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ARTICLE

Diffuse expression of MUC6 defines a distinct clinicopathological subset of pulmonary invasive mucinous adenocarcinoma

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Abstract

Invasive mucinous adenocarcinoma (IMA) of the lung is a unique variant of lung adenocarcinoma. Aberrant mucin expression is associated with cancer development and metastasis. However, the clinicopathological significance of mucin expression in IMA is not fully understood. Herein, we evaluated the clinicopathological, immunohistochemical, and molecular characteristics of 70 IMA tumors. *EGFR, KRAS, GNAS*, and *TP53* mutations were assessed by PCR-based sequencing. Next-generation sequencing was used to assess cases without *EGFR/KRAS* mutations. A NanoString-based screening for fusions was performed in all IMAs without mitogenic driver mutations. Expression of mucins (MUC1, MUC2, MUC4, MUC5AC, and MUC6) was evaluated by immunohistochemistry and categorized as follows: negative (<10% of tumor cells), patchy expression (<90% of tumor cells), or diffuse expression (≥90% of tumor cells). Immunohistochemical testing for transcription factors (TTF-1, CDX2, HNF1β, HNF3α, HNF3β, and HNF4α) was also performed. As expected, *KRAS* mutations were the most common (in 67% of cases), followed by small numbers of other alterations. Patchy or diffuse expression of MUC1, MUC2, MUC4, MUC5AC, and MUC6 was observed in 52% or 6%, 3% or 0%, 30% or 3%, 26% or 73%, and 59% or 27% of cases, respectively. Furthermore, all IMAs were generally positive for HNF1β (100%), HNF3α (100%), HNF3β (100%), and HNF4α (99%) but were positive less often for TTF-1 (6%) and CDX2 (9%). Overall, there was no significant correlation between mucin expression and transcription factor expression. Unexpectedly, diffuse expression of MUC6 was significantly associated with *KRAS*-wild-type tumors ($p = 0.0008$), smaller tumor size ($p = 0.0073$), and tumors in female patients ($p = 0.0359$) in multivariate analyses. Furthermore, patients with tumors exhibiting diffuse MUC6 expression had significantly favorable outcomes. Notably, none of these patients died of the disease. Our data suggested that diffuse expression of MUC6 defines a distinct clinicopathological subset of IMA characterized by wild-type *KRAS* and possibly less aggressive clinical course.

Introduction

Invasive mucinous adenocarcinoma (IMA) of the lung, which constitutes from 2 to 10% of all lung adenocarcinomas [1–3], is classified as a variant of invasive adenocarcinoma as per the 2015 World Health Organization (WHO) classification. IMA has unique histopathologic and genetic

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characteristics: tumor cells have a goblet or columnar cell morphologic pattern with abundant intracytoplasmic mucins and frequently harbor *KRAS* mutations [4]. However, IMA can manifest in a wide array of clinical presentations: some tumors present as a solitary mass, while others present in a multifocal or even bilateral fashion, which is thought to be due to aerogenous spread in most cases. Some IMAs mimic pneumonia by exhibiting lobar consolidation, sometimes with abundant bronchorrhea [5–7]. Although IMA is considered to be an intermediate-grade tumor lung adenocarcinoma, the prognosis of IMA is not as well characterized as that of non-mucinous adenocarcinomas, with conflicting results regarding its prognosis [8–14].

Mucins are heavily glycosylated proteins with a high molecular weight and are expressed in epithelial cells or various organs. Mucins are classified into two major groups: membrane-bound mucins and secretory mucins. The former includes MUC1 and MUC4, and the latter includes MUC2, MUC5AC, and MUC6 [15]. Mucins are involved in a wide range of biological activities under both normal and pathological conditions. Furthermore, altered mucin glycosylation patterns during malignant transformation enable their interaction with various receptors and thereby promote cancer cell differentiation, proliferation, invasion, and metastasis [16]. In lung adenocarcinoma, several mucins have been analyzed, and IMA has been shown to express MUC5AC [17]. Furthermore, IMAs commonly lack the transcription factor NKX2-1 (also known as TTF-1) that normally suppresses mucin genes. However, they do express hepatocyte nuclear factor 4α (HNF4α), a nuclear transcription factor important for goblet cell maturation in the colonic mucosa [18, 19]. Our knowledge of mucin involvement in other diseases is rapidly expanding. However, the role and clinical significance of mucin expression in IMA is not fully understood.

Herein, we evaluated the prevalence of MUC1, MUC2, MUC5AC, and MUC6 expression in 70 IMAs, as well as the association of mucin expression with various clinicopathological parameters and genetic alterations.

Materials and methods

Study population and histological analysis

We screened the archives of the Department of Human Pathology, Juntendo University School of Medicine, for all patients who had undergone a complete resection of primary lung adenocarcinoma between January 2010 and December 2018. We obtained clinicopathological data, including age, gender, smoking status, tumor size, lymphovascular invasion, lymph node and distant metastases, resection type, and adjuvant therapy. All tumors were resected at the Department of General Thoracic Surgery, Juntendo University Hospital. All tissues were fixed in 10% buffered formalin and embedded in paraffin after routine processing. Hematoxylin and eosin-stained slides and Elastica van Gieson-stained slides from all patients were available. All tumors measuring 3 cm in diameter or less were submitted in their entirety, and larger tumors were sampled extensively. Two pathologists (SK and TH) reviewed the slides, and pathological diagnoses were based on the most recent WHO classification [4]. Our archives contained data for 70 IMAs. Follow-up had been conducted for all patients via regular physical and blood examinations, with mandatory X-ray, computed tomography, or magnetic resonance imaging. Approval for this study was obtained from the Ethics Committee of Juntendo University School of Medicine (no. 2019067).

Analysis of genetic alterations

First, analyses of genetic alterations in a total of 70 IMAs were performed according to previously reported methods. Briefly, *EGFR* and *KRAS* mutations were analyzed using the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method [20] and the peptide nucleic acid-mediated PCR clamping method [20], respectively, and *TP53* and *GNAS* mutations were analyzed using PCR followed by direct sequencing [21]. Samples without *EGFR/KRAS* mutations were subsequently analyzed by next-generation sequencing (NGS) including whole-exome sequencing and whole-transcriptome sequencing, as previously described [22], or targeted sequencing, as described below. Four cases without *EGFR/KRAS* mutations could not be analyzed by NGS due to insufficient or unavailable material. Samples without any mitogenic driver mutations were further analyzed by NanoString assays to identify kinase fusions.

Targeted sequencing

Genomic DNA was isolated from tumors, and normal formalin-fixed and paraffin-embedded (FFPE) tissues were prepared using the QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, the Netherlands). NGS was carried out using a custom 50 gene Ion AmpliSeq panel, which was based on the Ion Ampliseq Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. In brief, library preparation and barcoding were carried out on Ion Chef (Thermo Fisher Scientific), utilizing the Ion AmpliSeq Kit for Chef DL8 (Thermo Fischer Scientific). The products were purified from the other reaction components using Agencourt AMPure XP (Beckman Coulter, Inc., Brea, CA, USA) and then quantified by qPCR and diluted to 30 pM. Template

preparation and chip loading were also performed on Ion Chef using Ion 530 Kit-Chef (Thermo Fisher Scientific). A maximum of 24 barcoded samples were used on the Ion 530 chip (Thermo Fisher Scientific). Sequencing was performed on Ion GeneStudio S5 (Thermo Fisher Scientific), utilizing the Variant Caller plugin v.5.10.1.20, which refers to the COSMIC database. The resulting BAM files were visualized using the Integrative Genomics Viewer (IGV; http://www.broadinstitute.org/igv/). The variant caller files were subjected to further analysis with the Ion Reporter v.5.10 (Thermo Fisher Scientific). Coverage analysis was assessed to evaluate the quality of the sequencing run, using the coverage analysis plug-in v.5.10.0.3. We considered the NGS amplification to be successful if an average minimum of 500 reads or greater was achieved across all target regions, and the number of mapped reads was >15,000. We excluded mutations with <5% allele frequency.

NanoString assay for kinase fusions

The NanoString assay design (NanoString Technologies, Seattle, WA, USA) is based upon the known genomic properties of existing tyrosine kinase fusions, namely, that these fusions typically occur upstream of the exons encoding the kinase domain. The exons encoding the kinase domain GXGXXG motif for all 90 tyrosine kinases were identified as previously described [23]. All exons were labeled according to ENSEMBL numbering. On the basis of this mapping, two 100 bp regions were selected for each gene transcript, a 5′ probe pair located far upstream of the kinase domain exons and a second probe located within those exons or further in the 3′ direction. The 100 bp regions were selected to straddle exon boundaries and reduce the risk of interfering with signals from gDNA. Each RNA sample extracted from FFPE tissue was analyzed as previously described [24].

Immunohistochemistry

All tissues were fixed in 10% buffered formalin and embedded in paraffin after routine processing. Tissue sections (thickness: 4 μm) were deparaffinized and hydrated. Immunohistochemical examinations were performed using the following antibodies against MUC1 (Ma695, Leica Biosystems, Wetzlar, Germany), MUC2 (Ccp58, Leica Biosystems), MUC4 (8G7, Santa Cruz Biotechnology, Dallas, TX, USA), MUC5AC (CLH2, Abcam, Cambridge, United Kingdom), MUC6 (MUC6/916, Abcam), TTF-1 (8G7G3/1, Dakopatts, Glostrup, Denmark), CDX2 (CDX2-88, Bio Genex, Fremont, CA, USA), HNF1β (CL0374, Abcam), HNF3α (FOXA1) (Q-6, Santa Cruz Biotechnology), HNF3β (FOXA2) (RY-7, Santa Cruz Biotechnology), HNF4α (H1415, Perseus Proteomics Inc, Tokyo, Japan), and p53 (1801, Bio Genex), following the manufacturer's recommendations. Two pathologists (SK and TH), blinded to clinical data, reviewed the whole stained sections. For the immunohistochemical analyses for MUC1, MUC2, MUC4, MUC5AC, and MUC6, immunoreactivity was semi-quantitatively categorized as negative (<10% of tumor cells were stained), patchy expression (<90% of tumor cells were stained), or diffuse expression (≥90% of tumor cells were stained). For the immunohistochemical analyses for TTF-1, CDX2, HNF1β, HNF3α, HNF3β, and HNF4α, samples in which ≥10% of tumor cells were stained with a nuclear pattern were considered positive.

Statistical analysis

Categorical variables were analyzed using a Fisher's exact or chi-square test. To determine prognosis, we performed Kaplan–Meier survival analysis. These statistical analyses were performed with GraphPad Prism® software version 7.0a (GraphPad, San Diego, CA). Multivariate analysis was performed using logistic regression analysis with JMP statistical software version 14 (SAS Institute, Cary, NC). P values < 0.05 were considered statistically significant.

Results

Clinicopathological and molecular characterization of IMA

The clinicopathological characteristics of the 70 IMAs examined in this study are shown in Tables 1, S1. Forty-one (59%) were from male patients. Patients were 60 years old (y/o) or older in 61 cases (87%) and were smokers in 44 cases (63%). IMAs were 20 mm or less in 31 cases (44%), and of pathological stage I in 49 cases (70%). Lymphovascular invasion and lymph node metastasis were detected in six cases (9%) and 1 case (1.4%), respectively. Histologically, 60 IMAs (86%) exhibited a pure mucinous pattern, and the other 10 displayed a mixed mucinous/non-mucinous pattern. Genomic data revealed that *KRAS* mutations (47 cases) were the most frequently detected alteration, followed by *ERBB2* mutations (three cases; each case harbored Y772_A775dup, G776delinsAVGC, and L775P), *CD74-NRG1* fusions (two cases), *BRAF* mutations (1 case; V600E, 1 case; K601E), and *MET* exon 14 skipping (1 case) (Fig. 1). Among the *KRAS* mutations, G12V was the most frequent mutation type (20 cases; 43% of *KRAS* mutation cases), and *KRAS* G12C, a variant for which several covalent inhibitors have been developed, was found in six cases (13% of *KRAS* mutation cases). Comparison among *KRAS* G12D, G12V, and G12C revealed no significance differences in clinical features, such as age,

Table 1 Clinicopathological characteristics of patients with IMA.

Median age (range)	70.5 (41–85)
Sex	
Female	29
Male	41
Smoking history	
Never	26
Ever (current/former)	44
Tumor size	
≤20 mm	31
>20 mm	39
Nodal status	
N0	69
N1/N2	1
TNM stage	
I	50
II–IV	20
Lymphovascular invasion	
Absent	64
Present	6
Histological subtype	
Pure	60
Mixed	10
KRAS mutation	
Wild type	23
Mutated	47
KRAS subtype	
G12V	20
G12D	15
G12C	6
Others	6
TP53 mutation	
Wild type	63
Mutated	7

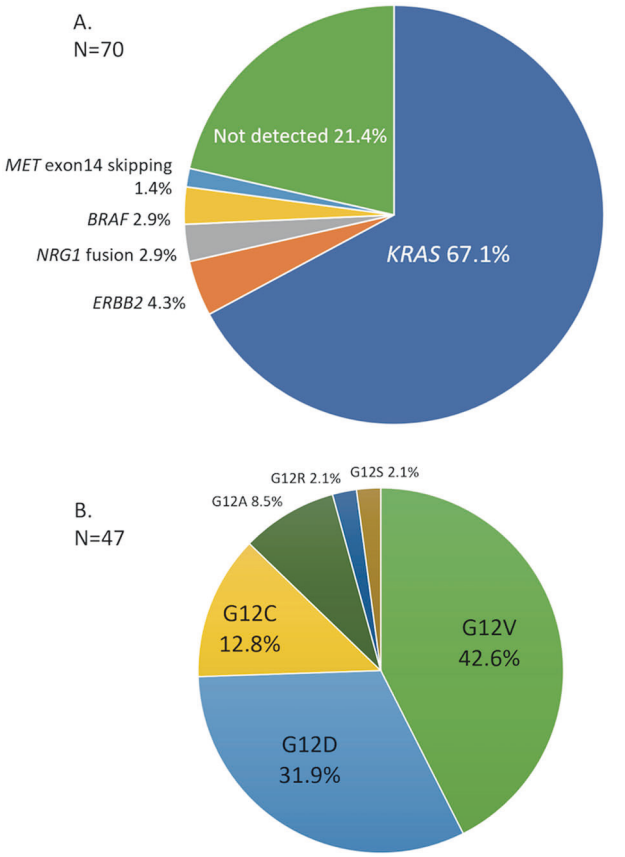

Fig. 1 Frequency of driver mutations in invasive mucinous adenocarcinoma. Pie charts showing the fraction of invasive mucinous adenocarcinomas that harbored the indicated drivers (**a**) and the fractions of *KRAS* mutation subtypes (**b**).

gender, and smoking status. *TP53* mutations were detected in seven cases (10%), which were immunohistochemically confirmed. Among these, five cases (70%) harbored a co-mutation of *KRAS*. *GNAS* mutations were detected in two samples from females (2.9%), who had either never smoked or had been light smokers. In 1 case, the *GNAS* R201H mutation co-occurred with a *KRAS* G12D mutation, while another case did not exhibit co-occurrence with a *KRAS* mutation. No cases harbored mutated *EGFR* or rearranged *ALK*, *ROS1*, and *RET*.

Expression of mucins in IMA

We evaluated the results of immunohistochemical staining for mucins, including MUC1, MUC2, MUC4, MUC5AC, and MUC6. With respect to staining pattern, MUC1 was mainly localized on the surface of tumor cells. MUC2 was detected in tumor cells with goblet cell morphology. MUC4 was mainly observed in mucous-containing tumor cells with a granular cytoplasmic pattern. Both MUC5AC and MUC6 were present in mucous-containing tumor cells, including in cells with goblet cell morphology, and displayed a cytoplasmic pattern. Patchy expression of MUC1, MUC2, MUC4, MUC5AC, and MUC6 were observed in 37 tumors (52%), 2 tumors (3%), 21 tumors (30%), 18 tumors (26%), and 41 tumors (59%), respectively. Furthermore, diffuse expression of MUC5AC and MUC6 were observed in 51 tumors (73%) and 19 tumors (27%), respectively, while diffuse MUC1 and MUC4 expression were observed in only 4 (6%) and 2 (3%) tumors, respectively. The prevalence of mucin expression was as follows: MUC1, 59% of cases; MUC2, 3% of cases; MUC4, 23% of cases; MUC5AC, 99% of cases (Fig. 2); and MUC6, 86% of cases (Fig. 3). With respect to the interrelation of different mucins, MUC1 expression was significantly correlated with the expression of MUC4 ($p = 0.0224$), and inversely correlated with a diffuse expression of MUC6 ($p = 0.0069$)

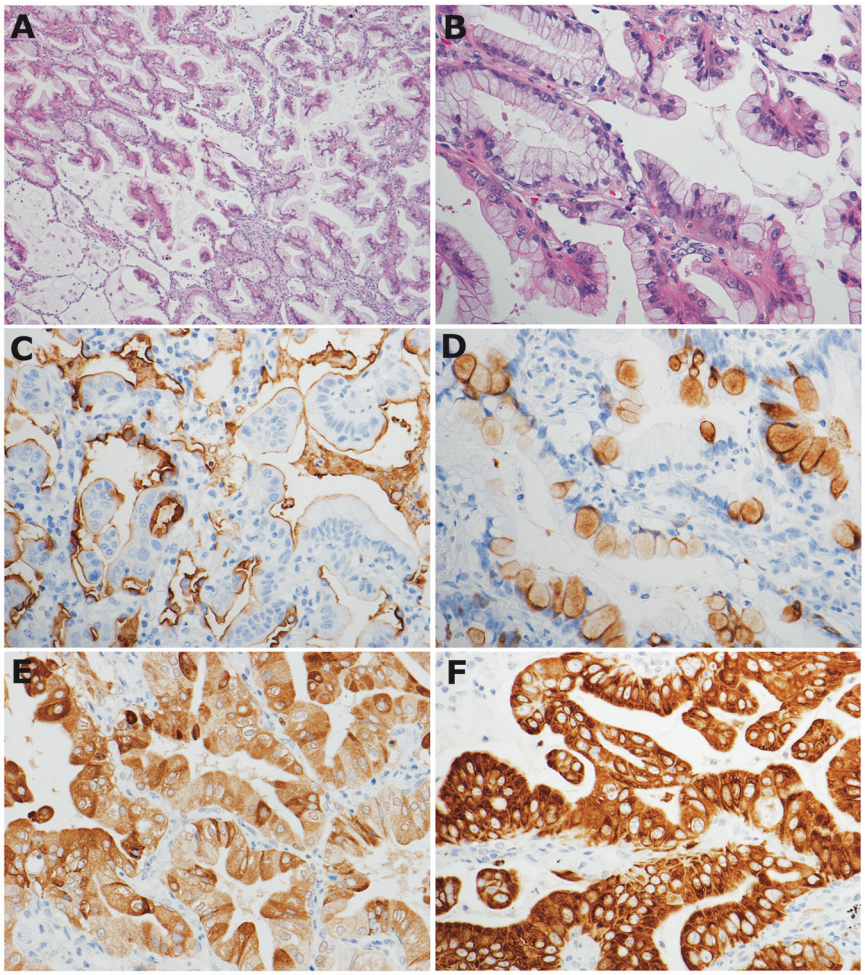

Fig. 2 Hisological findings and mucin expression of invasive mucinous adenocarcinoma. Representative H&E staining of invasive mucinous adenocarcinoma in low-power view (**a**) and high-power view (**b**). The tumor cells lined the alveolar walls or proliferated with a papillary pattern and consisted of columnar cells with abundant cytoplasmic mucin and basally oriented nuclei. Representative examples of immunohistochemical staining of MUC1 (**c**), MUC2 (**d**), MUC4 (**e**), and MUC5AC (**f**).

(Table S2). In a tumor lacking MUC5AC expression, tumor cells were negative for other mucins, including MUC1, MUC2, and MUC4. However, these cells exhibited diffuse MUC6 expression. With regard to clinicopathological parameters, the expression of MUC1 ($p = 0.0384$) and MUC4 ($p = 0.0128$) was significantly associated with tumors displaying a mixed pattern. Furthermore, expression of MUC1 was significantly associated with larger tumor size ($p = 0.015$). A comparison of MUC6-positive (both patchy and diffuse expression) and MUC6-negative tumors revealed no significant differences in clinicopathological features. Histograms of the distribution of MUC6 expression showed that MUC6 reactivity had two peak values: the higher peak was 90–100%, and the second was 10–19%, although other mucins had one peak value (MUC1: 0–9%; MUC4:0–9%; MUC5AC: 90–100%), suggesting that MUC6-positive tumors consisted of two groups (Fig. 4). Further, diffuse MUC6 expression was significantly associated with smaller tumor size ($p = 0.0007$) and tumors in female patients ($p = 0.0069$), relative to negative or patchy MUC6 expression (Table 2).

Expression of transcription factors in IMA

To further clarify the characteristics of IMA, we next evaluated the results of immunohistochemical staining for transcription factors, including TTF-1, CDX2, HNF1β, HNF3α, HNF3β, and HNF4α. TTF-1 and CDX2 were detected in four (6%) and six (9%) tumors, respectively. No tumor expressed both TTF-1 and CDX2 simultaneously. HNF4α was detected in 69 tumors (99%), while HNF1β, HNF3α, and HNF3β were present in all tumors examined (Fig. 5). With respect to staining pattern, TTF-1 and CDX2 showed focal positivity, while HNF1β, HNF3α, HNF3β, and HNF4α showed diffuse positivity with varying intensity. Two of the four TTF-1-positive tumors (50%) showed diffuse MUC6 expression, while one of the six CDX2-positive tumors (17%) exhibited diffuse MUC6 expression. Overall, there was no significant correlation between the expression of transcription factors and that of mucins, with the exception of a correlation between HNF4α and MUC5AC (Table S3).

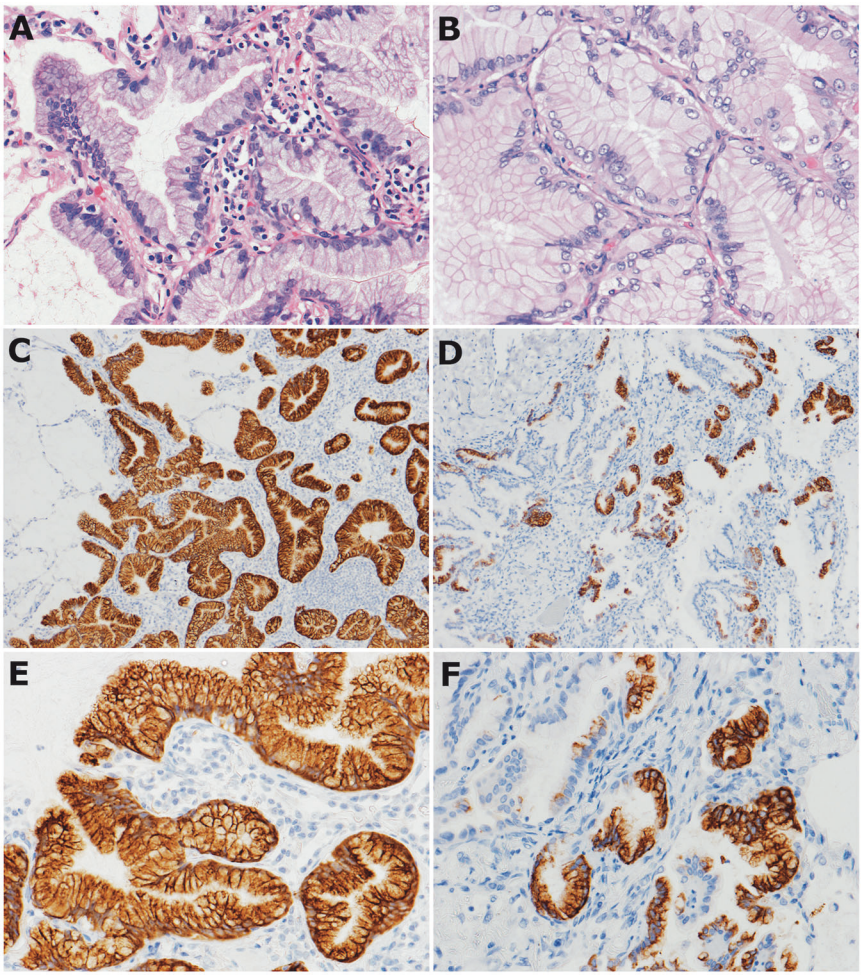

Fig. 3 Diffuse and patchy MUC6 expression in invasive mucinous adenocarcinoma. Representative H&E staining of invasive mucinous adenocarcinoma (IMA) with diffuse (**a**) and patchy (**b**) MUC6 expression. Low-power view showed that almost all tumor cells were stained with diffuse type MUC6 in IMA with rearranged *CD74-NRG1* (**c**), while scattered tumor cells stained with patchy type MUC6 in IMA with mutated *KRAS* (**d**). High-power view showed a cytoplasmic pattern in IMA with diffuse (**e**) and patchy (**f**) MUC6 expression.

Expression of mucins and transcription factors based on oncogenic driver status

KRAS-wild-type tumors were significantly associated with diffuse MUC6 expression ($p = 0.0003$) (Table 2). Furthermore, multivariate analysis showed that diffuse expression of MUC6 in IMA was associated with *KRAS*-wild-type tumors ($p = 0.0008$), smaller tumor size ($p = 0.0073$), and tumors in female patients ($p = 0.0359$) (Table 3). Tumors with positive diffuse MUC6 expression harbored potentially actionable alterations such as *NRG1* fusion (two cases), *ERBB2* (two cases) and *BRAFV600E* (1 case), mutations. Two tumors with an *NRG1-CD74* rearrangement exhibited similar mucin expression: diffuse expression of both of MUC5AC and MUC6, no expression of MUC2 and MUC4 in both cases. Additionally, one of the three tumors with *ERBB2* mutations exhibited diffuse expression of both of MUC5AC and MUC6. With respect to transcription factors, 2 of the 4 TTF-1-positive tumors harbored *ERBB2* mutations, and 4 of the 6 tumors with CDX2 expression harbored *KRAS* mutations.

Clinical outcomes

The median follow-up period after surgery for all patients was 37 months. All recurrences were limited to the lungs. Both cancer-specific survival (CSS) and recurrence-free survival (RFS) rates were significantly associated with pathological stage ($p < 0.0001$). The CSS rate was significantly higher in patients with wild-type *TP53* than those with mutated *TP53* ($p < 0.0001$). Among patients with *KRAS* mutations, RFS was significantly shorter in those with *KRAS* G12D-mutated tumors than in those with other *KRAS* mutation subtypes ($p = 0.0187$), suggesting that *KRAS* G12D represents an aggressive subset of IMA (Fig. S1). With respect to mucin expression, patients with MUC4-positive tumors had significantly worse CSS ($p = 0.0305$) and RFS ($p = 0.0454$). Moreover, patients with MUC1-positive tumors had worse CSS and RFS, but these differences were not statistically significant. In contrast, patients whose tumors had diffuse MUC6 expression had a significantly better CSS ($p = 0.0495$) and RFS ($p = 0.0094$) (Fig. 6). Of note, no patient with diffuse MUC6 expression

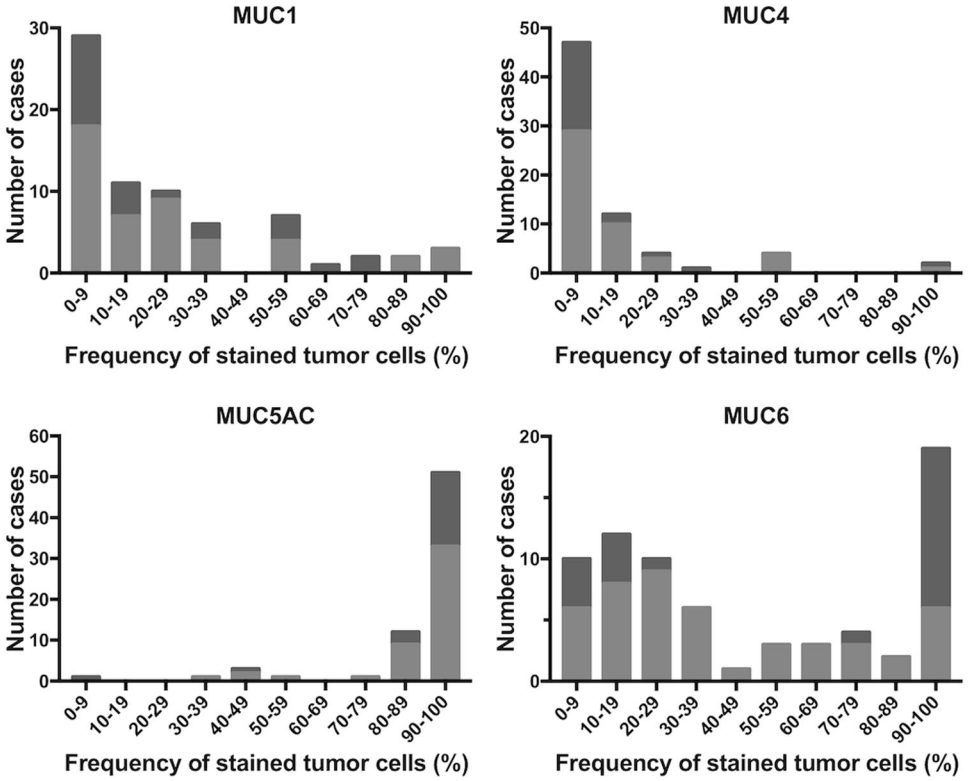

Fig. 4 The histograms of the distribution of MUC1, MUC4, MUC5AC, and MUC6. Light and dark gray bars indicate the number of *KRAS*-mutated and wild-type tumors, respectively. Note that MUC6 reactivity had two peak values; other mucins had one peak.

tumor died of the disease, although two patients died due to an acute exacerbation of interstitial pneumonia or aortic dissection.

Discussion

To the best of our knowledge, this is the first cohort study to assess the association between mucin expression and various clinicopathological and molecular parameters in IMA. Mucin expression patterns are helpful for understanding the pathogenesis of cancer in various organs [15]. Our immunohistochemical analysis results revealed that IMAs almost always expressed MUC5AC, frequently expressed MUC6, less frequently expressed MUC1 and MUC4, and hardly expressed MUC2, suggesting that IMA exhibits a gastric mucin phenotype, which is consistent with a previous report [17, 25]. This gastric phenotype has been observed in tumors besides those of digestive organs, including breast, salivary gland, and uterine tumors [26–29]. Interestingly, genetic alterations differ between lMA and gastric type of uterine cervix adenocarcinoma: the former frequently harbor *KRAS* mutations, which gastric adenocarcinomas rarely harbors; while the latter exhibits genetic similarity with gastric adenocarcinoma, including *TP53* mutations [30], suggesting that tumors in various organs with gastric mucin phenotype may not be analogous. Moreover, while the IMAs in our study consistently exhibited diffuse MUC5AC expression, we found that approximately one-third of IMAs had diffuse MUC6 expression and displayed distinct clinicopathological characteristics, such as wild-type *KRAS*, smaller tumor size, and female origin, differing from the common clinicopathological features of IMA. Of note, diffuse MUC6 expression was associated with a significantly favorable RFS in patients with IMA. Additionally, we found that patients with MUC4-positive IMAs had significantly worse outcomes. The poor prognostic value of MUC4 has been widely reported in malignancies of various organs, including in lung adenocarcinomas [31].

Although mucins exhibit a spatiotemporal-specific expression pattern in different regions of the respiratory epithelium during lung development, MUC6 is not expressed in normal lung tissue [32]. Additionally, MUC6 expression levels increase significantly during the progression from atypical adenomatous hyperplasia, through adenocarcinoma in situ, to invasive adenocarcinoma of the lung [33]. These data, along with the results of our immunohistochemical analysis, demonstrate that MUC6 is aberrantly expressed during the carcinogenesis of lung adenocarcinomas including IMA. While human lung adenocarcinomas express MUC6, and its expression correlated with that of transcription factors CDX1 and CDX2 in a xenograft model [34], our results showed that a limited number of IMAs expressed CDX2. Additionally, there was no significant correlation between MUC6 and CDX2 in IMA, despite IMAs generally expressing a number of different hepatocyte

Table 2 Association of mucin expression with other clinicopathological findings.

	MUC1 diffuse expression ($n = 4$)	MUC1 patchy expression ($n = 37$)	MUC1 negative ($n = 29$)	Correlation (p) Expression vs. negative	MUC4 diffuse expression ($n = 2$)	MUC4 patchy expression ($n = 21$)	MUC4 negative ($n = 47$)	Correlation (p) Expression vs. negative	MUC6 diffuse expression ($n = 19$)	MUC6 patchy expression ($n = 41$)	MUC6 negative ($n = 10$)	Correlation (p) Expression vs. negative	Correlation (p) Diffuse vs. patchy or negative
Median age	72.5	74	69		82	75	69		69	75	69		
(Range)	(66–81)	(41–85)	(45–83)		(81–83)	(41–84)	(42–85)		(41–76)	(42–85)	(45–83)		
Sex				0.2179				0.3015				0.5065	0.0069
Female	1	13	15		0	12	17		13	13	3		
Male	3	24	14		2	9	30		6	28	7		
Smoking history				0.1346				>0.9999				>0.9999	0.9746
Never	1	11	14		0	9	17		7	15	4		
Ever (Current/ former)	3	26	15		2	12	30		12	26	6		
Size				0.015				0.27				0.2783	0.0007
≤20 mm	0	12	19		1	8	22		16	12	3		
21–30 mm	1	9	4		1	2	11		1	9	4		
31–50 mm	1	8	1		0	3	7		1	7	2		
≥51 mm	2	8	5		0	8	7		1	13	1		
Nodal status				>0.9999				0.3286				>0.9999	>0.9999
N0	3	37	29		2	20	47		19	40	10		
N1/N2	1	0	0		0	1	0		0	1	0		
TNM stage				0.5951				0.0893				0.713	0.0717
I	2	26	22		1	12	37		17	25	8		
II–IV	2	11	7		1	9	10		2	16	2		
Histological pattern				0.0384				0.0128				0.1471	0.0522
Pure mucinous pattern	2	30	28		1	17	42		19	34	7		
Mixed pattern	2	7	1		1	4	5		0	7	3		
Lymphovascular invasion				0.3893				0.3867				0.5835	>0.9999
Absent	2	34	28		2	18	44		18	36	10		
Present	2	3	1		0	3	3		1	5	0		
KRAS mutation				0.601				0.117				0.7192	0.0003
Wild type	1	11	11		1	4	18		13	6	4		
Mutated	3	26	18		1	17	29		6	35	6		

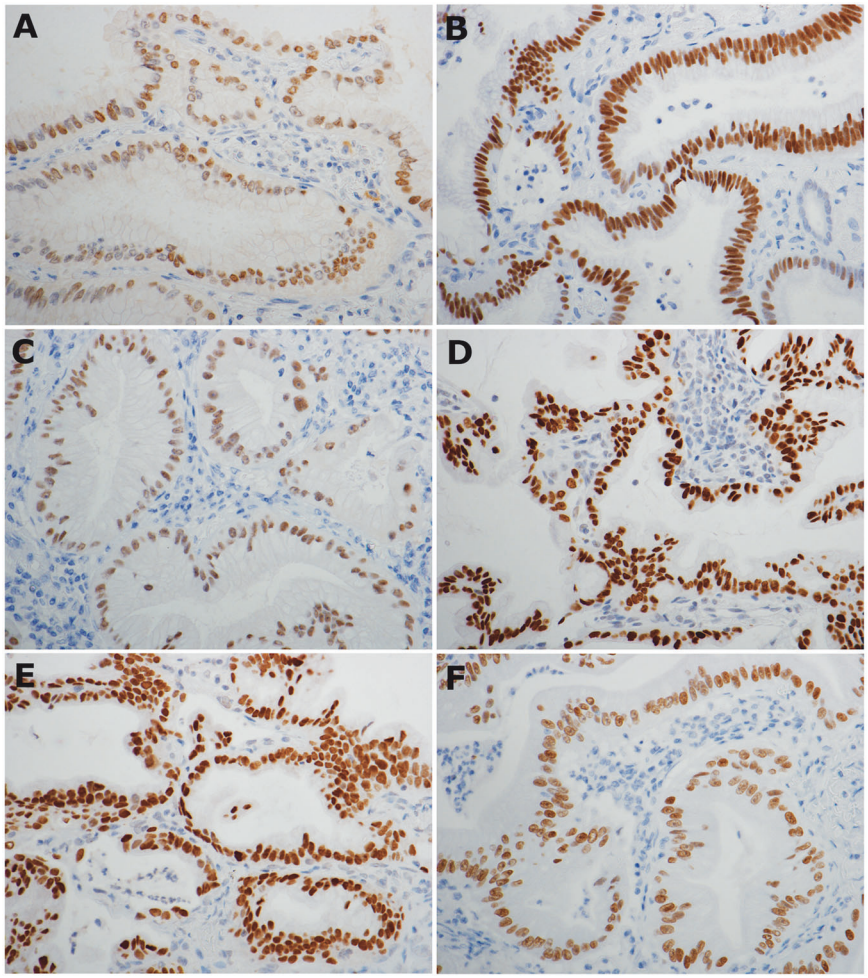

Fig. 5 Expression of transcription factors in invasive mucinous adenocarcnoma. Representative examples of immunohistochemical staining for TTF-1 (**a**), CDX2 (**b**), HNF1β (**c**), HNF3α (**d**), HNF3β (**e**), and HNF4α (**f**). Nuclear staining pattern of tumor cells.

Table 3 Univariate and multivariate logistic regression analysis of MUC6 diffuse expression.

	Univariate			Multivariate		
	Odds ratio	95% CI	*P* value	Odds ratio	95% CI	*P* value
Age (<70 vs. ≥70)	2.856	0.937–8.708	0.0650			
Sex (female vs. male)	4.740	1.525–14.729	0.007	4.833	1.107–25.605	0.0359
Smoking (never vs. ever)	0.982	0.330–2.926	0.975			
Size (≤20 mm vs. >20 mm)	12.800	3.633–61.130	<0.0001	8.158	1.728–51.965	0.0073
KRAS (wild vs. mutant)	8.883	2.705–29.169	0.0003	11.743	2.666–71.946	0.0008
MUC1 (<10% vs. ≥10%)	4.740	1.525–14.728	0.0072	2.764	0.554–14.886	0.2119
MUC4 (<10% vs. ≥10%)	3.441	0.887–13.342	0.0739			

nuclear factors (HNFs). Thus, the HNF cross-regulatory network may play a key role in mucin production in IMA. Notably, HNF1β has been described as a strong inducer of HNF4α when acting together with GATA6 [35], which is associated with mucin production in lung adenocarcinoma [36].

Although the specific mechanisms of production and function of MUC6 in IMA remain unclear at present, mucin genes are regulated through various signaling pathway in lung cancer cells. In particular, EGFR and its downstream signaling molecule ERK1/2 are necessary for mucin gene expression in lung cancer cells [37]. Besides, the acidic tumor microenvironment could lead to regional or patchy MUC6 expression, since acid conditions can upregulate MUC6 [38]. Such intrinsic and extrinsic control of MUC6 expression could have led to the observed patchy or diffuse MUC6 expression in IMA. In particular, mucin expression in lung adenocarcinoma, which is generally regional or of a patchy pattern, may be caused by acidic tumor microenvironment. Moreover, diffuse expression of MUC6 might

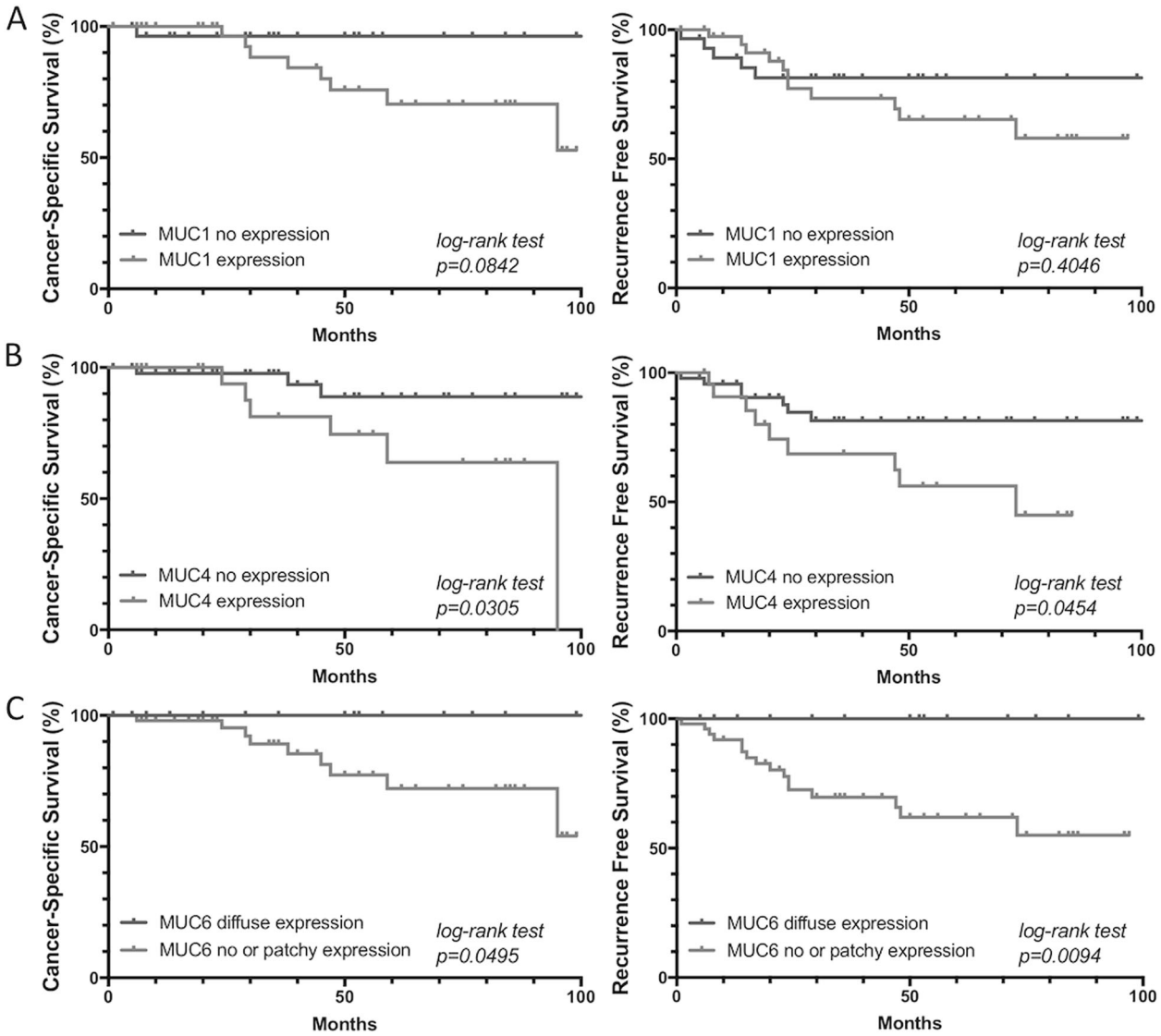

Fig. 6 Kaplan–Meier curve of cancer-specific survival (CSS) and recurrence-free survival (RFS) of 70 patients with invasive mucinous adenocarcinoma of the lungs after surgical resection. Comparison of CSS and RFS in patients with MUC1-expressing tumors (**a**), MUC4-expressing tumors (**b**), and tumors expressing diffuse MUC6 (**c**).

have been caused by oncogenic signals that were *KRAS*-independent. Nevertheless, overexpression of MUC6 is known to alter cell adhesion properties and decrease cell invasion in pancreatic cancer cell lines, which supports our survival data [39]. Alternatively, the expression of MUC1, which is associated with lung cancer aggressiveness and metastasis [40], was inversely correlated with high expression levels of MUC6 in this study and may contribute to poor survival in IMA. Further studies are needed to elucidate the role of mucin in IMA of different genetic and transcriptional background.

The genomic landscape of IMA is dominated by *KRAS* mutations. Recent high-throughput analyses, along with our NGS analysis, have revealed several rare alterations, including *BRAF, ERBB2*, and *PIK3CA* mutations, *NRG1, BRAF, NTRK, ALK, RET*, and *ERBB4* rearrangements, and *MET* exon 14 skipping [41]. *PIK3CA* mutations and some other rearrangements were not identified in our cohort, while previous reports have not identified *MET* exon 14 skipping. These observations may be due to the low frequency of these alterations in IMA. Furthermore, our analysis confirmed that the rate of *TP53* mutations in IMA was much lower than that observed in lung adenocarcinomas in general, which may reflect the lower mutational burden of IMA [12, 42]. With respect to *KRAS* amino acid changes in IMA, we showed that G12V and G12D were the most common variants in IMA, which is consistent with a previous report [12], and more closely resembles the mutational pattern observed in colorectal or pancreatobiliary tumors than that observed in lung adenocarcinomas [43]. In

this study, we further revealed that IMAs rarely harbored *GNAS* mutations, which have been frequently identified in indolent and slow-growing mucinous epithelial neoplasms such as intraductal papillary mucinous neoplasms of the pancreas and low-grade appendiceal mucinous neoplasms. This suggests that IMA is distinct from the slow-growing mucinous epithelial neoplasms characterized by *GNAS* mutations in several organs. Of note, large genomic databases show that a subset of mucinous lung adenocarcinomas harbor *GNAS* mutations [44].

There are discrepancies in the existing literature regarding the prognosis of IMA, owing to its low incidence [8–14]. In one of the largest cohorts, consisting of 72 patients with IMA, all recurrences were limited to the lungs without extrapulmonary metastases [12], as was the case in our cohort of 70 patients. Moreover, our clinicopathological results expanded the characterization of IMA, describing a lower rate of nodal metastases and less lymphovascular invasion [13, 45]. These findings suggest that IMAs may not be aggressive tumors. Some studies suggest that IMAs are associated with poor survival outcomes [9, 10]. We demonstrated that IMAs from different patients had similar histological presentation and expression of transcription factors, while being heterogenous with regard to genetic alterations and mucin expression. Notably, we found that patients with *TP53*- or *KRAS* G12D-mutated tumors had worse outcomes, while patients with tumors of high MUC6 expression had more favorable outcomes. Additional studies are required to clarify the clinicopathological impacts of mucin expression in IMA through external validation. However, our results may have some clinicopathological implications, since they are based on a relatively large number of IMAs that were strictly diagnosed according to the WHO classification. Thus, patient stratification by mucin expression as well as by genetic alterations could be useful for resolving conflicting IMA survival data.

In summary, our clinicopathological, immunohistochemical, and genetic analyses revealed diffuse expression of MUC6 as a distinctive phenotype that accounted for one-third of the IMAs and was characterized by wild-type *KRAS*, smaller tumor size, and female origin. It is likely that patients with tumors of this distinct phenotype had favorable outcomes. Further prospective studies evaluating clinical outcomes in IMA should include mucin expression analysis, including of MUC6, for patient stratification.

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Compliance with ethical standards

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

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