2.10	0.6047	1.099	0.57-2.07	0.7735
Vascular invasion (absent vs. present)	1.401	0.74-2.70	0.3055	1.197	0.56-2.60	0.6426
Lymphatic invasion (absent vs. present)	1.245	0.63-2.47	0.5268	0.768	0.35-1.63	0.4925
pStage (I vs. II, III)	5.222	2.75-9.85	$<0.0001^a$	6.475	3.28-12.8	$<0.0001^a$

^aStatistically significant. CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; HR, hazard ratio; MUC21, mucin 21.

(Fig. 5). The expression of CRABP2, CEACAM15, and MUC21 was not detected in non-neoplastic bronchiolar and alveolar epithelial cells.

Cut-off values for 'high' and 'low' immunohistochemical scores. The optimal cut-off immunohistochemical scores for CRABP2, CEACAM5, and MUC21, determined using ROC curves, were 100, 50, and 80, respectively. We then categorized the samples as 'high' or 'low expressors' in subsequent correlation analyses.

Relationship among CRABP2, CEACAM5, and MUC21 immunohistochemical levels and clinicopathological characteristics. The clinicopathological characteristics of the 207 LUAD cases are presented in Table I. High CRABP2, CEACAM5, and MUC21 expression levels were significantly related to *EGFR* mutations (Table I). Additionally, high MUC21 expression was significantly associated with a non-smoking status (Table I).

Relationship between CRABP2, CEACAM5, and MUC21 levels and highly malignant pathological factors. High

CRABP2, CEACAM5, and MUC21 expression levels were significantly associated with lymphatic canal invasion [CRABP2, 78 (66.7%) of 117 vs. 35 (38.9%) of 90; Pearson χ^2 test, $P<0.0001$; CEACAM5, 71 (62.3%) of 114 vs. 42 (45.2%) of 93, $P=0.0171$; MUC21, 28 (73.7%) of 38 vs. 85 (50.3%) of 169, $P=0.0112$; Table I]. Both CRABP2 and CEACAM5 were significantly associated with vascular invasion [47 (40.2%) of 117 vs. 16 (17.8%) of 90; $P=0.0007$ Pearson χ^2 tests and 42 (36.8%) of 114 vs. 21 (22.6%) of 93; $P=0.0334$, respectively (Table I)]. These results support the notion that frequent lymphatic canal invasion is a biological basis for the aggressiveness of mPAP elements (1,7-10).

Relationships between CRABP2, CEACAM5, and MUC21 levels and postoperative outcomes. Individuals with high CRABP2, CEACAM5, and MUC21 expression had significantly worse 5-year RFS rates [CRABP2: 50.7% vs. 73.8%, $P=0.0002$; CEACAM5: 55.6% vs. 86.5%, $P<0.0001$; MUC21: 55.8% vs. 72.2%, $P=0.0475$; (Fig. 6)]. Individuals with high CRABP2 and CEACAM5 expression had significantly worse 5-year OS rates [72.3% vs. 92.0%, $P=0.0019$, and 75.1% vs. 86.9%, $P=0.0039$, respectively]. All P-values were determined using log-rank tests.

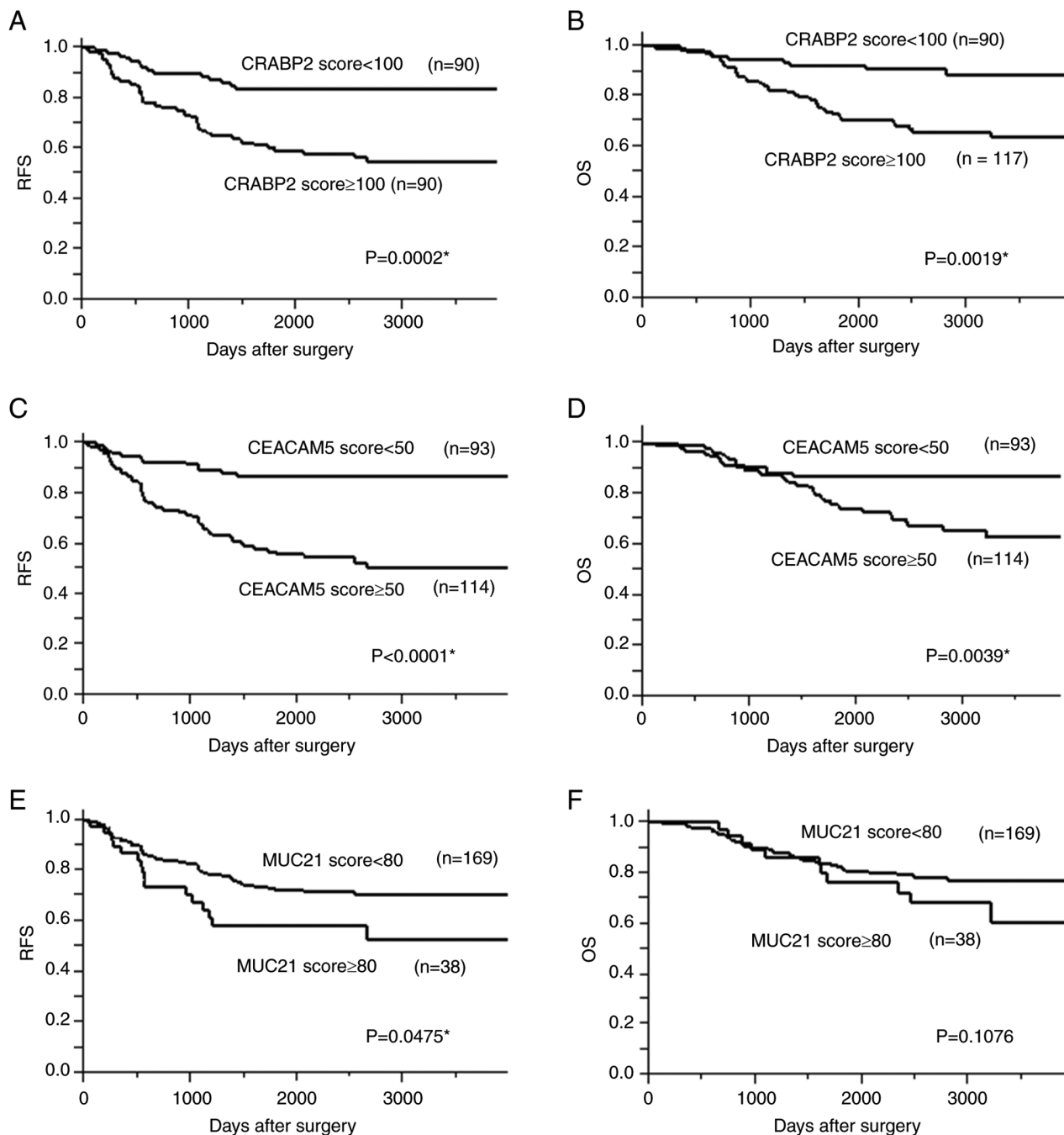


Figure 6. Kaplan-Meier RFS and OS curves of the associations between CRABP2, CEACAM5 and MUC21 and disease recurrence and survival. RFS rates were significantly worse in samples with high (A) CRABP2, (C) CEACAM5 and (E) MUC21 expression levels. OS rates were significantly worse in samples with high (B) CRABP2 and (D) CEACAM5 expression levels. (F) No significant difference in OS rates was observed between patients with high and low MUC21 expression levels. $P<0.05$, log-rank test. *Statistically significant. A total of 207 patients with lung adenocarcinoma were followed up for a median of 84 (4-201) months. CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CRABP2, cellular retinoic acid binding protein 2; MUC21, mucin 21; n, number of tumors; OS, overall survival; RFS, recurrence-free survival.

In addition, multivariate analysis revealed CRABP2 ($P=0.0325$) and CEACAM5 expression ($P=0.0002$) as independent predictors of disease recurrence and stage (Table II).

A sub-analysis of stage I LUAD revealed that individuals with high CRABP2 and CEACAM5 expression had significantly worse 5-year RFS rates of 71.9% vs. 88.3% ($P=0.009$) and 68.6% vs. 91.1% ($P=0.002$), respectively (Fig. S1). Moreover, individuals with high CRABP2 and CEACAM5 expression had significantly worse 5-year OS rates of 72.7% vs. 92.0% ($P=0.033$) and 87.2% vs. 92.6% ($P=0.0345$), respectively. All

P-values were determined using log-rank tests. These results indicate that CRABP2 and CEACAM5 could serve as excellent prognostic markers for recurrence and mortality in the early stages of LUAD.

Discussion

In the present study, we identified three molecules-CRABP2, CEACAM5, and MUC21-that play crucial roles in promoting the lepidic-filigree-mPAP progression. These molecules

may be important in conferring aggressiveness to micropapillary, as their high expression levels were associated with lymphatic canal invasion, high recurrence rates, and poor 5-year survival rates. However, the stage at which these molecules exert their effects appears to vary. Specifically, CRABP2 is likely involved in the early transition from lepidic to filigree, whereas CEACAM5 and MUC21 are implicated in the later transition from filigree to mPAP elements. No significant differences in *CEACAM5* and *CRABP2* mRNA expression levels were observed between the filigree and mPAP elements. However, an increase in the protein expression of CEACAM5 and CRABP2 was observed between these elements in the immunohistochemical analysis. This discrepancy may be attributed to post-translational modifications.

To regulate the transcription of downstream genes, CRABP2 transports retinoic acid to the nucleus, where it binds to transcription factors. CRABP2 expression has been detected in lung cancer (14), breast cancer (15), and glioblastoma (16). Furthermore, CRABP2 enhances the migration, invasion, and anoikis resistance of lung cancer cells *via* the HuR and integrin β 1/FAK/ERK signaling pathways and promotes metastasis *in vivo* (14). Integrins play an important role in cell adhesion, and *CRABP2* overexpression activates the integrin β 1/FAK/ERK signaling pathway. These data suggest that CRABP2 is involved in decreasing tumor cell adhesion and promoting the morphological progression to filigree and mPAP patterns during the early stages of LUAD development. CRABP2 is reportedly significantly upregulated in LUAD with micropapillary elements (17) and in small invasive adenocarcinoma (18). Moreover, high CRABP2 levels are correlated with lymph node metastases, poor OS, and increased recurrence (18). Collectively, these findings further support our findings.

The surface glycoprotein CEACAM5 is involved in cell adhesion, intracellular signaling, and tumor progression. This protein promotes cell proliferation and invasion *via* p38/Smad2/3 signaling in non-small-cell lung cancer (NSCLC) (19). The induced overexpression of CEA is associated with anoikis, a form of apoptosis caused by detachment from the cell matrix, which enhances metastasis of colorectal cancer (20). These data implicate CEACAM5 in the morphological progression of the micropapillary pattern (21,22).

MUC21 is a transmembrane mucin expressed in various human neoplasms, including lung carcinomas (13,23). It is associated with the aggressive behavior of neoplastic cells (13). We have previously revealed that MUC21, characterized by short glycosylated sugar chains, is associated with highly malignant histological components, such as micropapillary elements (13). Moreover, mouse *Muc21*/epiglycanin disrupts cell-extracellular matrix interactions and interferes with intercellular adhesions, suggesting that *Muc21*/epiglycanin inhibits surface integrins and intercellular adhesion molecules (24,25). Miyoshi *et al.* (26) reported that the micropapillary element exhibits a loss of integrin-mediated adhesion to the basal membrane, which may help explain the mechanism by which MUC21 contributes to the micropapillary morphology.

In the present study, we identified CRABP2 and CEACAM5 as independent prognostic markers for LUAD

recurrence. Clinically, these molecules have various applications. First, immunohistochemical examination of CRABP2 and CEACAM5 in preoperative biopsied tissue or surgical resected tissue serves as predictive markers to determine the suitability of neo-adjuvant or adjuvant chemotherapy. Second, the plasma concentrations of these proteins may be used as a screening assay for the early detection of lung cancer (27,28). Increased plasma CRABP2 and CEACAM5 levels are correlated with decreased survival rates in patients with LUAD (28,29). Third, targeting these molecules for molecular therapy is becoming increasingly feasible. Specifically, CEACAM5 has recently emerged as a promising target for antibody-drug conjugate therapy of NSCLC. Tusamitamab ravtansine (SAR408701), a humanized antibody-drug conjugate targeting CEACAM5, is in clinical development for nonsquamous NSCLC with high CEACAM5 expression (30).

This study has some limitations. First, the quality of RNA extracted from FFPE specimens may deteriorate due to long-term storage or poor storage conditions (such as high temperature and humidity). Japan Pathology Quality Assurance System recommends using FFPE specimens within 2.5 to 3 years of preparation to ensure reliable genetic testing. We use FFPE specimens prepared within 3 years for the Visium Spatial Gene Expression Profiling System. Additionally, these FFPE specimens were stored at temperatures below 25°C. Second, the spatial resolution of the Visium platform was insufficient to analyze the microenvironment surrounding tissue elements (such as lepidic, filigree, and mPAP elements) each in LUAD. However, in recent years, the Xenium spatial transcriptomics platform, which offers higher resolution than the Visium spatial transcriptomics platform, has emerged. This technology will enable the analysis of the microenvironment for different tissue elements of LUAD in the future.

In conclusion, we investigated the potential molecular basis of the lepidic-filigree-mPAP progression using the Visium Spatial Gene Expression Solution and identified three molecules that promote this progression. These molecules may have clinical utility as prognostic markers and as potential targets of molecular therapy.

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Availability of data and materials

The raw sequencing data generated in the present study may be found in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE 300676 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE300676>. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

MM and KO wrote the majority of the manuscript. TW and HA collected patient information, compiled a clinical database and conducted formal analysis. TW and HA made substantial contributions to analysis and interpretation of data. TK was responsible for the statistical analysis. MM and KO designed the study and suggested the contents of the manuscript. MM, CK and KO examined the tissue sections and made pathological diagnoses. MM and CK contributed to funding acquisition. TS and HM cut tissue sections and stained them with hematoxylin and eosin. DM and KF performed Visium experiments, and analyzed and interpreted the data. MM and KO confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees of Kanagawa Prefectural Cardiovascular and Respiratory Center (Yokohama, Japan; approval no. KCRC-17-0016; October 1, 2018). All patients provided written informed consent for the use of their samples for research purposes. For prospective observational studies, written informed consent has been obtained from study participants. For retrospective observational studies, existing specimens obtained for clinical purposes (pathological specimens not additionally collected for research purposes) were used. In principle, informed consent was sought for these specimens; however, when obtaining informed consent was difficult, opt-out consent was taken.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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