Original contribution

Early KRAS oncogenic driver mutations in nonmucinous tissue of congenital pulmonary airway malformations as an indicator of potential malignant behavior☆

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Summary The potential for malignant degeneration is the most common reason for some practitioners to resect asymptomatic congenital pulmonary airway malformations (CPAMs). We aimed to investigate the potential of various immunohistochemical (IHC) and genomic biomarkers to predict the presence of mucinous proliferations (MPs) in CPAM. Archival CPAM tissue samples were re-assessed and underwent IHC analysis using a panel of differentiating markers (TTF1/CDX2/CC10/MUC2/MUC5AC/p16/p53/DICER1). In each sample, intensity of IHC staining was assessed separately in normal lung tissue, CPAM, and MP tissue, using a semiquantitative approach. Likewise, next-generation targeted sequencing of known adult lung driver mutations, including KRAS/BRAF/EGFR/ERBB2, was performed in all samples with MP and in control samples of CPAM tissue without MP. We analyzed samples of 25 CPAM type 1 and 25 CPAM type 2 and found MPs in 11 samples. They were all characterized by strong MUC5AC expression, and all carried a KRAS mutation in the MP and adjacent nonmucinous CPAM tissue, whereas the surrounding normal lung tissue was negative. By contrast, in less than half (5 out of 12) control samples lacking MP, the CPAM tissue also carried a KRAS mutation. KRAS mutations in nonmucinous CPAM tissue may identify lesions with a potential for malignant degeneration and may guide histopathological assessment and patient follow-up.
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1. Introduction

Congenital pulmonary airway malformation (CPAM), formerly known as congenital cystic adenomatoid malformation, is the most common congenital lung abnormality (CLA), comprising up to 30% of all CLA [1]. Advances in prenatal ultrasound are leading to an increase in the incidence which suggests this abnormality is more common than originally thought [2]. Additionally, in recent years, an increasing amount of evidence suggesting malignant degeneration has been found in CPAM, and the conservative management of these lesions is debated [3−5].

Five histological CPAM subtypes can be distinguished according to the level of origin in the tracheobronchial tree, each of which has its own histological features. The diagnosis is either confirmed by a computed tomography scan (CT) or on pathology assessment after surgical resection (Fig. 1) [6,7]. Current concepts claim CPAM to be part of a spectrum of which the phenotype is dependent on the timing and level of occurrence in the embryological development of the lungs. However, the current classification by Stocker is criticized because of the large overlap of lesions, their concurrent occurrence, and the inconsistent use of definitions. Multiple other classification systems have been proposed, which are either based on clinical relevance, histological appearance, or pathogenesis [8−12]. To date, the exact pathogenesis remains unknown, and

although the potential of malignant degeneration has been described in certain subtypes, no definitive evidence has been found for this. The inconsistent use of the classification raises the question whether the malignant potential is only confined to certain subtypes.

The most compelling evidence for malignant potential has been found for the rare and distal CPAM type 4. Thus, multiple clusters of nonciliated cuboidal epithelium with underlying primitive mesenchymal cells in CPAM type 4 should arouse suspicion of pleuropulmonary blastoma (PPB), which is a very rare but aggressive childhood lung tumor [6]. PPB is associated with high morbidity and poor outcome when detected in a late stage and is radiologically indistinguishable from CPAM type 4 [13,14], causing some to believe these are the same entity [4,15−18]. A pathognomonic molecular marker for PPB has not yet been discovered, but association with DICER1 germline mutation is seen in up to 66% [18,19]. This is a genetic syndrome caused by the heterozygous loss-of-function mutation of the DICER1 gene, which is associated with unique disorders, including several malignancies [18,19].

Approximately one-third of the more common CPAM type 1 lesions harbor one or multiple areas of mucinous proliferation (MP), which in turn are thought to be a precursor of mucinous adenocarcinoma in situ [20,21]. Some argue these proliferations to be invasive mucinous adenocarcinoma, if assessed according to ATS/ERS guidelines [22,23]. These assumptions are supported by the overlap in

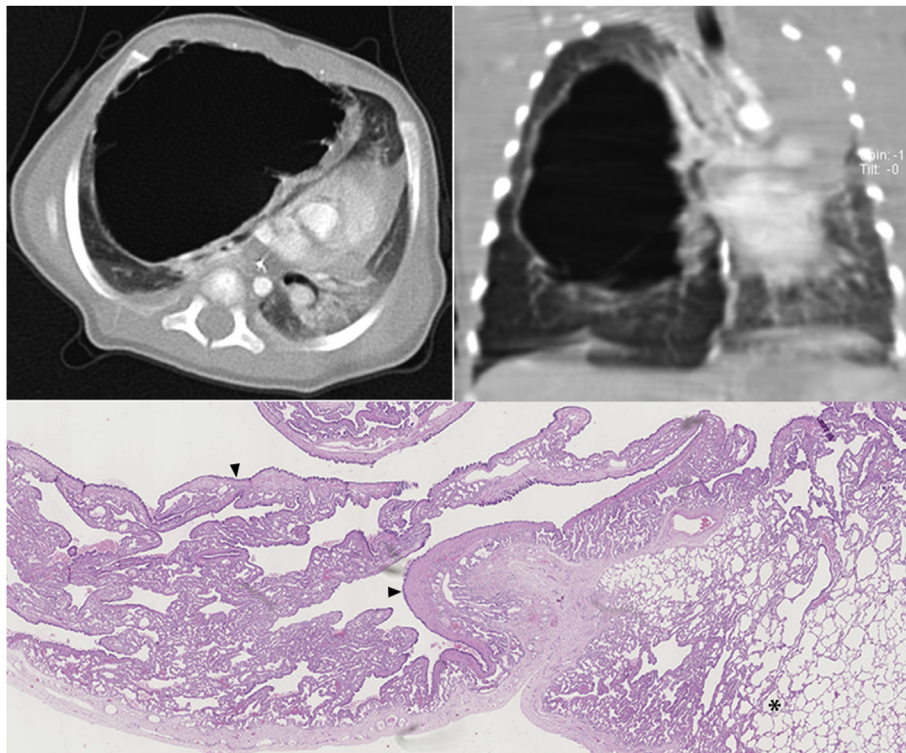

Fig. 1 Axial and coronal CT-scan image of CPAM type 1 and corresponding microscopic image (1.25×) showing the cyst lining (ars) and adjacent normal alveolar tissue (*). CT, computed tomography; CPAM, congenital pulmonary airway malformation.

somatic gene mutations between MP in CPAM type 1 and lung adenocarcinomas [13,20,24]. For example, identical KRAS mutations have previously been reported in patients with MPs [22,23,25] and in CPAMs with malignancy [26−29]. Approximately 32% of all lung adenocarcinomas harbor KRAS mutations, mostly G12V and G12D mutations [30,31]. However, in mucinous lung adenocarcinoma, approximately 75% harbor a KRAS mutation [32−34].

Resection of CPAM lesions is indicated for symptomatic patients presenting with respiratory distress or recurrent infections. However, the majority remain asymptomatic, and a worldwide lack of consensus regarding the optimal management exists [35]. Some argue a wait-and-see policy in asymptomatic patients is better because the surgical risk exceeds the risk of infection [36]. Others advocate a surgical resection in all patients to avert the risk of infection with subsequent increased surgical difficulty and to prevent the risk of malignant degeneration [37].

While a low threshold for surgical resection of CPAM type 4 can be justified because of its close association with PPB, the situation is more equivocal for other CPAM phenotypes. Triage of patients based on the likelihood of malignant progression could prove a valuable strategy for determining the need for close follow-up and/or surgical resection. To this end, identification of robust tissue biomarkers for the likelihood of synchronous or metachronous malignant change in CPAM lesions is required. We therefore aimed to investigate the potential of various immunohistochemical (IHC) and genomic biomarkers to predict the presence of MPs in CPAM.

2. Material and methods

The institutional review board approved this study and waived informed consent (MEC-2018-1355). A search of the pathology database in our tertiary care center was done to identify CPAM cases diagnosed between January 1990 and January 2019. All cases with a CPAM diagnosis, collected following correct consent procedures using opt-out criteria, were included. Formalin-fixed paraffin-embedded (FFPE) slides were retrieved and independently reviewed by 2 pathologists (J.W. and J.vd.T). Tissue was routinely fixed in 4% buffered formalin and then paraffin embedded; routinely hematoxylin and eosin (HE) stained sections, prepared for diagnosis, were used for review. Both pathologists were blinded to the patient data, including histological diagnosis.

2.1. Morphological reassessment

Each slide was evaluated under a light-microscope and assessed for morphology, architecture, and cell types, as shown in Table 1. After independent review of all cases, discrepancies between pathologists concerning the histological findings were resolved by consensus using a double-headed microscope. Final pathology diagnosis was based on morphology and architecture of the cysts as well as the predominant cell type.

2.2. Immunohistochemical analysis

We used a panel of IHC markers (TTF1/CDX2/CC10/MUC2/MUC5AC/p16/p53/DICER1), which are used in routine diagnostics of lung malignancies, as well as markers we considered useful in distinguishing between CPAM types and abnormalities (Table 2). For IHC analysis, 4 μm sections of FFPE tissue were mounted on adhesive glass slides. Deparaffinization was done according to the Benchmark Ultra protocol, and antigen retrieval was performed by CC1 antigen retrieval solution.

All IHC staining was performed with antibodies on a Benchmark Ultra system, using an Ultraview Dab kit (ref. 760-500) for visualization and amplified using an amplification Kit (ref. 760-080). The samples were counterstained with hematoxylin II (ref. 790-2208) and cover-slipped (all antibodies from Ventana Medical Systems, Oro Valley, AZ). On each slide, a positive tissue control was also included and stained.

The evaluation of the slides was done using a semi-quantitative approach, according to the H-scoring method [38−40]. The staining intensity was scored as the estimated percentage of cells (in 10th percentiles) for each intensity level (0 = negative, 1 = weak, 2 = moderate, and 3 = strong) by one pathologist (J.W.) and confirmed by another (J.vd,T.) by means of random sampling. For each IHC stain, a total score ranging from 0 to 300 was calculated by multiplying the percentage of cells with the intensity level. The mean score of three high power fields (40×) per slide was calculated.

2.3. Next-generation sequencing

A targeted panel used in the diagnosis of lung malignancies, containing 40 commonly mutated genes, was performed on samples with MP tissue and samples lacking MP as a control group (Table 3). In each sample, manual microdissection was performed, and next-generation sequencing (NGS) was done separately for MP and non-mucinous CPAM tissue. In samples with MP, Sanger sequencing of the KRAS gene was performed on normal lung tissue as described below (see Supplemental Digital Content 3, for Molecular analysis details).

DNA isolation was performed using standard procedures after microdissection of normal and affected tissue from 10 HE-slides (4 μm) of FFPE tissue [41]. DNA was extracted using proteinase K and 5% Chelex 100 resin and lesional areas composed of 10−50% anomalous cells. NGS was performed by semiconductor sequencing on the Ion Torrent Personal Genome Machine (LifeTechnologies, Carlsbad, CA) according to the manufacturer's protocols and subsequently analyzed and annotated with Seqnext, version 4.2

Table 1   Morphological assessment of CPAM samples.

Low-power view (5×):

Confirmation of CPAM diagnosis: Cystic formations lined by bronchial epithelium and/or in the absence of smooth muscle and cartilage tissue.

Classification based on cyst size (in combination with previously described macroscopic findings):
- Type 1: Cyst(s) > 2 cm
- Type 2: Cysts < 2 cm

High-power view (20x, 40x):

Cell morphology and predominant cell type:
- Bronchial epithelium: tall columnar ciliated cells
- Proximal bronchiolar epithelium: nonciliated columnar cells
- Distal bronchiolar epithelium: cuboidal cells
- Alveolar epithelium: flattened alveolar cells

Classification based on predominant cell type:
- Type 1: Cystic lesions lined by pseudostratified epithelium with abundant papillary infoldings and abrupt transitions to thinner alveolar spaces.
- Type 2: Cystic lesions with multiple, uniform, thin-walled cysts lined by columnar epithelium.

Presence of mucinous proliferation: Cluster(s) of mucinous cells partially lining cyst without invasion of the cyst wall or surrounding parenchyma.

Presence of inflammation:
- Chronic active: presence of neutrophilic granulocytes and lymphocytes
- Chronic: presence of lymphocytes
- Mucostasis: presence of mucus and foamy macrophages

Abbreviation: CPAM, congenital pulmonary airway malformation.

(JSI Medical Systems). Variants were called when the position was covered at least 100 times. To validate the mutations detected by NGS, specific PCR fragments were analyzed by conventional Sanger sequencing, and sequence analyses were performed on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). Samples were analyzed using Mutation Surveyor software (SoftGenetics) and were compared with the public sequence of GenBank (NM_004985.3). Sequence analysis of KRAS exon 2 was performed by bidirectional sequencing of PCR-amplified fragments using M13-tailed forward and reverse primers.

2.4. Statistical analysis

Statistical analysis was performed using SPSS (version 25, IBM Corp., Armonk, NY) and RStudio (version 1.0.153, RStudio, Inc., Boston, MA). We used the "glmnet" and "Ridge" packages for our analyses. Differences

Table 2   Immunohistochemistry details.

Antibody	Gene	Clone	Staining pattern	Supplier	Reference number	Incubation time
TTF1	NKX2-1	SP141	Nuclear	Ventana	790−4756	32 min.
CDX2	CDX2	EPR2764Y	Nuclear	Cell Marque	760−4380	16 min.
P16	CDKN2A	E6H4	Nuclear	Ventana	805−4713	4 min.
P53	TP53	Bp53-11	Nuclear	Ventana	760−2542	4 min.
MUC2	MUC2	CCP59	Cytoplasm	Novus Bio	NBP2-25221	32 min.
MUC5AC*	MUC5AC	45M1	Cytoplasm	Antibodies/online	V2198	32 min.
DICER1	DICER1	CLO378	Cytoplasm	Novus Bio	NBP2-30699	32 min.
CC-10	SCGB1A1	polyclonal	Cytoplasm	Antibodies/online	ABIN2776862	32 min.

NOTE. All antibodies were detected using Ultraview with CC1 64 antigen retrieval without amplification except for * in which Optiview was used with CC1 32 antigen retrieval with amplification.

Table 3 Genes included in the lung cancer panel.

AKT1 exon 3	GNA11 exon 4 & 5	PIK3CA exon 10 & 21
ALK exon 20, 22, 23, 24 & 25	GNAQ exon 4 & 5	POLD1 exon 12
APC exon 14	GNAS exon 8 & 9	POLE exon 9 & 13
ARAF exon 7	HER2 (ERBB2) exon 19, 20 & 21	PTEN all exons
BRAF exon 11 & 15	HRAS exon 2, 3 & 4	RAF1 exon 7
CDKN2A all exons	IDH1 exon 4	RET exon 11 & 16
CTNNB1 exon 3, 7 & 8	IDH2 exon 4	RNF43 exon 3, 4 & 9
EGFR (ERBB1) exon 18, 19, 20 & 21	KIT exon 8, 9, 11, 13, 14 & 17	ROS1 exon 38 & 41
EZH2 exon 16	KRAS exon 2, 3 & 4	SMAD4 exon 3, 9 & 12
FBWX7 exon 9 & 10	MET exon 2, 14 & 19	STK11 exon 4, 5 & 8
FOXL2 exon 3	MYD88 exon 5	TP53 all exons
FGFR1 exon 4, 7 & 12	NOTCH1 exon 26 & 27	MAP2K1 exon 2 & 3
FGFR2 exon 7, 9 & 12	NRAS exon 2, 3 & 4	
FGFR3 exon 7 & 9	PDGFRα exon 12, 14 & 18	

between groups were assessed using the Mann-Whitney U test for continuous variables and Chi-square test for categorical variables. Ridge regression was performed to assess the predictive ability of histological and IHC components when adjusting for all others and to correct for multicollinearity. A logistic ridge regression was performed to either predict the presence of MP or KRAS mutations. Ridge regression is a form of penalized regression in which coefficients with unimportant terms are driven toward zero. The penalization parameter or ridge parameter was chosen using 10-fold cross-validation, and we used the lambda with the minimum mean cross-validated error for our ridge regression. Coefficients are thus slightly biased downward but have smaller standard errors and are therefore more precise [42]. The two-tailed statistical significance was set at a P-value <0.05, unless Bonferroni's correction for multiple testing was applied.

3. Results

Fifty eligible archival CPAM samples were obtained from the pathology department, of which 31(62%) were male, and the median age at diagnosis was 1.5 months (0−48 years). All samples were obtained from symptomatic patients undergoing surgery. In half of the samples, the final pathology diagnosis was CPAM type 1 and the other half type 2.

In 37 (74%) cases, a presurgical chest CT-scan was available. A discordance between CT diagnosis and
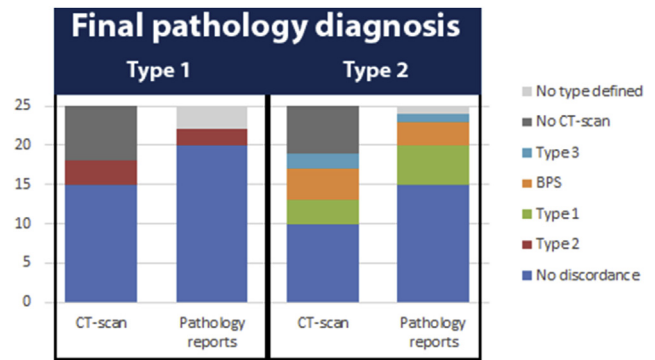

Fig. 2 Diagnosis discordance between final pathology diagnosis CPAM type 1 or 2 and original diagnosis in pathology reports and on CT-scan. BPS, bronchopulmonary sequestration; CT, computed tomography; CPAM, congenital pulmonary airway malformation.

pathological diagnosis was found in 12 (32%) cases (Fig. 2). After careful morphological reassessment, 11 (22%) cases were discordant with the original routine diagnosis, of which most cases were reclassified to type 2.

3.1. Morphological reassessment

We found no difference in CPAM type when basing the classification on either cyst size or cell type, and our final pathology diagnosis based on both resulted in 25 (50%) samples with CPAM type 1 and 25 (50%) with CPAM type 2. The predominant cell type in CPAM type 1 was bronchial epithelium in 21 (84%) samples, followed by proximal bronchiolar epithelium in 4 (16%) samples. In CPAM type 2, proximal bronchiolar epithelium was seen in 14 (56%) cases, followed by bronchial epithelium in 10 (40%) and distal bronchiolar epithelium in 1 (4%) case. MPs were found in 11 (22%) samples, of which 6 (12%) in CPAM type 1 and 5 (10%) in CPAM type 2. Of the MP cases, 5 (45%) samples demonstrated predominantly bronchial cell types, and 6 (55%) had proximal bronchiolar cell types. The median age at diagnosis was significantly lower in cases with MP opposed to cases without MP (10 days versus 5 months, $P = 0.01$).

Signs of inflammation were found in 17 (34%) samples, of which 4 (8%) showed chronic inflammation, 8 (16%) chronic active inflammation, and 5 (10%) mucostasis. In 8 (47%) of these samples, clinical signs of infection were present as well. Two (12%) of 17 samples with signs of inflammation had a MP.

3.2. Immunohistochemistry

The staining intensity in H-scores is summarized individually for normal lung tissue, nonmucinous CPAM tissue, and MP in Table 4. MUC5AC was strongly expressed in MP, whereas other markers showed a wide variety in staining intensity between aforementioned tissue types.

Table 4 Immunohistochemistry H-scores for each tissue type.

IHC stain	Normal lung	CPAM	MP
TTF1[a,b,c]	283 (276−293)	293 (276−296)	107 (80−210)
CDX2[b,c]	0 (0−0)	0 (0−0)	13 (0−27)
p16[a,b]	0 (0−0)	42 (13−63)	20 (0−50)
p53[a]	33 (17−57)	53 (30−77)	70 (40−93)
MUC2[a]	0 (0−0)	0 (0−0)	0 (0−0)
MUC5AC[b,c]	3 (0−33)	13 (0−50)	300 (300−300)
DICER1[a]	10 (0−30)	35 (13−80)	40 (0−60)
CC10[a,c]	0 (0−0)	60 (25−140)	0 (0−0)

Abbreviations: CPAM, congenital pulmonary airway malformation; MP, mucinous proliferation.

NOTE. Data presented as Median (IQR). Signs indicate significant ($P < 0.05$) difference between.

[a] Normal lung & CPAM
[b] Normal lung & MP
[c] CPAM & MP

This variety was dependent on the cellular target of the IHC stain. We found no significant difference in IHC staining when comparing samples based on CPAM type or on predominant cell type. When comparing samples with and without MP, TTF1 and MUC2 did not add any further information, and tumor markers p16, DICER1, and p53 did not show a mutant profile.

However, we did find significant differences in staining intensity between samples with and without MP for some of the markers (Table 5). A significantly higher staining intensity for MUC5AC and DICER1 was seen in CPAM

Table 5 Immunohistochemistry H-scores for samples with and without a mucinous proliferation.

IHC stain	Tissue type	Mucinous proliferation	
		Yes	No
TTF1	normal	283 (266−293)	285 (276−293)
	CPAM	286 (273−296)	293 (286−296)
CDX2	normal	0 (0−0)	0 (0−0)
	CPAM	0 (0−0)	0 (0−0)
P16	normal	0 (0−0)	0 (0−0)
	CPAM	47 (0−63)	40 (17−63)
P53	normal	53 (37−83)	33 (13−57)*
	CPAM	77 (40−90)	50 (27−67)
MUC2	normal	0 (0−0)	0 (0−0)
	CPAM	0 (0−3)	0 (0 - 0)*
MUC5AC	normal	43 (7−87)	0 (0−13)*
	CPAM	30 (10−87)	10 (0−43)*
DICER1	normal	17 (10−33)	3 (0−20)*
	CPAM	62 (33−100)	30 (10−70)*
CC10	normal	0 (0−0)	0 (0−0)
	CPAM	15 (15−30)	90 (45−150)*

NOTE. Data presented as Median (IQR).
Abbreviation: CPAM, congenital pulmonary airway malformation.
*$P < 0.05$.

tissue of samples with MP, whereas this was significantly lower for CC10. The normal lung tissue in these samples showed a higher intensity for P53, MUC5AC, and DICER1 and a lower intensity for CC10. A logistic ridge regression (Table 6) identified DICER1 H-score in CPAM tissue, and P53 and MUC5AC H-scores in normal lung tissue as significant predictors for MP, with all increasing the odds by 1% for every unit increase in H-score. The CC10 H-score in CPAM tissue decreases the odds of MP by 1% for every unit increase.

In 2 cases, a CPAM type 3 diagnosis was made based on CT-imaging (Fig. 3.) of which one was initially histologically classified as type 3. After re-assessment for this study, both were assigned a type 2 diagnosis, and no MPs were detected. We did not find a significant difference in IHC expression for these cases; one underwent NGS which showed a wild-type expression for KRAS.

3.3. Next-generation sequencing

Targeted NGS findings are summarized in Fig. 4. We analyzed 23 (46%) samples with NGS of which 11 (22%) samples contained MP. Of the 40 genes in our targeted sequencing panel, only mutations of the KRAS gene were found. Other genes, commonly mutated in adult lung cancer, including CDKN2A, BRAF, EGFR, HER2, and P53, showed no alterations (Table 3).

KRAS mutations were found in the isolated MPs of which 3 (27%) G12V, 3 (27%) G12D, and in 5 (46%) samples an insufficient amount of tissue was available for NGS. The allelic frequency (AF) ranged between 24 and 55% with approximately 10−50% of the slide deemed to contain MP tissue. In nonmucinous CPAM tissue of all samples with sufficient MP tissue identical KRAS were found, of which 3 (27%) G12V and 3 (27%) G12D mutations. The nonmucinous CPAM tissue of the remaining samples with insufficient MP tissue contained 2 (18%) G12V, 2 (18%) G12D, and 1 (8%) G12R mutation. In these samples, the AF ranged between 16 and 43%, and approximately 10−60% of the slide was deemed to contain CPAM tissue. No KRAS mutations were found in the surrounding normal lung tissue.

In the 12 control samples of nonmucinous CPAM without MP, KRAS mutations were found in 5 (42%) samples, of which 4 (33%) G12V and 1 (8%) G12R. In these samples, the AF ranged between 6 and 39%.

KRAS mutations were more common in CPAM type 1 (83%) as opposed to type 2 (55%), whereas mutations occurred equally in samples with predominantly bronchial (67%) and proximal bronchiolar (80%) cell types, although differences were not statistically significant (Fig. 5).

A logistic ridge regression (Table 6) to predict KRAS mutations in CPAM tissue did not identify significant predictors, although a trend toward significance was seen for CPAM P53 H-score (p = 0.056), which increased the odds for KRAS mutations by 1% for every unit H-score increase.

Table 6 Logistic ridge regression parameters.

IHC stain	Tissue type	Mucinous proliferation			KRAS mutation		
		Beta	Exp(Beta)	P-value	Beta	Exp(Beta)	P-value
(Intercept)		1.66	5.25	NA	1.37	3.95	NA
TTF1	normal	0.00	1.00	0.51	0.00	1.00	0.22
	CPAM	−0.01	0.99	0.30	0.00	1.00	0.57
P16	CPAM	0.00	1.00	0.78	0.00	1.00	0.27
P53	normal	0.01	1.01	0.03*	0.00	1.00	0.45
	CPAM	0.00	1.00	0.28	0.00	1.00	0.06
MUC5AC	normal	0.01	1.01	0.01*	0.00	1.00	0.26
	CPAM	0.00	1.00	0.28	0.00	1.00	0.81
DICER1	normal	0.01	1.01	0.10	0.00	1.00	0.39
	CPAM	0.01	1.01	0.03*	0.00	1.00	0.44
CC10	CPAM	0.00	1.00	0.01*	0.00	1.00	0.13
Distal bronchiolar cell type		−0.55	0.58	0.52	−0.41	0.67	0.16
Proximal bronchiolar cell type		0.33	1.39	0.19	0.10	1.11	0.34
Inflammation present		−0.21	0.81	0.40	−0.20	0.82	0.08
CPAM type 2 final diagnosis		−0.03	0.97	0.90	−0.17	0.85	0.12

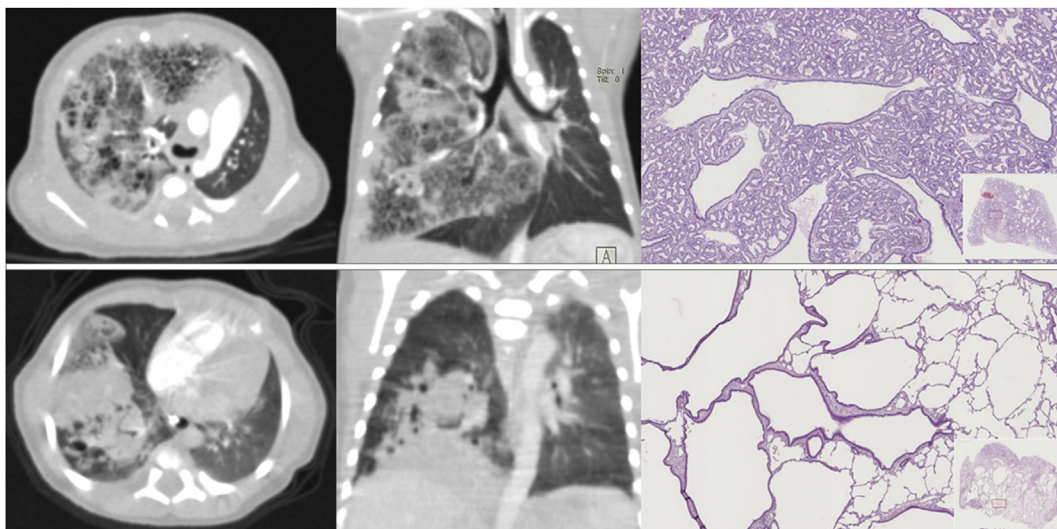Abbreviation: CPAM, congenital pulmonary airway malformations.
*$P < 0.05$.

Fig. 3 Both cases with a CT-diagnosis of CPAM type 3 on axial and coronal CT-scan images and corresponding microscopic images (5×) showing a histological diagnosis of CPAM type 2. The cellular composition and architecture of the top case showed characteristics of both CPAM type 2 and type 3, but ultimately better fit a type 2 diagnosis. CT, computed tomography; CPAM, congenital pulmonary airway malformation.

4. Discussion

We aimed to investigate clues for the malignant potential of CPAM lesions and to identify predictive factors and useful diagnostic tools. We found identical KRAS mutations in all isolated MP tissue as well as the surrounding nonmucinous CPAM tissue, whereas adjacent normal lung tissue showed no mutations. MUC5AC IHC staining may be helpful for identification of the frequently small and bland appearing MP.

4.1. Morphological reassessment

We found no difference in CPAM type when basing the classification on either the predominant cell type or on cyst size with a 2 cm cut-off. Still, use of this cut-off is questionable as it is based on a series of only 38 cases in the original article and may not be reproducible as cyst size varies because of hyperinflation and infection [43]. Likewise, we found a 32% discordance when comparing the pathology and CT diagnosis, which also discourages CT
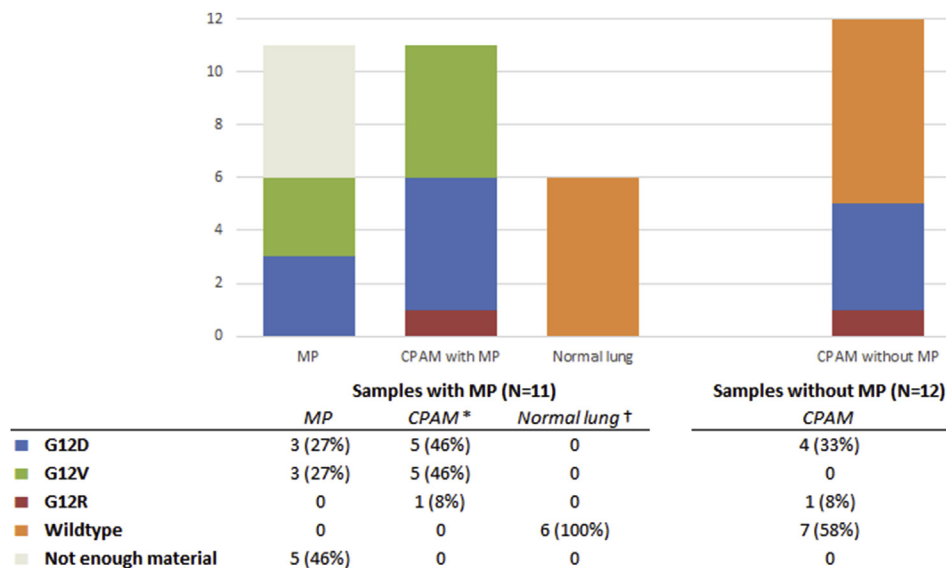
| | | Samples with MP (N=11) | | | Samples without MP (N=12) |
		MP	CPAM *	Normal lung †	CPAM
■	G12D	3 (27%)	5 (46%)	0	4 (33%)
■	G12V	3 (27%)	5 (46%)	0	0
■	G12R	0	1 (8%)	0	1 (8%)
■	Wildtype	0	0	6 (100%)	7 (58%)
■	Not enough material	5 (46%)	0	0	0

Fig. 4 Location of KRAS mutations separately for each tissue type, in samples with and without MP. * Mutations identical to MP in the same sample. † Only 6 samples sequenced.
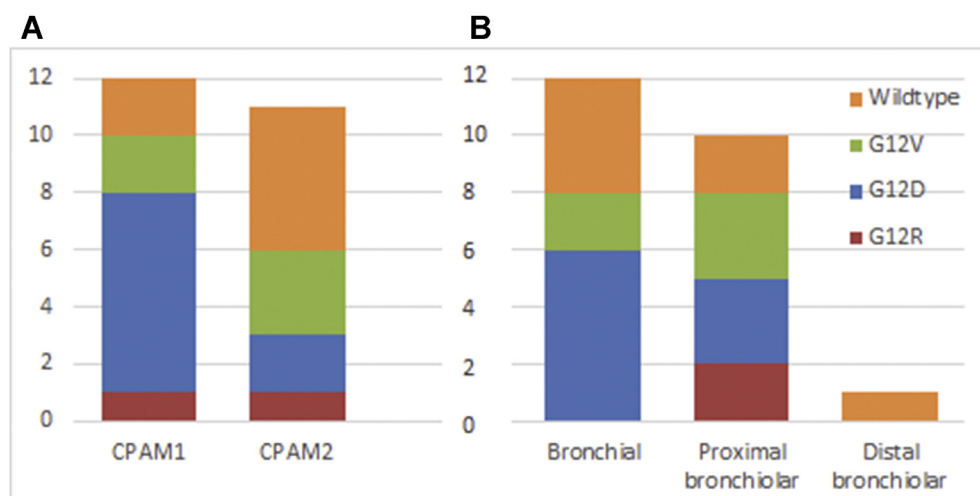

Fig. 5 Distribution of KRAS mutation in CPAM tissue between CPAM types (A) and predominant cell types (B). CPAM, congenital pulmonary airway malformation.

classification based on cyst size, as this is unreliable because of overlap in features and ventilation differences. Furthermore, airspace collapse, direction of sectioning, and mechanical traction during preparation may influence cyst size measurements on histological examination [3]. The updated classification, based on cell type ranging from proximal bronchial epithelium to alveolar cells, may be a better fit, although overlap is unavoidable [6]. Our series show both CPAM type 1 and type 2 can have a predominance of bronchial and proximal bronchiolar epithelium, whereas distal bronchiolar epithelium is only found in CPAM type 2. Although this may aid in distinction between subtypes, the clinical significance of this distinction is still arguable. Traditionally, type 1 is thought to be associated with malignant degeneration, whereas type 2 showed association with other CLA. However, we found MP as well as KRAS mutations in both CPAM type 1 (44%) and type 2 (26%). We speculate some CPAMs arise from a transition zone, in which some lesions harbor characteristics and histological features of both CPAM types. If overlap exists between subtypes, it may exist within aforementioned associations as well, rendering clinical use of this distinction unnecessary. The definitive diagnosis should therefore combine both radiological and histological features and management based solely on CT appearance should be done with due caution.

MPs were found in 22% of our samples, with equal frequency in CPAM types 1 and 2, as well as histologically predominant bronchial and proximal bronchiolar cell types. In previous reports, these MP have been mostly reported within type 1 CPAM, although the majority have been published in case reports [25,44]. Two larger studies reported 12% of patients in their cohort had MP, all in type 1 CPAM [20,23]. We show a higher frequency of MP, which may be because of detection being highly dependent on sampling and attention, as these lesions are small, bland areas that are easily missed. Detection within CPAM type 2 has previously only been reported in one case report [27] and in one with features of both type 1 and type 2 CPAM [45]. In fact, CPAM type 2 has only been reported in combination with malignancy in 2 other case reports with rhabdomyosarcoma [46,47]. As our cohort shows type 2 CPAM harbor MP as well, analogous to type 1, a careful examination of type 2 samples should be done to detect possible MP foci. As mentioned before, this finding might suggest distinction between these subtypes may not be clinically relevant as they may share the same associations. Decisions on management should therefore not be based on the type of CPAM.

We suggest structured reporting of CPAM tissue samples using well-defined terms based on objective histological elements rather than the subjective interpretation and diagnosis of one observer (Online supplementary file 1). In our opinion, this will improve the clarity of reports and can aid in clinical decision-making as the course of management may be based on various histological findings. In addition, structured reports may facilitate objective comparison of study findings as definitions in this field may change with the use of novel diagnostic tools.

4.2. Immunohistochemistry

In general, the use of IHC in routine pathological examination can aid diagnosis because of expression of specific proteins. Our cohort identified P53 and MUC5AC intensity in normal lung tissue as significant predictors for MP, whereas CC10 in nonmucinous CPAM tissue decreased the odds. However, the clinical use of these findings is limited. Previous studies have already shown MUC5AC to be a valuable marker for MP [23], and similar IHC expression patterns as mucinous lung adenocarcinoma in situ were seen in MP tissue of our set [20,22,48−51].

A higher p53 expression in normal lung tissue of samples with MP might be explained by an increased stimulation of the antiproliferative effects of the p53 protein in tissues adjacent to the potentially proliferative CPAM tissue [52].

CC10 protein is secreted by club cells which are mainly located in the terminal bronchioles and have a function similar to stem cells [53]. We hypothesized CC10 immunohistochemistry would be helpful in differentiation

between CPAM type 1 and type 2, as the latter subtype is comprised of more distal epithelium, but unfortunately no association was found.

No aberrant expression was seen for the tumor markers p53, p16, and DICER 1 in MP and adjacent CPAM tissue, which is in agreement with the lack of genomic alterations in the corresponding genes with NGS.

4.3. Next-generation sequencing

We are the first to report a separate NGS analysis of MP and adjacent nonmucinous CPAM and normal lung tissue with use of microdissection. We found KRAS mutations in all samples with MP. Identical mutations were present in MP tissue as well as adjacent nonmucinous CPAM tissue, whereas the surrounding normal lung tissue showed a wild-type profile of the genes tested in a large targeted diagnostic panel. The AF percentage in the MP tissue (16-43%) may be explained by careful isolation with manual microdissection and thus minimal contamination from surrounding tissue. Conversely, the presence of identical mutations with a similar AF (24-55%) in the surrounding CPAM tissue confirms the true presence of KRAS mutations in the CPAM tissue, because contamination of a small number of morphologically undetected mucinous cells is highly unlikely to lead to such a high AF. The absence of KRAS mutations in normal lung argues against a germline defect but cannot exclude somatic mosaicism.

We hypothesize that CPAM lesions harboring KRAS mutations have an increased malignant potential and may either harbor undetected MP or these may develop over time. This is supported by our data showing identical KRAS mutations in the CPAM tissue of all samples with a MP. Even though 5 (42%) samples of our control set without apparent MP harbored KRAS mutations, such MP may have been missed because of a sampling error. In contrast to adult lung cancer, in which a KRAS mutation confirms malignancy, the clinical relevance of these mutations within pediatric lung specimens still need to be investigated [30,54]. Long-term follow-up of these lung lesions may help in understanding their clinical behavior, including their malignant potential. If MP are considered a malignant or premalignant finding, long-term follow-up of patients with KRAS mutations in CPAM tissue may be indicated, especially if resection margins contain KRAS positive CPAM tissue. However, our findings need to be validated in a larger set, and the predictive value of KRAS positivity in children with CPAM for the development of mucinous adenocarcinoma remains to be investigated.

In our set, only one case with CPAM type 1 showed signs of malignant degeneration (Fig. 6, online supl). A CPAM was diagnosed as an incidental finding in a 30-year-old, pregnant, nonsmoker without any previous medical history who underwent a chest CT-scan because of dyspnea and oxygen requirement after an emergency caesarian section. A video-assisted thoracoscopic lobectomy of the
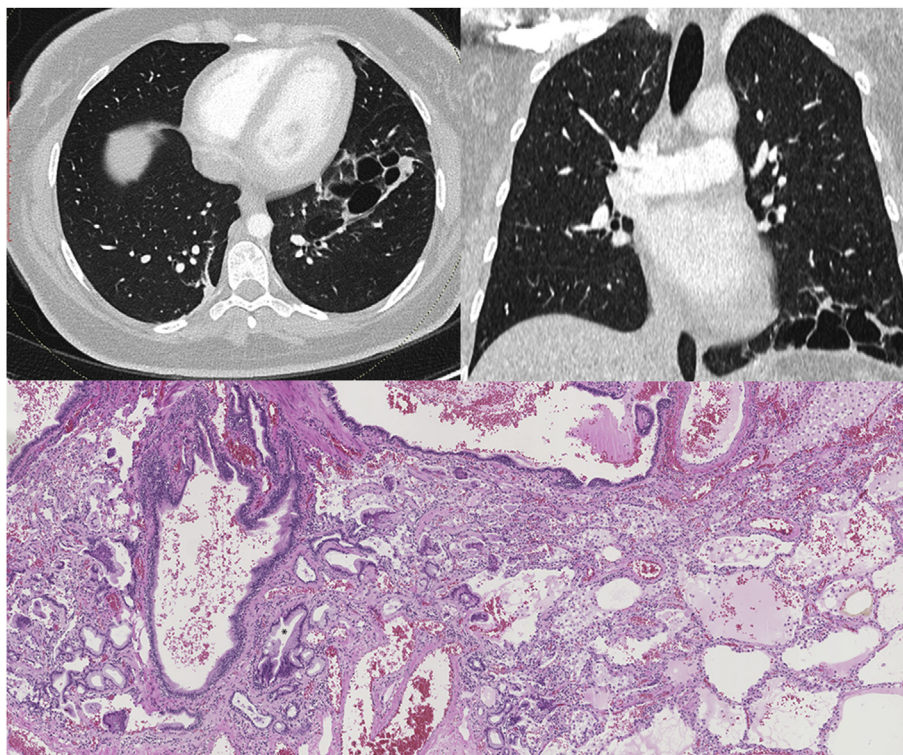
Fig. 6 Axial and coronal CT-scan image of CPAM type 2 and corresponding microscopic image (5×) showing multiple mucinous proliferations, focally suggestive of invasion (*). A high-resolution image showing invasive mucinous adenocarcinoma is available as eSlide: VM000xx. CT, computed tomography; CPAM, congenital pulmonary airway malformation.

left lower lobe was performed 3 months later, following a multidisciplinary team meeting. The procedure as well as the postoperative clinical course was uncomplicated, and she was discharged 5 days after surgery.

Histological examination of the specimen showed a CPAM type 1 lesion with multiple atypical MPs and a maximal diameter of 7 cm, confirming the diagnosis of a lepidic mucinous adenocarcinoma. No hilar lymph node involvement was seen, and resection margins were clear. Separate NGS was done on a slide with 30% CPAM tissue and another with 50% MP, which both showed a G12D KRAS mutation. A follow-up chest CT-scan 3 months after the operation showed no abnormalities, and she remained asymptomatic at the outpatient clinic at 1 year follow-up.

Similar to conventional adult practice, targeted mutation analysis on biopsy material or bronchoalveolar lavage cytology may identify high-risk CPAM variants, in which close follow-up and/or a surgical resection may be warranted [55].

A third of lung adenocarcinomas show KRAS mutations with a higher frequency found in smokers showing G12C mutations, whereas nonsmokers often present mutations in G12D and G12V. The latter is believed to be associated with more aggressive tumor growth and angiogenesis [56,57]. Reports of CPAM samples containing carcinoma describe the same mutations in G12V [28,29] and G12D [26,27]. We found G12D and G12V KRAS mutations in isolated MP tissue and adjacent CPAM tissue of our set. This corresponds to previous studies which also reported G12D [22], G12V [23,25], and G12C [23] mutations in MP. One previous report describes no mutations in the KRAS gene of CPAM tissue with malignancy [58]. We found KRAS mutations in position G12D in isolated CPAM tissue without MP, a finding not previously reported. One study reported the absence of KRAS mutations but analyzed CPAM and adjacent normal lung tissue which might have diluted the DNA material [59]. Another novel finding in our set was the KRAS mutation in position G12R in one CPAM tissue sample with MP and one sample without.

KRAS mutations are nearly always mutually exclusive with EGFR and BRAF mutations as is shown in our cohort as well. In line with previous studies, none of our samples showed mutations in EGFR [22,23,25,29] or HER2 genes [23]. Expression of p53 was present in our set, corresponding with an absence of mutant-type overexpression or "null-mutation" patterns [60]. CDKN2A/P16INK4 mutations were absent in our set, whereas this was a common finding in a previous study [22].

This study is limited by the small sample size, although a larger cohort may be difficult to achieve in a single-center setting, when taking the rarity of this disease into account. However, our findings need to be confirmed in larger multicenter studies. Long-term follow-up of these lesions is necessary to determine the likelihood of progression to

invasive mucinous adenocarcinoma, and to this end, a retrospective analysis should be done on cases with invasive mucinous adenocarcinoma for evidence of pre-existing CPAM lesions. Another limitation is the fact that all samples in our cohort were obtained from symptomatic patients, which might introduce a bias toward patients with unfavorable lesion characteristics. Finally, because of the retrospective nature of our study, we were only able to examine representative tissue blocks, which adds a selection bias on top of the inevitable sampling error. Future studies utilizing a more comprehensive molecular assessment such as whole exome sequencing may reveal novel findings.

5. Conclusions

In conclusion, we report the first study of a separate NGS analysis of MP and adjacent nonmucinous CPAM and normal lung tissue with the use of microdissection in CPAM type 1 and 2. Of the 40 genes in our targeted sequencing panel, only mutations of the KRAS gene were found. These KRAS mutations were present in MP of all samples as well as in adjacent nonmucinous CPAM tissue, whereas the surrounding normal lung tissue was negative. CPAM lesions harboring this mutation may have an increased potential for malignant degeneration, and this could determine the need for total analysis of resection specimens, completion of resection following subtotal CPAM surgery, as well as the need for close follow-up. Noninvasive techniques to determine the mutational status could guide indications and the extent of surgical management of CPAM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2020.07.015.

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