Diplomarbeit

Atypical goblet cell hyperplasia occurs in CPAM 1, 2, and 3, and is a probable precursor lesion for childhood adenocarcinoma

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Fabian Fakler

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unter der Anleitung von Univ.-Prof. Dr. Helmut Popper und

Priv.-Doz. DDr. Luka Brcic

Graz, 23.03.2020

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 23.03.2020 *Fabian Fakler eh*

Vorwort

Motiviert durch meine Diplomarbeitsbetreuer und mit Ihrer Hilfe war es möglich meine Arbeit an der Pathologie in eine Form zu bringen, in der an eine Publikation zu denken war. Dafür möchte ich mich herzlich bei Univ.-Prof. Dr. Helmut Popper und Priv.-Doz. DDr. Luka Brcic bedanken.
Aus einer Idee von Prof. Popper entstand das erste gemeinsame Manus. Darauf folgte eine intensive Literaturrecherche. Diese führte ich im Rahmen eines Speziellen Forschungsmoduls (SFM) unter Supervision durch. Als Abschluss des SFM konnte ich ein Konzept und eine richtungsweisende Auswahl an immunhistochemischen Markern vorlegen.
Als nächster Schritt folgte eine systematische Suche in unserer Datenbank nach Fällen, die im weiteren Sinn als CPAM, Sequester oder „zystische Fehlbildung", klassifiziert wurden. Mit diesen Daten konnte ich im Archiv der pathologischen Abteilung sämtliche Paraffinblöcke und Schnitte finden. Einige der Fälle forderten wir bei der Biobank an. Es beteiligten sich auch pathologische Abteilungen aus anderen Ländern an der Studie und schickten uns ihre Fälle zur Durchsicht (Department of Pathology, Erzurum Regional Training and Research Hospital, Erzurum, Turkey, 2nd Department of Pathology, Semmelweis University Budapest, Budapest, Hungary, Department of Pathology and Medical Biology, University of Groningen, University Medical Center, Groningen, The Netherlands). Auch hier möchte ich mich für die Mithilfe bedanken.
Nachdem im nächsten Schritt alle Blöcke geschnitten und gefärbt wurden, konnte mit der mikroskopischen Evaluierung begonnen werden. Gemeinsam mit Dr. Brcic konnten wir zuerst eine grobe Einteilung der Fälle machen, auch wurden viele aussortiert, da sich keine CPAM gezeigt hat. In den von mir zuvor angefertigten Listen und Tabellen dokumentierten wir sämtliche Ergebnisse. Zum einen klassifizierten wir den Subtyp, zum anderen bestimmten wir zu jeder Färbung die Intensität der immunhistochemischen Expression der jeweiligen Marker.
Nachdem ich sämtliche Ergebnisse aufgearbeitet habe, konnte ich sie Prof. Popper vorlegen und wir legten unseren Fokus fest. Danach mikroskopierten Prof. Popper und ich sämtliche Schnitte, welche in die Studie eingeschlossen wurden. Nach diesem sehr ergiebigen und intensiven Prozess der Datenerhebung konnte ich mit der Auswertung beginnen. Zeitgleich begann ich die Einleitung der Arbeit zu schreiben.
Unter der Supervision meiner beiden Betreuer entstand im Anschluss die erste Version des Manuskripts. Bis dieses eine Form erreichte, wie es zur Publikation nötig ist, dauerte es noch einige Zeit. Die Arbeit wurde nach einer Revision im Virchows Archiv publiziert:
Fakler, F., Aykutlu, U., Brcic, L. et al. Atypical goblet cell hyperplasia occurs in CPAM 1, 2, and 3, and is a probable precursor lesion for childhood adenocarcinoma. Virchows Arch(2019). https://doi.org/10.1007/s00428-019-02732-4
Der Prozess vom ersten Gespräch bis zum Erscheinen der Arbeit war arbeitsintensiv. Ich bin sehr dankbar für die Möglichkeit diese Forschungsarbeit in einem so tollen Team durchführen zu dürfen. Durch diese Zusammenarbeit konnte ich viel Erfahrung für meine weitere berufliche Laufbahn sammeln.

Zusammenfassung

Die congenital pulmonary airway malformation (CPAM) ist eine angeborene Erkrankung. Die häufigsten Subtypen sind CPAM 1, 2 und 3. Es ist möglich, dass die atypische Becherzellhyperplasie (atypical goblet cell hyperplasia AGCH), die in einer CPAM zu finden ist, eine Vorstufe eines Adenocarcinoms der Lunge ist. 9 von 33 CPAM 1-3 Fälle zeigten Bereiche mit Becherzellenhyperplasie. Da diese Zellen das normale Epithel komplett ersetzen bevorzugen wir für diese Proliferation den Namen AGCH. Adenocarcinome (AC) wurden in fünf Fällen gesehen. Alle Fälle wurden auf das Vorhandensein von speziellen Proteinen getestet, die im Verdacht stehen, mit der Entwicklung von CPAM zu korrelieren: fibroblast growth factor 10 (FGF10), fibroblast growth factor receptor 2 (FGFR2), forkhead box A1 (FOXA1) und A2 (FOXA2), MUC protein 5 AC (MUC5AC), human epidermal growth factor receptor 2 (erbB2, HER2/neu), hepatocyte nuclear factor 4α (HNF4α), SOX2 and Ying Yang protein 1 (YY1). Mittels next generation sequencing wurden AGCH und Adenocarcinome auf sogenannte driver mutations (Verstärker Mutationen) untersucht.
Im CPAM Epithel und Stroma konnte die Expression von FGF10, FGFR2, FOXA1 und FOXA2 nachgewiesen werden. AGCH und AC zeigten im Gegensatz dazu keine Unterschiede im Expressionsmuster. SOX2 war positiv im CPAM Epithel und AGCH, aber nur schwach im AC. YY1 und MUC5AC zeigten eine stärkere Expression in AGCH und AC als im CPAM Epithel. HER2 exprimierte stark im AC, weniger stark in AGCH und nicht im CPAM Epithel. In allen AGCH und AC Fällen konnte eine KRAS Mutation am Exon 2 nachgewiesen werden, diese fehlte im CPAM Epithel.
AGCH kann sich in den CPAM Typen 1-3 entwickeln. Eine KRAS Mutation scheint bereits eine onkogene Bedeutung in der AGCH zu spielen und beweist seine Rolle als Vorstufe des AC. Weiters könnte HER2 nach oben regulieren werden, auch YY1 scheint in der Karzinogenese von Bedeutung zu sein.

Inhaltsverzeichnis – Table of content

Tabellenverzeichnis - Index of tables

Abbildungsverzeichnis – Table of figures

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ORIGINAL ARTICLE

Atypical goblet cell hyperplasia occurs in CPAM 1, 2, and 3, and is a probable precursor lesion for childhood adenocarcinoma

Fabian Fakler[1] · Umut Aykutlu[2] · Luka Brcic[1] · Sylvia Eidenhammer[1] · Andrea Thueringer[1] · Karl Kashofer[1] · Janina Kulka[3] · Wim Timens[4] · Helmut Popper[1]

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Abstract

Congenital pulmonary airway malformation (CPAM) is a developmental disorder. Types 1-2-3 are the more common ones. Atypical goblet cell hyperplasia (AGCH) in CPAM might be a precursor lesion for pulmonary adenocarcinomas. In nine out of 33 CPAM cases, types 1–3 showed foci of goblet cell proliferations. As these cells completely replace normal epithelium, we prefer to name these proliferations AGCH. In 5 cases, adenocarcinomas were seen (AC). All cases were analyzed for proteins possibly being associated with CPAM development: fibroblast growth factor 10 (FGF10) and receptor 2 (FGFR2), forkhead box A1 (FOXA1) and A2 (FOXA2), MUC protein 5AC (MUC5AC), human epidermal growth factor receptor 2 (erbB2, HER2/neu), hepatocyte nuclear factor 4α (HNF4α), SOX2, and Ying Yang protein 1 (YY1). By next generation sequencing, AGCH and adenocarcinomas were evaluated for driver mutations. Expression for FGF10, FGFR2, FOXA1, and FOXA2 was seen in CPAM epithelium and stroma, but not differently in AGCH and AC. SOX2 was positive in CPAM epithelium and AGCH, however weakly in AC. YY1 and MUC5AC showed more intense staining in AGCH and AC than in CPAM epithelium. HER2 was intensely expressed in AC and less intensely in AGCH, but not in CPAM epithelium. KRAS mutation in exon 2 was detected in all AGCH and AC, but was absent in CPAM epithelia. AGCH can arise in CPAM types 1–3. Oncogenic KRAS mutation seems to be the oncogenic driver already in AGCH, proving its role as a precursor lesion for adenocarcinoma. It might upregulate HER2 at the protein level. YY1 seems to be involved in carcinogenesis.

Keywords Congenital pulmonary airway malformation · Types 1–3 · Atypical goblet cell hyperplasia · Mucinous adenocarcinoma · KRAS mutation · HER2 · Ying Yang 1 protein upregulation

Introduction

Congenital pulmonary airway malformation (CPAM) has been classified into 5 types by Stocker [42]. CPAM types 1–3 are the most common types found in neonates, but occasionally are diagnosed in young adults. Whereas CPAM 1 and 2 are cystic lesions, CPAM 3 is solid [8, 27, 42, 44]. They represent localized developmental defects of airway formation at different times of lung development. Type 1 seems to occur early during the branching morphogenesis, resulting in large cystic bronchi, which are not connected to the alveolar periphery, whereas CPAM 2 seems to occur later, preventing the connection with the mesenchyme during branching of segmental bronchi [2, 41]. Type 1 is often diagnosed early on, because the cysts compress the normal lung and causes symptoms of hypoxia. CPAM 2 is less symptomatic and sometimes

Fabian Fakler and Umut Aykutlu contributed equally to this work.

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✉ Helmut Popper
helmut.popper@medunigraz.at

[1] Diagnostic and Research Institute of Pathology, Medical University Graz, Neue Stiftingtalstrasse 6, 8036 Graz, Austria

[2] Department of Pathology, Erzurum Regional Training and Research Hospital, Erzurum, Turkey

[3] 2nd Department of Pathology, Semmelweis University Budapest, Budapest, Hungary

[4] Department of Pathology and Medical Biology, University of Groningen, University Medical Center, Groningen, The Netherlands

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detected by chance. Bronchial atresia has been frequently reported in CPAM 1 and 2, but is most often missed in the specimen sent to pathology, because surgeons prefer to dissect and sew at this site. Probably mucus accumulation might guide one to atresia. Other developmental disorders are also seen in connection with CPAM 2, especially sequestration [11, 20, 35]. CPAM 3 is different, as it is composed of bronchiolar structures not connected to the mesenchyme, thus lacking alveolar tissue. Goblet cell proliferations have been so far described in CPAM 1 and have been proposed as possible precursor lesions of a childhood adenocarcinoma [17, 39, 43].

Different genes have been reported of being associated with CPAM 1–3. In animal models, fibroblast growth factor 10 (FGF10) overexpression caused CPAM-like changes. When overexpressed during pseudo-glandular phase, it results in large cysts similar to CPAM 1, and overexpression during canalicular phase in smaller cysts similar to CPAM 2. FGF10, as well as the fibroblast growth factor receptor 2 (FGFR2) were demonstrated by immunohistochemistry in human CPAM cases [6, 15, 29]. In these reports, FGF10 was linked to CPAM development, whereas in the report by Jancelewicz, FGF9 was said to be essential for CPAM development [23]. Forkhead box proteins (FOX) are associated with differentiation in the developing fetal lung. Branching morphogenesis was shown to require downregulation of FOXA1, whereas downregulation of both FOXA1 and 2 inhibit cell proliferation, epithelial differentiation, and bronchial branching [50]. We have previously reported that goblet cell hyperplasia seen in CPAM seems to be driven by the expression of interleukin 4 receptor and interleukin 13, by human epidermal growth factor receptor 2 (erbB2, HER2/neu), MUC2 and 5AC genes [39]. We, and others, have demonstrated the association of MUC5AC and HER2/neu also in the development of adenocarcinoma [4, 37, 39]. However, molecular analysis was limited at that time. SOX2 has been shown to independently act in CPAM formation. SOX2 differentiate naïve epithelial cells into the proximal lineage irrespective of FGF10. Premature differentiation by SOX2 resulted in cyst formation [33]. In contrast to this findings, another investigation demonstrated association of SOX2 with a reduction of ventral differentiation gene NKX2 (known as TTF1) and expression of dorsal markers in primary bronchi [9]. Ying Yang 1 (YY1) is a multifunctional zinc-finger-containing transcription factor that plays crucial roles in numerous biological processes. YY1 inactivating mutations in lung epithelium resulted in neonatal death due to respiratory failure. It impaired tracheal cartilage formation, altered cell differentiation, abrogated lung branching, and caused airway dilation similar to that seen in human congenital cystic lung diseases. A decreased expression of YY1 was seen in pleuropulmonary blastoma [3]. No valid data exist, what effect an overexpression of YY1 might cause.

KRAS mutations have been reported in CPAM type 1 and several reports also investigated abnormalities in HER2 expression. Some were case reports [25, 38, 40, 46], others investigated case series, and reviewed the literature [28, 35, 37]. Interestingly, all KRAS mutations were found in codon 12. HER2 expression, but not amplification or mutation was reported. Abnormalities of p16, FHIT, and RB1 were found in single cases, while no mutations in TP53 were detected [28]. No other CPAM types showed atypical goblet cell hyperplasia and adenocarcinoma (the findings of previous reports are summarized in Table 1).

In our study, we aimed to analyze CPAM type 1–3 cases for goblet cell proliferations, and also adenocarcinomas arising within these proliferations. We also evaluated the above described markers. The role of different genes for the progression of goblet cell proliferation into adenocarcinoma was investigated using next generation sequencing.

Materials and methods

Case selection

Twenty-eight CPAM types 1-2-3 were retrieved from the lung archive of one of the authors; within these cases, there were 4 cases of AGCH, and 3 of them with adenocarcinoma. Five additional cases with AGCH, with or without adenocarcinoma, were provided by co-authors (for details see Table 2). For this investigation, cases with AGCH, with or without adenocarcinoma, were selected. The other 24 cases were used as controls in the immunohistochemical analysis. All slides were independently re-evaluated by 4 of the authors (FF, LB, UA, HP).

Definition of CPAM and AGCH

CPAM 1 and 2 were separated according to the classification of Stocker [43] and Kitaichi and Yousem [26], using the size of the cysts (> 2 cm), a bronchial type of epithelium, and the presence of bronchial structures in the wall of the cysts (for example, layers of smooth muscle cells) for CPAM 1. CPAM 2 was defined by smaller cysts, ≤ 2 cm, and a bronchiolar and/or ductal epithelium type within walls and a thin layer of smooth muscle cells (< 3 layers), sometimes only few smooth muscle cells. CPAM 3 is a solid lesion occasionally with small cysts, < 1 cm, composed of bronchiolar, rarely ductular epithelium, without a connection to peripheral alveolar tissue.

Mucus producing or goblet cells were found in all three types of CPAM. These lesions have been referred as goblet or mucus cell proliferation, or atypical goblet cell hyperplasia (in our previous publication). However, in contrast to hyperplasia of goblet cells in adults, where goblet cells are interspersed within normal columnar and ciliated

Table 1 Presentation of KRAS, EGFR, and Her2 status in published cases

Author	Cases	Age	CPAM type	Carcinoma	KRAS	EGFR	HER2	Other abnormalities
Lantuejoul S. et al., 2007 [28]	7	6 mo-63 yrs	1	4/7 with BAC, 6/7 intracystic mucinous cell clusters, 3/7 with extracystic mucinous cell clusters	Present in 3/3 intracystic and 2/3 extracystic mucinous cell clusters and 3/4 BAC, one G12A, others G12D	No	NA	P16, FHIT, RB1 in single cases
Guo H et al., 2007 [19]	23	26GW-10 yrs	1: 12 2: 7 3: 4	2 cases of CPAM type 1 with mucinous cell hyperplasia	None (all cases analyzed)	IHC expression absent in mucinous cell areas	NA	NA
Summers RJ et al., 2010 [46]	1	8 yrs	1	Well differentiated multifocal/metastatic mucinous AC	G12V	NA	NA	NA
Rossi G et al., 2012 [37]	19	NA	1	Mucinous cell proliferations in 5 cases, no AC	G12C in 4 patients G12V in 1 patient	No	Positive expression (IHC) in mucinous cells, no mutation	NA
Ishida M et al., 2013 [22]	1	9 yrs	1	BAC	G12D	NA	NA	NA
Kim MY et al., 2014 [25]	1	23 mo	1	Mucinous AC with multiple foci of mucinous hyperplasia	G12V		Positive expression (IHC) in mucinous cells	NA
Singh G et al., 2016 [38]	1	18 yrs	1 and 2 combined	Invasive mucinous AC	G12V	NA	NA	NA
Stephanov O et al., 2018 [40]	1	Newborn (diagnosed prenatally)	1	Lepidic mucinous AC	G12D	No	No	NA

Yrs, years of age; *mo*, months; *GW*, gestational week; *BAC*, bronchioloalveolar carcinoma (this cannot be translated due to different definitions used); *AC*, adenocarcinoma; *IHC*, immunohistochemistry; *NA*, not available

Table 2 Patient characteristics (investigated cases only)

Age, years	Gender	Resection type	CPAM type	AGCH/ AC	Follow-up
8	m	Wedge	2	1/0	14 yrs., noR
18	m	Lobar	2	1/0	5 yrs., ltf, noR
3	f	Lobar	3	1/0	4 yrs., noR
5	m	Wedge	1	1/1	6 yrs., noR
1	f	Lobar	1	1/1	15 yrs., noR
13	m	Lobar	1	1/1	6 yrs., noR, ltf
14	m	Wedge	1	1/0	20 yrs., noR
12	m	Lobar	2	1/1	4 yrs., noR
12	m	Lobar	2	1/1	6 yrs., noR

Age, age at the time of surgery; *m*, male; *f*, female; *AGCH*, atypical goblet cell hyperplasia; *AC*, adenocarcinoma; *R*, recurrence, *noR*, no recurrence during observation period; *ltf*, lost to follow-up; observation time as given by clinicians

cells, in CPAM, these cells replace the CPAM epithelium entirely. Sometimes, these cells form small clusters, but are still confined to the surface epithelium of the CPAM cyst (Fig. 1). We therefore do not use terms such as mucus cell proliferation or hyperplasia, but atypical goblet cell hyperplasia, to avoid a misinterpretation. Of note, cuboidal and cylindrical cells are making up the majority of cells in all CPAM cases, which do not show atypical goblet cell hyperplasia, and we refer to them as CPAM epithelium.

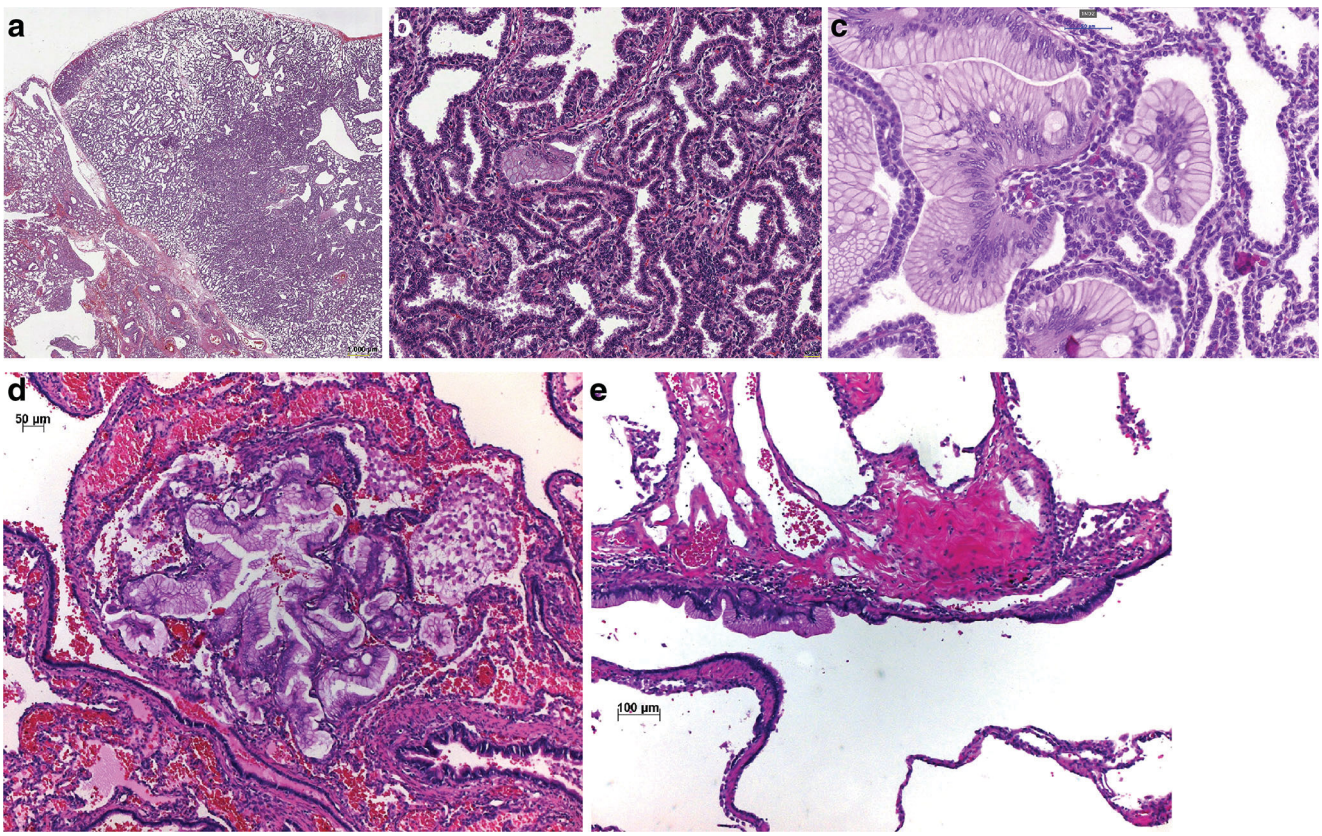

Fig. 1 CPAM types 3 and 2. **a** Overview of CPAM 3 showing to the right side the almost solid lesion, whereas to the left another segment is seen which is normal. **b** The lesion is entirely composed of bronchiolar structures without any larger bronchi and no peripheral lung parenchyma; a small focus of atypical goblet cell hyperplasia is seem; **c** atypical goblet cell hyperplasia in this case of CPAM 3—note the thin walls covered by bronchiolar cells. **d**, **e** Shows two cases of CPAM 2 with atypical goblet cell hyperplasia; in (**d**) the lesion is within one cyst, non-goblet cell CPAM epithelium is seen below, a normal small bronchus is to the right; in (**e**) the atypical goblet cell hyperplasia has replaced part of the "normal" CPAM epithelium, which is cuboidal to cylindrical. HE, bars 1 mm, 20, 50, 50, and 100 µm

Cases where the goblet cells entirely replaced the CPAM epithelium, forming a solid structure, replacing the CPAM cysts and leaving only small strands of stroma, we call in situ adenocarcinoma. If stroma invasion with disruption of smooth muscle cell layers and/or desmoplastic stroma reaction is seen, these were regarded as minimally invasive adenocarcinoma. As for nuclear atypia, normally very useful in evaluating carcinomas, this is difficult in mucinous adenocarcinomas of goblet cell type in general, and even more complicated in adenocarcinomas in CPAM. The nuclei are compressed at the basal portion of cell, the chromatin is condensed, and nucleoli are usually not prominent.

Immunohistochemistry

Serial sections were taken from the tissue blocks and stained by hematoxylin and eosin (HE). In addition, sections were incubated with antibodies for FGF10, FGFR2, FOXA1, FOXA2, MUC5AC, HER2/neu, hepatocyte nuclear factor 4α (HNF4α), SOX2, and Ying Yang protein 1 (YY1) (details in Table 3). Staining for other MUC proteins and IL4Rα were not done, as this was already available for the cases.

Immunohistochemical staining was evaluated for intensity (0 negative − 1 mild/moderate − 2 strong) and percentage of positive cells. The epithelial layer, the stroma cells, the goblet cells, and the carcinoma lesions were evaluated separately. This was done independently by four authors (FF, UA, LB, and HP). In case of differences, these cases were discussed on a multiheaded microscope or via digitized slides, and consensus was reached.

Next generation sequencing

All five cases harboring adenocarcinomas together with atypical goblet cell hyperplasia and 4 cases with atypical goblet cell hyperplasia only were investigated for mutations. Several sections were taken, the goblet cell proliferations and the concomitant adenocarcinoma were marked and macrodissected. DNA was extracted using the Maxwell RSC DNA FFPE Kit (Promega, Mannheim, Germany) and quantified with Picogreen on a Qbit fluorometer (Life Tech Austria, Vienna, Austria). NGS libraries were prepared using the AmpliSeq library kit 2.0 (Thermo Fisher Scientific) and the Ion Ampliseq Cancer Hotspot Panel V2 (CatNr: 4475346) primer pool covering hotspot mutations in 50 genes implicated in cancer. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo Fisher Scientific) to a length of 200 base pairs. Initial data analysis was done using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, https://github.com/iontorrent/). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. Called variants were annotated using open source software ANNOVAR [52] and SnpEff [5]. All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (http://www.broadinstitute.org/igv/), and variant calls resulting from technical read errors or sequence effects were excluded from the analysis.

Table 3 Antibodies and protocols used in the study

Name	Company/clone	Dilution	Pretreatment	Detection
FGF10	Abcam/ab80064	1:1000	MW9.0 (S2367 DAKO)	ENV DAB (K5007 DAKO)
FGFR2	Abcam/ab58201	1:1000	MW9.0 (S2367 DAKO)	ENV DAB (K5007 DAKO)
FOXA1	Abcam/ab173287	1:1000	CC1mild (Ventana 5424534001)	ultraView Ventana (Ventana 5269806001)
FOXA2	Abcam/ab108422	1:500	CC1mild	ultraView Ventana (Ventana 5269806001)
MUC5AC	Eubio/MS-145-P	1:100	MW Tris HCL Urea (Gatt–Koller 403210131)	ENV DAB (K5007 DAKO)
erbB2/HER2	Ventana/790-2991	Rtu	CC1mild	ultraView Ventana (Ventana 5269806001)
HNF4α	Sigma/SAB1412164	1:100	CC1mild	ultraView Ventana (Ventana 5269806001)
SOX2	Abcam/ab92494	1:100	CC1mild	ultraView Ventana (Ventana 5269806001)
YY1	Abcam/ab232573	1:500	CC1mild	ultraView Ventana (Ventana 5269806001)

Rtu, ready to use

Results

Morphology

Goblet cell proliferation/atypical goblet cell hyperplasia (AGCH) was seen in 9 cases. Two of them were reinvestigated from the previous study [39], and 4 cases were contributed by the coauthors. In 5/9 cases, there was a concomitant adenocarcinoma. In contrast to previous reports, AGCH was not only found in CPAM 1 but also in CPAM 2 and even in one case of CPAM 3 (Fig. 1).

In 5 cases, the goblet cells replaced not only the CPAM epithelium but also formed a solid structure, obscuring the cystic structure of CPAM. In these cases, there was not anymore a mixture of different types of epithelia within the CPAM, only the goblet cell proliferation (Fig. 2). Small strands of stroma were present. These are the cases we regarded as in situ, mucinous acinar adenocarcinoma. Invasion into the stroma was seen in 2 of the 5 cases, in one proven by a desmoplastic reaction of myofibroblasts and tumor cells splitting from the main tumor. In the other case, there was a dense lymphocytic infiltration confined to the invasion, and also separation of small tumor cell complexes. A disruption of the smooth muscle cell layer was seen in one case (Fig. 3). In 3 cases, no invasion could be demonstrated despite step sections. There are no similarities to adult non-mucinous adenocarcinomas, where most carcinomas arise from the bronchioloalveolar junction and grow along alveolar septa. However, in adult mucinous adenocarcinomas of goblet cell type, there are cases, which come close to these CPAM-associated adenocarcinomas (suppl. Table 1).

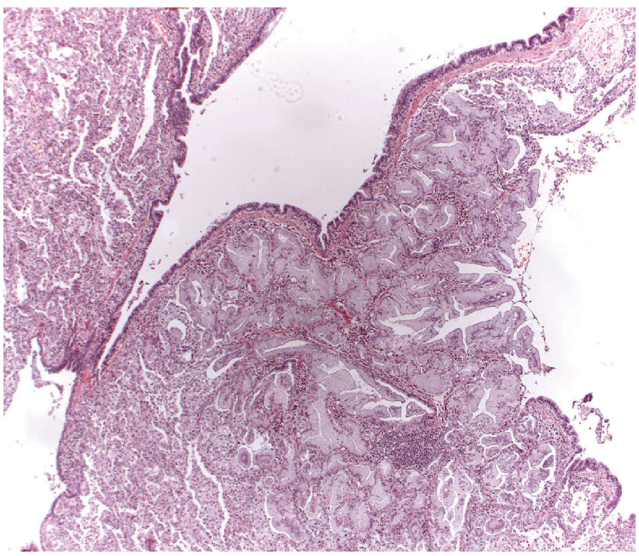

Fig. 2 Adenocarcinoma in situ; the goblet cell proliferation has occupied the whole peripheral lung and also parts of the bronchial mucosa. On the upper left side, another focus of CPAM is seen, but without an atypical goblet cell hyperplasia. Invasion was not seen in this case. H&E, × 100

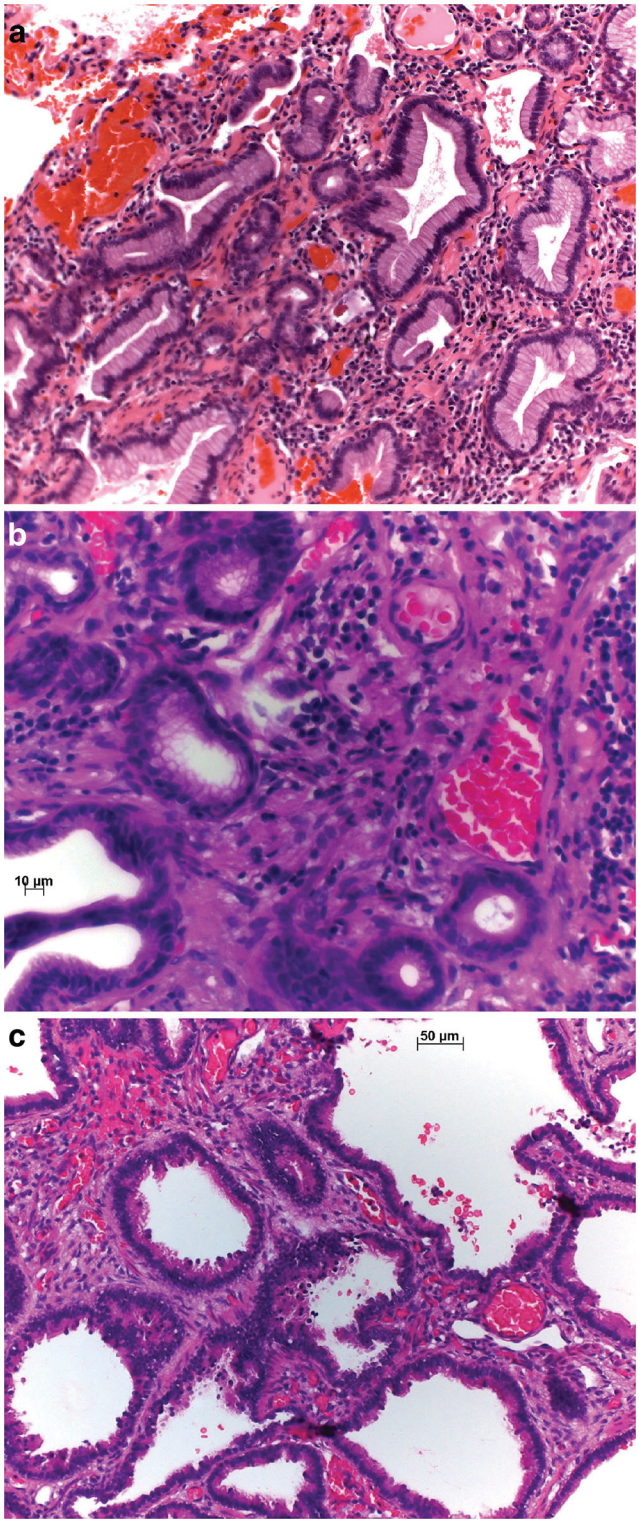

Fig. 3 In these two cases, the adenocarcinomas have already invaded the stroma (minimally invasive adenocarcinoma); **a** in case one, the invasion is seen in the center, characterized by small clusters of tumor cells and a dense lymphocytic reaction; **b** in the second case, the invasive portion is seen; here is a reaction by myofibroblasts, and a disruption of the smooth muscle layer; **c** for comparison, a pseudo-invasion is shown: the smooth muscle layer surrounds each individual bronchiolar cyst. HE, × 200, bars 10 and 50 µm

Immunohistochemistry

Expression of the different immunohistochemical markers was evaluated in the CPAM epithelium (cases with and without AGCH), in the goblet cells (AGCH), the adenocarcinoma cells, and in pneumocytes and bronchial/bronchiolar cells in adjacent normal lung (see a summary in Table 4). The stroma of the CPAM lesions was also evaluated.

Mild expression (1+) for FGF10 was seen in 19/28 cases in the CPAM and normal epithelium; in 17 cases, there were foci with strong expression (2+). Expression in goblet cells was present in 4 (1+) and 5 cases (2+). CPAM stroma also expressed FGF10 in 25 (1+) and 6 cases (2+). Expression was weak in adenocarcinomas (Fig. 4a).

The expression for FGFR2 was seen in normal bronchial and alveolar epithelium in 32 (2+) and 1 cases (1+). All goblet cells expressed FGFR2 moderately to strong: the intensity was less in most carcinomas and the stroma cells were negative (Fig. 4b).

HER2 antibodies stained only scattered cells in CPAM epithelium, but all goblet and carcinoma cells demonstrated a 2+ cytoplasmic and membranous staining (Fig. 5).

YY1 antibodies stained the nuclei of CPAM epithelium in all 9 cases with AGCH, the goblet cells were more intensely decorated compared to the other CPAM epithelia, whereas the carcinoma cells were more intensely decorated compared to the goblet cells (Fig. 6). YY1 was weakly positive only in basal cells, a few pneumocytes, and some stroma cells.

FOXA1 expression in the CPAM epithelium was seen in 21 and 14 cases (without AGCH), 1+ and 2+ respectively. Expression in goblet cells was focal in 4/9 cases (1+), stroma cells were all negative.

FOXA2 antibodies stained normal adjacent lung epithelium in 25 and 7 cases, with an intensity of 1+ and 2+, respectively. Stroma cells were negative, goblet cells and carcinoma cells were all 1+ positively stained (Suppl. Fig. 1ab).

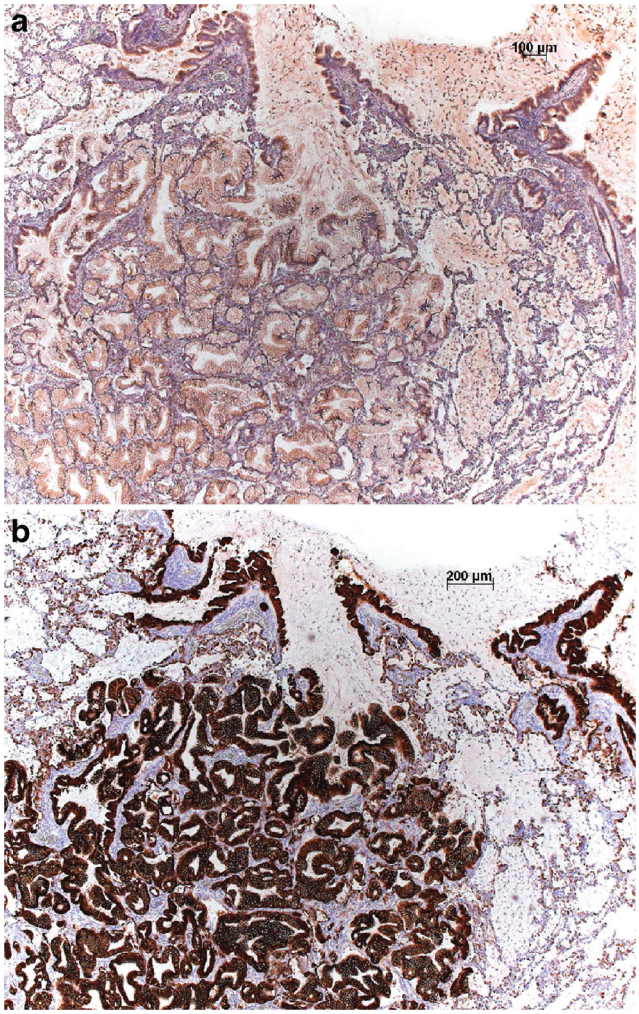

Fig. 4 **a** Moderate expression of FGF10 in CPAM epithelia (top and left), but much less in the carcinoma (center); **b** moderate to strong expression of FGFR2 in CPAM epithelia and in adenocarcinoma in this case. Bars, 100 and 200 µm

MUC5AC antibodies stained single scattered CPAM epithelial cells, whereas all goblet cells were stained moderately, and the carcinoma cells more intensely (2+).

Table 4 Immunohistochemistry of CPAM cases

Marker	CPAM epithelium	AGCH	Adenocarcinoma	Normal bronchial/alveolar epithelium
FGF10	19 +/17 ++	9 cases + to ++	± in all	All cases + to ++
FGFR2	++ to + in all	+ to ++ in all	+ in all	33 cases ++ to +
HER2	± single cells	++ in all	++ in all	-
YY1	+ nuclear	+ to ++ in all	++ in all	±
FOXA1	+ to ++	Focal +	Focal +	-
FOXA2	+ in all	+ in all	+ in all	+ to ++
MUC5AC	Focal ±	+ in all	++ in all	-
SOX2	++ in all	4/9 + to ++	± in all	-

CPAM epithelium—cases without AGCH; *AGCH*, atypical goblet cell hyperplasia; ±, only single positive cells are seen

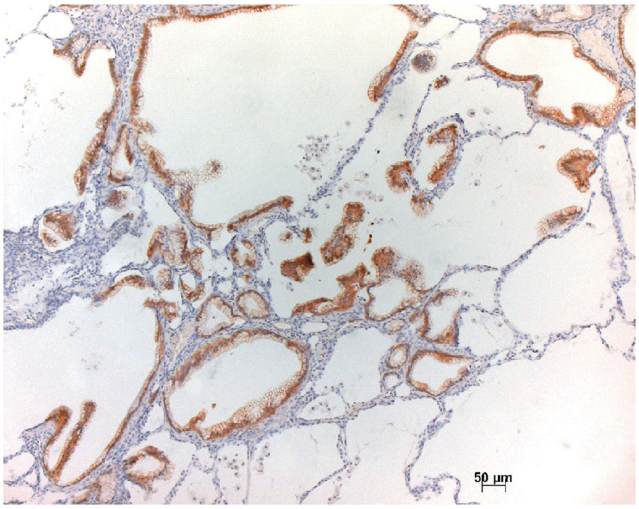

Fig. 5 Expression of HER2 protein in atypical goblet cell hyperplasia. All goblet cells express HER2 in a membranous pattern, whereas adjacent normal lung cells as well as non-goblet type CPAM epithelia were negative. Bar 50 µm

SOX2 was positive in all CPAM epithelial cells (2+), in 4/9 cases with atypical goblet cell hyperplasia (1+ or 2+). The carcinoma cases were all stained but only weakly (Suppl. Fig. 2).

HNF4α was negative in all cases.

Mutation analysis

A mutation analysis could be performed in 7 of 9 cases with AGCH and all 5 adenocarcinoma cases. In 2 cases of AGCH, there was not enough tissue left to perform next generation sequencing. A mutation in the KRAS gene was seen in all 7 AGCH cases, and in all five cases with adenocarcinoma. In all cases, a pG12D mutation in exon 2 was detected. No other mutations were found.

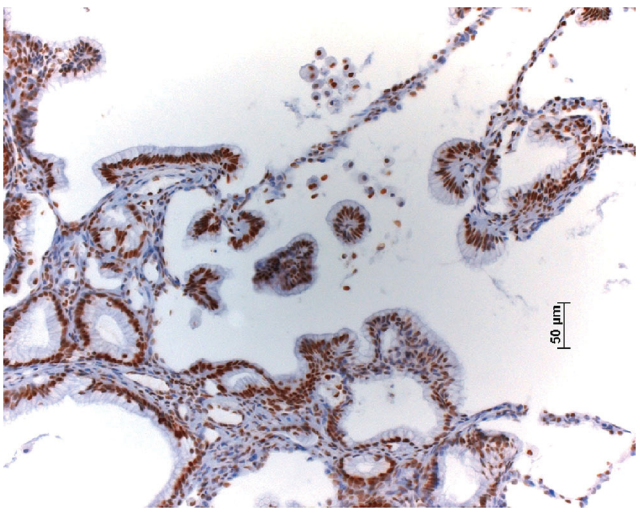

Fig. 6 Strong nuclear expression of Ying Yang 1 protein in atypical goblet cell hyperplasia; stroma cells also expressed YY1 but less intense. Bar 50 µm.

Comparison to mucinous adenocarcinomas in adults (see supplementary table 1)

In mucinous adenocarcinomas, KRAS mutations are overrepresented, as in AC within CPAM. The second common mutation in adult, AC might be the fusion of NRG1 with different partner genes. In adults, this results in activation of ERBB3 and HER2 forming dimers. In AC of CPAM, this has not been evaluated; however, HER2 is activated too. In adults, AC showed a similar MUC5B and AC expression as in childhood AC. HER2 amplifications have been seen in adult AC, but not in childhood AC. Other alterations seen in adult AC such as ROS1, BRAF, and ALK are not found in AC/CPAM. EGFR mutations in mucinous AC are exceedingly rare, but so far have not been seen in AC arising in CPAM.

Discussion

Atypical goblet cell proliferations were initially described in CPAM 1 and regarded as a possible forerunner for pediatric pulmonary adenocarcinomas [22, 39]. Previously, we have analyzed few cases of goblet cell proliferations in CPAM and showed that HER2, MUC5AC, and interleukin receptor 4 might be driving the goblet cell proliferation into adenocarcinoma [39]. At that time, we could not evaluate the status of HER2 with respect to mutation, amplification, or posttranslational modification. In the present investigation, we demonstrated for the first time, that atypical goblet cell hyperplasia occurs not only in CPAM 1 but also in CPAM 2 and CPAM 3. Carcinomas might therefore arise in all three. HER2 and MUC5AC, but also YY1, seem to play an important role for the development of carcinomas out of AGCH. Assessing the diagnosis of adenocarcinoma in the setting of CPAM is difficult: nuclear atypia in goblet cells is not easy to evaluate, as nuclei are often compressed by the intracytoplasmic mucin vacuoles. Therefore, pattern is the most reliable feature. AGCH usually replaces completely the other epithelial cells in CPAM, but within the CPAM cysts there are still areas of cuboidal and cylindrical cells present. In AGCH, the goblet cell complexes always stay within the CPAM cysts. In carcinomas, the goblet cells not only form layers within the cyst but completely outgrow the CPAM lesion forming a solid tumor composed of acinar structures. These cell complexes furthermore expand into adjacent lung structures (Figs. 2 and 3). Invasion can only be confirmed, if a desmoplastic stroma reaction is seen. This stroma reaction is similar to that in adult adenocarcinomas, with myofibroblast proliferation and/or lymphocytic infiltration. In addition, the smooth muscle layer is disrupted in areas of invasion. Small tumor cell complexes

also separate from the main tumor. We have not seen cases outside CPAM lesions, but such cases have been reported, and had similar molecular abnormalities [28].

Expression of proteins by itself cannot be used to differentiate preneoplastic and true cancer cells. However, in all carcinoma cases, an activating mutation of the KRAS gene could be proven, and moreover, in all analyzed cases of preneoplastic AGCH, the same KRAS mutation was found. This KRAS mutation is well-known: It activates KRAS constitutively. No upstream activation is necessary. In experimental models of genetically engineered adenocarcinoma in mice the same KRAS mutation drives the proliferation from papillary preneoplasia at the bronchioloalveolar junction zone into in situ adenocarcinoma. In human adenocarcinoma, especially in invasive mucinous AC, the codon 12 mutations are the preferentially ones. In genetically engineered AC for invasion, another hit, such as TP53 mutation, is required [16, 32, 36]. This is in accordance with our findings of in situ AC in the CPAM cases. So, what might be the driving force for the invasive adenocarcinoma in CPAM?

Mutations of KRAS were already reported, either as cases or small series [22, 25, 28, 35, 37, 38, 40, 46], only one report found no mutation in 23 cases including only 2 cases with AGCH [19]. Interestingly, in all cases, codon 12 mutations are seen, contrary to adult AC, which also show mutations in codons 13 and 61. The role of different codons of the RAS gene in binding to DNA, activating different downstream pathways, are not clear, but some findings point to different sensitivity of codon mutations of the RAS genes towards carcinogens. In addition, different mutations might also activate downstream pathways differently [18, 48].

HER2, a receptor tyrosine kinase, is a driver for many carcinomas. Although it was neither mutated nor amplified in our cases, increased expression was seen from AGCH to AC, whereas only few scattered cells in normal epithelia were positively stained. It might assist in the progression from the precursor to the carcinoma. A similar finding was reported in pulmonary carcinoma mutated for KRAS: EGFR protein was upregulated without genetic modification [12, 30]. Similarly, Rossi et al. reported HER2 expression exclusively in mucinous lesions in CPAM [37]. It might be speculated that HER2 is translationally upregulated via KRAS; however, this can only be answered by further experimental investigations. This is very different to adult mucinous AC, where an upregulation of HER2 confers a worse prognosis [24]. A comparison of molecular and protein expression patterns for adult and childhood AC is provided in supplementary table 1.

The upregulation of YY1 was unexpected. YY1 plays a role in the transcription machinery, as it is known to either repress or promote transcription [13]. If inactivated, it causes lung defects and interferes with lung growth and maturation. Cystic lung malformation results in mice similar to what is seen as CPAM in human lung [3]. On the contrary, in mesothelioma, a fusion transcript with YY1 as a partner was found, thus providing evidence of an oncogenic function of YY1 [34]. Another interesting function is the negative regulation of E-cathepsin in lung [7]. Downregulation of E-cathepsin by YY1 might inhibit a T-lymphocyte infiltration in lung cancer [49], and also decrease the adherence of the cells, and therefore promote the development of AC. HNF4α, a gene found in adult mucinous AC was negative in all our cases, and therefore play no role [45]. NRG1 is part of our fusion panel and was not altered.

Recurrence and death of diseases very rarely occur in AC arising in CPAM [39]. Also, in our cases, no recurrence was reported. The main reason might be that the precursor and the AC is completely resected together with CPAM in most cases, usually due to symptoms caused by CPAM 1–3. In addition, as we and others have shown, atypical goblet cell hyperplasia is rare in CPAM (less than 10% in all CPAM cases, and furthermore AC is rare in AGCH), thus further reducing the probability of an adenocarcinoma development. Minimally invasive adenocarcinoma, as we have seen in our cases, has the same good prognosis as adenocarcinoma in situ, when completely resected [1].

When compared to adult adenocarcinomas, we have to take into account that mucinous adenocarcinoma of goblet cell type has also a similar prognosis, if resected early on [14]. This however changes, if a multifocal colloid adenocarcinoma develops, which we had seen in one case of our previous report [39]. In this case, the patient died of disease 3 years after the initial resection. When looking up published reports, dead of disease seems to be exceptionally rare. In a case report an AGCH was found, which progressed into mucinous adenocarcinoma; the patient was followed for 15 years, and very late died of disease [21]. This case nicely illustrates the different biological behavior of adenocarcinomas in children when compared to adults. Even recurrences or metastatic spread seems to be very unusual [10, 31].

There are similarities, as well as differences, of mucinous adenocarcinomas in children and adults: KRAS mutation occurs in both AC types, but in adults different codons are involved (codon 12 in all childhood, but codon 12, 13, and 61 in adult cases). In addition, also other genes can be affected, and co-alterations do occur in adults (suppl.table 1).

In the last decades, there is an ongoing discussion if CPAM should be surgically removed. Therefore, some cases might have not been resected [10, 47]. However, the AGCH-AC sequence has also important clinical implications: Once CPAM is radiologically detected, it should be removed. Resection margins should be analyzed in all cases with AGCH and AC. A careful follow-up is recommended.

With respect to FGF10, FGFR2, FOXA1/A2, and SOX2, we can confirm previous findings [15, 29, 50, 51]. Interestingly, all these markers were negative in the CPAM

stroma, but confined to the epithelium. There was also some positivity in bronchial and alveolar epithelium outside the CPAM lesion but less pronounced. This might be used as an argument that CPAM arises out of the bronchial buds and does not involve stroma cells.

In conclusion, we confirmed FGF10, FGFR2, FOXA1/A2, and SOX2 as drivers for CPAM 1–3 development, acting exclusively in epithelial cells. We showed atypical goblet cell hyperplasia in all three CPAM types. We have identified KRAS mutations in the precursor as well as in the adenocarcinoma, assisted by posttranslational expression of HER2 and YY1. Based on our findings, we confirm previous reports that AGCH is very likely a precursor of adenocarcinoma in CPAM.

Compliance with ethical standards

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

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