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Next generation sequencing of lung adenocarcinoma subtypes with intestinal differentiation reveals distinct molecular signatures associated with histomorphology and therapeutic options

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ABSTRACT

Objectives: We aim to provide a better understanding of the molecular landscape of primary lung adenocarcinomas with intestinal differentiation.

Material and Methods: Five invasive mucinous adenocarcinomas (IMA) and seven pulmonary enteric adenocarcinomas (PEAD) were included in this study. Furthermore, we analyzed six pulmonary colloid adenocarcinomas (CAD), including one primary tumor, one metastasis, and two sample pairs consisting of the primary colloid lung tumor and a matching metastasis and an acinar component, respectively. All samples were characterized using immunohistochemistry (TTF-1, CK7, CK20, CDX2, Ki-67, ALK and PD-L1) and a next generation sequencing panel covering 404 cancer-related genes (FoundationOne® gene panel).

Results and Conclusion: While Ki-67 expression was comparably low in IMA (range: 8-15%) and in primary CAD (range: 5-8%), we observed considerably higher proliferation rates in the non-colloid tumor compartment (16%) and metastases (72%) from CAD, as well as in the PEAD-group (36-71%). The overall tumor mutational burden was lowest in IMA (2.5 mutations per megabase), intermediate in CAD (5.8 mutations per megabase) and highest in PEAD (16.8 mutations per megabase). *KRAS* mutations were frequent in all three tumor subtypes, but *TP53* mutations were mostly limited to PEAD. While chromosomal alterations were rare in IMA, we discovered *MYC* amplifications in three of four CAD. Comparing primary and metastatic CAD, we observed the acquisition of multiple mutations and chromosomal alterations. PEAD had a variety of chromosomal alterations, including two cases with RICTOR amplification. PD-L1 expression (20%, 50% and 80% of tumor cells) was limited to three PEAD samples, only.

In conclusion, we provide a detailed insight into the molecular alterations across and within the different subtypes of pulmonary adenocarcinomas with intestinal differentiation. From a clinical perspective, we provide data on potential treatment strategies for patients with PEAD, including immunotherapy.

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1. Introduction

For several decades, it is well-known that primary lung adenocarcinomas can also resemble intestinal cancers in their histomorphological and immunhistochemical characteristics [1]. The latest revision of the World Health Organization (WHO) Classification of Lung Tumors in 2015 describes three different subtypes of primary lung adenocarcinomas which typically show intestinal differentiation and resemble gastrointestinal cancer in histomorphology and immunohistochemistry (IHC): invasive mucinous adenocarcinoma (IMA), colloid adenocarcinoma (CAD) and pulmonary enteric adenocarcinoma (PEAD) [2]. IMA is the most common member of this subgroup, representing approximately 5% of all pulmonary adenocarcinomas [3]. This tumor entity consists of goblet or columnar cells with abundant intracellular mucin and a variable, but usually limited, amount of mucin secreted into the alveolar space [4]. CAD and PEAD are much less common. CAD are characterized by extensive accumulations of extracellular mucin [2,4]. PEAD typically share histomorphological features of colorectal adenocarcinomas such as tall columnar tumor cells and luminal necrosis, but may also show focal mucin production [1,4]. Regarding the immunoprofile, all of these variants frequently lack TTF-1 expression while being positive for intestinal markers such as CDX2, CK20 or MUC2 [5–7]. Therefore, the differentiation of a primary mucinous lung tumor from a metastatic lesion originating from the gastrointestinal tract can be challenging. We recently showed that PEAD can be distinguished from colorectal cancer based on their methylation profile [8]. However, IMA of the lung share many epigenetic and also RNA expression pattern with upper gastrointestinal cancers [8,9].

The knowledge about the mutational landscape of these three primary lung cancer subtypes differs. *KRAS* mutations and *CD74-NRG1* translocations have been identified as recurring alterations in IMA, the latter also representing a potential therapeutic target [10,11]. However, there are only few studies about the genetic alterations in CAD and PEAD and they are mostly limited to singles genes or small gene panels [6,12–17]. Therefore, we tried to broaden this view and performed next generation sequencing (NGS) on a cohort of IMA, CAD and PEAD samples, using a comprehensive pan-cancer gene panel. By integrating the results from molecular analysis with clinical, histomorphological and immunohistological data, we aimed to contribute to a better understanding of these lung adenocarcinoma subtypes, focusing on potential clinical applications as well as the link between molecular alterations and histomorphology.

2. Material and Methods

2.1. Patients and samples

24 tissue samples were obtained from the archives of the Institute of Pathology and the Department of Neuropathology of the Charité – University Hospital Berlin. FFPE tissue blocks were stored at temperatures between 15 °C and 20 °C and humidity levels of 20% to 60%. Additional details such as specimen type and year of resection are shown in Supplementary Table S1.

All cases were reevaluated and restaged according to the 8th Edition Lung Cancer Staging Classification [18]. We investigated a total of 18 adenocarcinomas specimens with intestinal differentiation, including five IMA (IMA-1 to IMA-5), seven PEAD (PEAD-1 to PEAD-7) and six CAD samples. The six CAD samples were derived from four different patients (CAD-1 to CAD-4), including two samples pairs from the same patient. One sample pair consisted of the primary lung tumor (CAD-1p) and a corresponding, surgically resected brain metastasis (CAD-1 m). The second sample pair included tissue from two morphologically different sites of the same primary lung tumor: one component with large, colloidal cysts (CAD-2c) and one non-colloid component with an acinar growth pattern (CAD-2nc). All patients with an adenocarcinoma of intestinal differentiation underwent thorough clinical, endoscopic and radiological investigation, without any evidence for an extrapulmonary primary site (Supplementary Table S1). Furthermore, methylation analysis for PEAD samples were supportive of a pulmonary origin [8]. For reference purposes, we also analyzed six non-intestinal adenocarcinomas (ADC-1 to ADC-6). This included common lung adenocarcinomas, including specimens with solid, acinar or papillary growth pattern. Cases with intracellular or extracellular mucin production were excluded. Furthermore, we ensured that these tumors did not harbor an ALK translocation.

2.2. IHC and slide analysis

Immunohistochemical staining for TTF-1, CK7, CK20, CDX2, Ki-67, ALK and PD-L1 was performed on the Leica BOND-MAX (Ki-67 and PD-L1) and the Ventana BenchMark XT (all other markers) automated slide stainer according to the instructions supplied by the manufacturer. Antibodies, their according manufacturers and concentrations are listed in Supplementary Table S2. All slides were digitalized using the Pannoramic SCAN II digital slide scanner (3DHISTECH, Budapest, Hungary). Automated Ki-67 quantification was performed using the Ki67Quantifier software, which is part of the CognitionMaster Professional Suite (VMscope GmbH, Germany) [19].

2.3. Next generation sequencing (NGS)

For genetic analysis, between five and ten FFPE tissue sections with a thickness of four micrometer were collected. Macrodisscetion was done using a reference hematoxylin and eosin (H&E) stained slide to maximize the tumor content. The tumor content per case was at least 20%. NGS using the FoundationOne® (Foundation Medicine, Cambridge, USA) gene panel covering 404 cancer-related genes was performed as described previously [20]. All NGS-results are also listed in Supplementary Table S3.

2.4. Fluorescence in-situ hybridization (FISH)

To validate the *MYC* copy number of selected cases, we used the Vysis LSI MYC SO Probe (Abbott Molecular, USA) as well as the Vysis CEP 8 (D8Z2) Aqua probe (Abbott Molecular, USA) according to previously described protocols [21].

2.5. Statistical analysis

Statistical analysis was performed using RStudio version 1.1.444 based on the statistical language R version 3.4.2 [22,23]. The Fisher's exact test and the Mann-Whitney U test were used for comparison of categorial and continuous data, respectively. Survival curves were generated using the Kaplan-Meier method and tested for significance using the log-rank test. Heatmaps for genetic alterations were plotted using the ComplexHeatmap package [24].

3. Results

3.1. Clinical data

The clinicopathological data are summarized in Table 1. None of the IMA cases showed lymph node or distant metastases. All five patients were former or active smokers with a mean smoking history of 26 pack years. Only one of five patients (20%) relapsed and died of disease (also refer to Fig. 2A).

With regards to CAD, two patients presented with symptomatic brain metastases at the time of initial diagnosis. We observed a diverse smoking history within these patients, ranging from never smokers to heavy smokers with a smoking history of 120 pack years. While all of them relapsed within a mean time of 16.8 month, there were no disease

Table 1
Table listing the clinicopathological data of the invasive mucinous (IMA), colloid (CAD) and pulmonary enteric adenocarcinoma (PEAD) samples included in this study. Disease free survival (DFS) and disease specific survival (DSS) times are given in months (m).

Case ID	Sex	Age	Stage	Smoking status	Pack years	DFS (m)	DSS (m)	Vital status
IMA-1	M	63	pT1a pN0 cM0 Pl0	Former smoker	20	87.4+	87.4+	Alive
IMA-2	M	72	pT3 pN0 cM0 Pl0	Former smoker	10	11.9+	11.9+	Alive
IMA-3	F	74	pT1b pN0 cM0 Pl0	Former smoker	10	31.2+	31.2+	Alive
IMA-4	M	56	pT2a pN0 cM0 Pl0	Active	30	50.4+	50.4+	Alive
IMA-5	M	60	pT2b pN0 cM0 Pl0	Former smoker	60	8.0	15.0	Dead
CAD-1p/m	M	39	pT2a pN0 pM1b Pl0	Former smoker	25	21.9	32.2+	Alive
CAD-2c/nc	M	80	pT2a pN0 cM0 Pl0	Never smoker	0	14.8	39.4+	Alive
CAD-3	F	75	pT2a pN0 pM1b Pl0	Active	120	12.1	45.5+	Alive
CAD-4	F	70	pT2b pN0 cM0 Pl0	Active	80	18.5	29.9+	Alive
PEAD-1	M	78	pT1b pN1 cM0 Pl0	Active	70	18.7	26.6	Lost to follow-up
PEAD-2	M	54	pT2b pN0 cM0 Pl0	Active	60	112+	112+	Alive
PEAD-3	M	59	cT3 cN0 pM1b Pl3	Active	50	NA	8.2	Dead
PEAD-4	F	46	pT3 pN0 cM0 Pl0	Active	35	8.4	21.1	Dead
PEAD-5	F	73	pT2b pN1 cM0 Pl0	Former smoker	50	16.2	19.5	Dead
PEAD-6	F	56	pT2b pN1 cM0 Pl1	Active	40	12.0	15.0+	Alive
PEAD-7	M	53	cT3 cN0 pM1b Pl2	Active	120	NA	19.6	Dead
ADC-1	M	46	pT2b pN1 cM0 Pl0	Former smoker	60	18.5+	18.5+	Alive
ADC-2	M	57	pT3 pN0 cM0 Pl2	Active	50	8.8	17.0	Dead
ADC-3	M	61	pT1b pN1 cM0 Pl0	Active	45	14.9	14.9	Lost to follow-up
ADC-4	F	58	pT2b pN0 cM0 Pl0	Never smoker	0	9.1	14.6+	Alive
ADC-5	M	76	pT1a pN0 cM0 Pl0	Former smoker	65	19.1+	19.1+	Alive
ADC-6	M	81	pT2a pN1 cM0 Pl0	Former smoker	70	15.5	16.8	Lost to follow-up

Abbreviations: NA = not available, Pl = pleural invasion.

related deaths within the follow-up interval .

Concerning PEAD, three patients initially presented with lymph node metastases and two patients with distant metastases, respectively. Most of them were active smokers with a mean smoking history of 60 pack years. Four patients died of their disease with a median survival time of 17.1 months.

3.2. Histomorphological and immunohistochemical analysis

Representative H&E and IHC stains for all three subtypes investigated in this study are displayed in Fig. 1.

All IMA samples consisted of goblet cells with PAS positive intracytoplasmic, apical mucin, growing along the alveolar septa in sense of a lepidic as well as acinar growth pattern. All tumors showed strong immunoreactivity against CK7, but none expressed TTF-1. CK20 and CDX2 expression was present in three of five samples (60%), respectively (Table 2). All IMA samples were negative for ALK and PD-L1. Ki-67 expression was comparably low with a mean of 11.4% positive cells.

Regarding CAD, all primary tumors (CAD-1p, CAD-2c, CAD-3) showed large colloidal cysts with abundant pools of PAS positive mucin. The two brain metastases (CAD-1 m and CAD-4) showed a papillary-like growth pattern with partial signet-ring cell formation, embedded in extensive extracellular PAS positive mucin accumulations. The non-colloid (CAD-2nc) compartment of CAD-2c showed smaller cysts, resembling an acinar growth pattern and accounting for approximately 20% of the total tumor mass. All CAD were negative for TTF-1 and CK20, while all tumors expressed CK7. CDX2 expression was observed in three of four individual cases (75%). Furthermore, all specimens were negative for ALK and PD-L1. With a mean of 6.3%, Ki-67 expression was low in all primary colloidal tumors. Of note, compared to the colloid component (Ki-67: 6%), the non-colloid tumor compartment showed a proliferation index of 16%. Furthermore, both metastases of CAD revealed significantly higher proliferation rates with 72% and 38%, respectively.

Concerning PEAD, all cases showed an acinar growth pattern, lined by tall columnar tumor cells with prominent luminal necrosis. TTF-1 expression was focally positive in one sample, while all other tumors were negative. Five of seven samples (71.4%) showed immunoreactivity against CK7 and all specimens were positive for CDX2. CK20 expression was observed in two cases (28.6%). All cases were negative for ALK, but three cases (42.9%) showed membranous PD-L1 staining in 20%, 50% and 80% of tumor cells. Furthermore, all samples showed high proliferation rates in Ki-67 IHC with a mean of 56%. For comparison, the mean Ki-67 proliferation index for the common adenocarcinomas investigated in this study was 31.2%.

3.3. Next generation sequencing

3.3.1. Comparison of overall tumor mutational burden and nucleotide substitution types

With regards to the overall tumor mutational burden (Fig. 2B), the lowest mean mutation rate was observed in IMA (2.5 mutations per megabase). Common adenocarcinoma samples (8.0 mutations per megabase) and CAD (5.8 mutations per megabase) revealed an intermediate, PEAD (16.8 mutations per megabase) the highest tumor mutational burden. When compared with common adenocarcinomas, the difference was only significant for IMA ($p = 0.027$). Of note, we observed no cases with microsatellite instability.

On comparison of the nucleotide substitution types across the investigated samples (Fig. 2C), we observed that C:G > A:T substitutions were significantly more common in PEAD samples ($p = 0.039$). Furthermore, C:G > G:C changes were an extraordinarily rare event in IMA ($p = 0.013$).

3.3.2. Invasive mucinous adenocarcinoma

The most commonly mutated gene in IMA was *KRAS* (80%, four of five patients), followed by *ARID1A*, *DICER1*, *DNMT3A*, *FAT3*, *SMAD3*, *SPAT1*, *STK11* and *ZNF703* (each 40%, two of five patients). *TP53* was mutated in only one sample (20%). With regards to *KRAS*, two mutations resulted in a G12 V and two alterations in a G12D amino acid change (Fig. 3A).

Overall, chromosomal alterations were comparatively rare and were only present in two IMA (Fig. 4A). One sample (IMA-3) showed loss of *CDKN2A/B* while amplification of *PIK3CA*, *PRKCI* and *TERC* was observed in another specimen (IMA-5).

For comparison, the results for the non-intestinal ADC samples are displayed in Supplementary Figure S1

3.3.3. Colloid adenocarcinoma

KRAS mutations were present in all six samples from the four

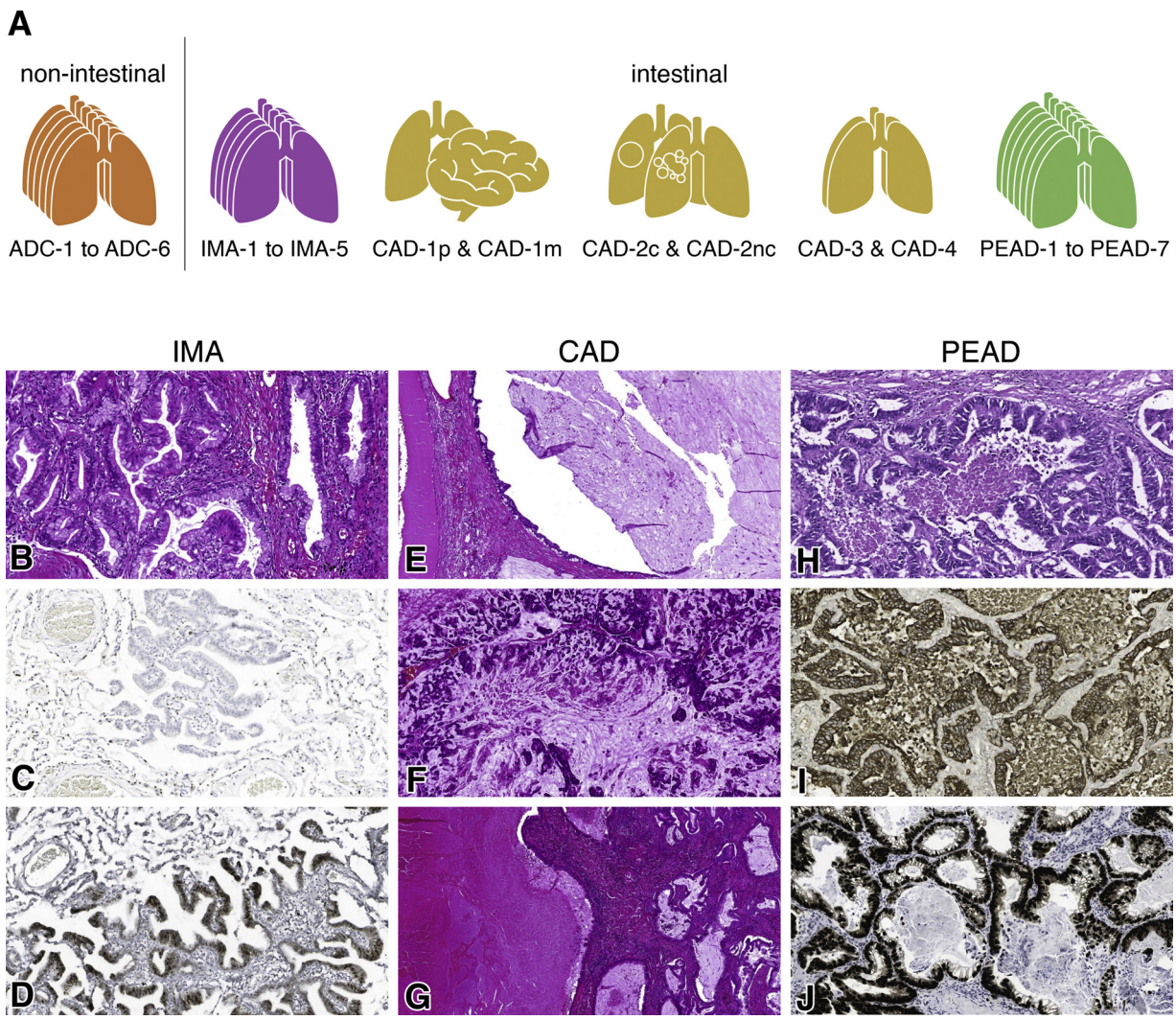

Fig. 1. Graphical summary of the study design as well as representative Hematoxylin and eosin (H&E) and immunhistochemical stainings of the three investigated tumor entities.

A We investigated a total of 17 adenocarcinomas specimens with intestinal differentiation, including five IMA (IMA-1 to IMA-5), seven PEAD (PEAD-1 to PEAD-7) and six CAD samples. The six CAD samples were derived from four different patients (CAD-1 to CAD-4), including two samples pairs from the same patient. One sample pair consisted of the primary lung tumor (CAD-1p) and a corresponding, surgically resected brain metastasis (CAD-1 m). The second sample pair included tissue from two morphologically different sites of the same primary lung tumor: one component with large, colloidal cysts (CAD-2c) and one non-colloid component with an acinar growth pattern (CAD-2nc). For reference purposes, we also analyzed six non-intestinal adenocarcinomas (ADC-1 to ADC-6).

B - D Invasive mucinous adenocarcinomas consist of goblet cells with intracytoplasmic mucin (**B**) and typically lack TTF-1 expression (**C**) while being positive for intestinal markers such as CDX2 (**D**).

E - G Colloid adenocarcinomas are characterized by abundant pools of mucins (**E**). However, the morphology may change when they metastasize, as shown in the matching brain metastasis (**F**). Furthermore, some colloid adenocarcinomas may also show non-colloid tumor components (**G**).

H – J Pulmonary enteric adenocarcinomas share histomorphological features of colorectal cancer, such as prominent luminal necrosis (**H**). They are typically TTF-1 negative, but CK7 expression is often preserved (**I**). By definition, they are positive for at least one intestinal marker, such as CDX2 (**J**).

different patients included in this study (Fig. 3B), including G12D, G12 V, G12R and G12C amino acid changes. Other frequently alterated genes included *STK11* (75%), and *PARP1* (50%). *TP53* mutations were observed in only one sample (25%).

With regards to chromosomal alterations, *MYC* amplification was most common and present in three samples from different patients, followed by loss *CDKN2A/B* in two samples. There were no further recurrent chromosomal alterations.

The comparison of a primary CAD (CAD-1p) with a matched brain metastasis (CAD-1 m) revealed that this tumor acquired multiple mutations and chromosomal alterations during the process of metastasis. This included known somatic mutations in the *EPHA3* and *MED12* gene as well as *MYC* amplification and loss of *CDKN2A/B*. The absence of the *MYC* amplification in the primary tumor and the presence in the matching metastasis was also confirmed by FISH (Supplementary Fig. S2).

The evaluation of two morphologically different tumor areas from one CAD patient revealed an identical mutational profile with the exception of an *ATRX* mutation, which was only present in the acinar (CAD-2nc) and not in the colloid (CAD-2c) tumor compartment. However, there was no overlap with regards to chromosomal alterations. In the acinar tumor component, we observed amplifications in ten different genes. However, these alterations were not shared with the colloid compartment. Instead, we observed two amplifications and two rearrangements in different genes.

3.3.4. Pulmonary enteric adenocarcinoma

In PEAD, we observed *TP53* mutations in six of seven specimens

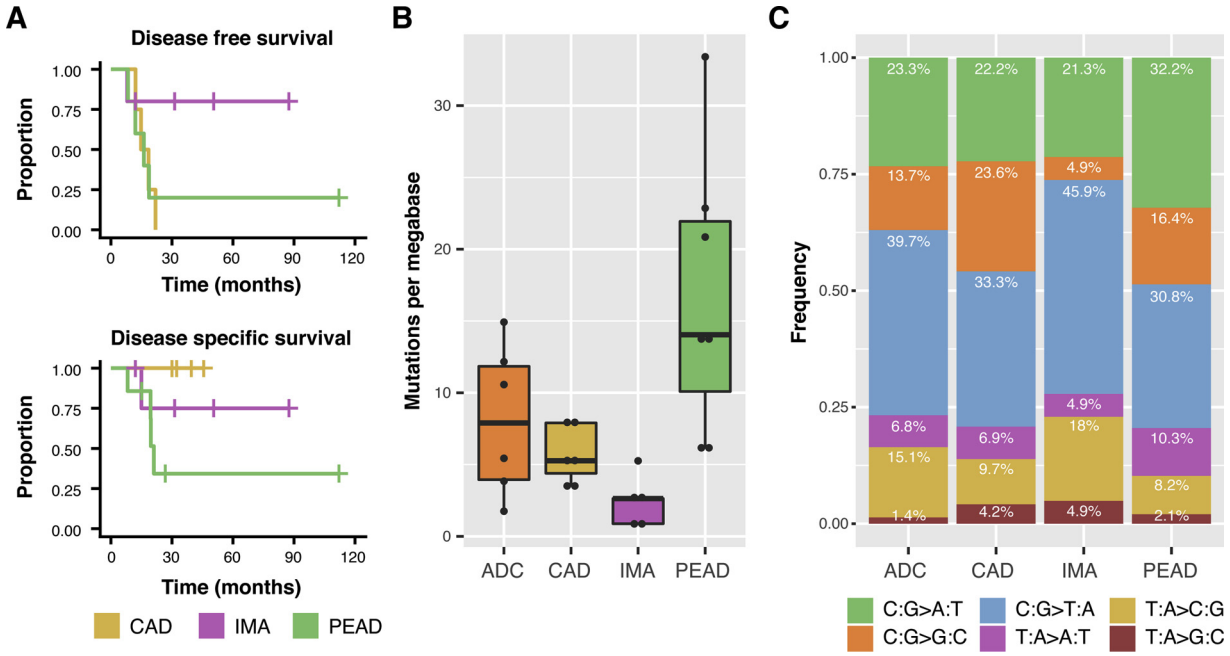

Fig. 2. Comparison of the survival times, the tumor mutational burden and the nucleotide substitution types across the different lung adenocarcinoma subtypes investigated in this study.

A Kaplan-Meier plots for invasive mucinous adenocarcinomas (IMA), colloid adenocarcinomas (CAD) and pulmonary enteric adenocarcinomas (PEAD) showing disease free and disease specific survival times.

B Boxplot showing the tumor mutational burden of the different subtypes investigated in this study compared to common non-mucinous adenocarcinoma samples (ADC). The lowest tumor mutational burden was observed in invasive mucinous adenocarcinomas while the most mutations were present in pulmonary enteric adenocarcinomas.

C Stacked barplots showing the nucleotide substitution rates of the different tumor entities. Pulmonary enteric adenocarcinomas were characterized by a C:G > A:T substitutions while C:G > G:C were a rare event in invasive mucinous adenocarcinomas.

Table 2
Results from immunohistochemistry (IHC) for the invasive mucinous (IMA), colloid (CAD) and pulmonary enteric adenocarcinoma (PEAD) samples as well as the common lung adenocarcinomas (ADC) included in this study.

Case ID	TTF-1	CK7	CK20	CDX2	ALK	PD-L1	Ki-67
IMA-1	–	+	+	+	–	0%	12%
IMA-2	–	+	+	+	–	0%	8%
IMA-3	–	+	–	+	–	0%	9%
IMA-4	–	+	–	–	–	0%	13%
IMA-5	–	+	+	–	–	0%	15%
CAD-1p	–	+	–	+	–	0%	5%
CAD-1 m	–	+	–	+	–	0%	72%
CAD-2c	–	+	–	–	–	0%	6%
CAD-2nc	–	+	–	–	–	0%	16%
CAD-3	–	+	–	+	–	0%	8%
CAD-4	–	+	–	+	–	0%	38%
PEAD-1	–	+	+	+	–	80%	71%
PEAD-2	–	+	–	+	–	20%	51%
PEAD-3	–	+	–	+	–	0%	64%
PEAD-4	–	–	+	+	–	0%	58%
PEAD-5	Focal	+	–	+	–	50%	36%
PEAD-6	–	+	–	+	–	0%	46%
PEAD-7	–	–	–	+	–	0%	66%
ADC-1	+	+	–	–	–	1%	21%
ADC-2	+	+	–	–	–	0%	46%
ADC-3	+	+	–	–	–	0%	28%
ADC-4	+	+	–	–	–	5%	32%
ADC-5	+	+	–	–	–	0%	41%
ADC-6	+	+	–	–	–	0%	19%

(85.7%) while *KRAS* mutations occurred in three cases (42.9%; Fig. 3C). Furthermore, we observed a variety of chromosomal alterations, including amplification of *RICTOR* in two samples (Fig. 4C).

4. Discussion

With this study, we aimed to provide a detailed insight into the molecular landscape of IMA, CAD and PEAD of the lung with potential implications for routine diagnostics.

Although the results from clinicopathological analysis cannot be generalized due to the low number of samples included in this study, it resembles the typical clinical course of the three investigated adenocarcinoma subtypes. Patients with IMA rarely develop distant metastases, but local relapses may occur frequently [25]. While some CAD progress very slowly and remain limited to the lung, others are able to spread to distant organs. Still, most patients show long-term survival despite metastatic disease [6,26]. PEAD often have already spread to lymph nodes or distant organs at the time of initial diagnosis [27,28]. In line with this, these patients seem to have the worst prognosis of all lung adenocarcinomas with intestinal differentiation [27].

In accordance with their intestinal differentiation, all tumors were positive for at least one intestinal marker. In line with previous studies, TTF-1 expression was lost in most tumors [5,6,16]. Even if the tumor showed focal expression of TTF-1, it was never co-expressed with CK20/CDX2 in the same cells. Furthermore, most tumors remained positive for CK7 with the exception of two PEAD. This may complicate the differentiation of a primary lung tumor from metastases from the lower gastrointestinal tract, as both were CDX2 positive.

As a reference for our further molecular analyses, we investigated the mutational landscape of IMA, which is already fairly well understood [9]. In line with previous results, we showed that this tumor entity is characterized by a low overall tumor mutational burden [25]. Although recent publications described *CD74-NRG1* translocations as a recurrent driving alteration in IMA [10,11], we provide first data that additional chromosomal alterations seem to be rare. Unfortunately, the NGS panel used in this study did not cover the *CD74* and *NRG1* genes.

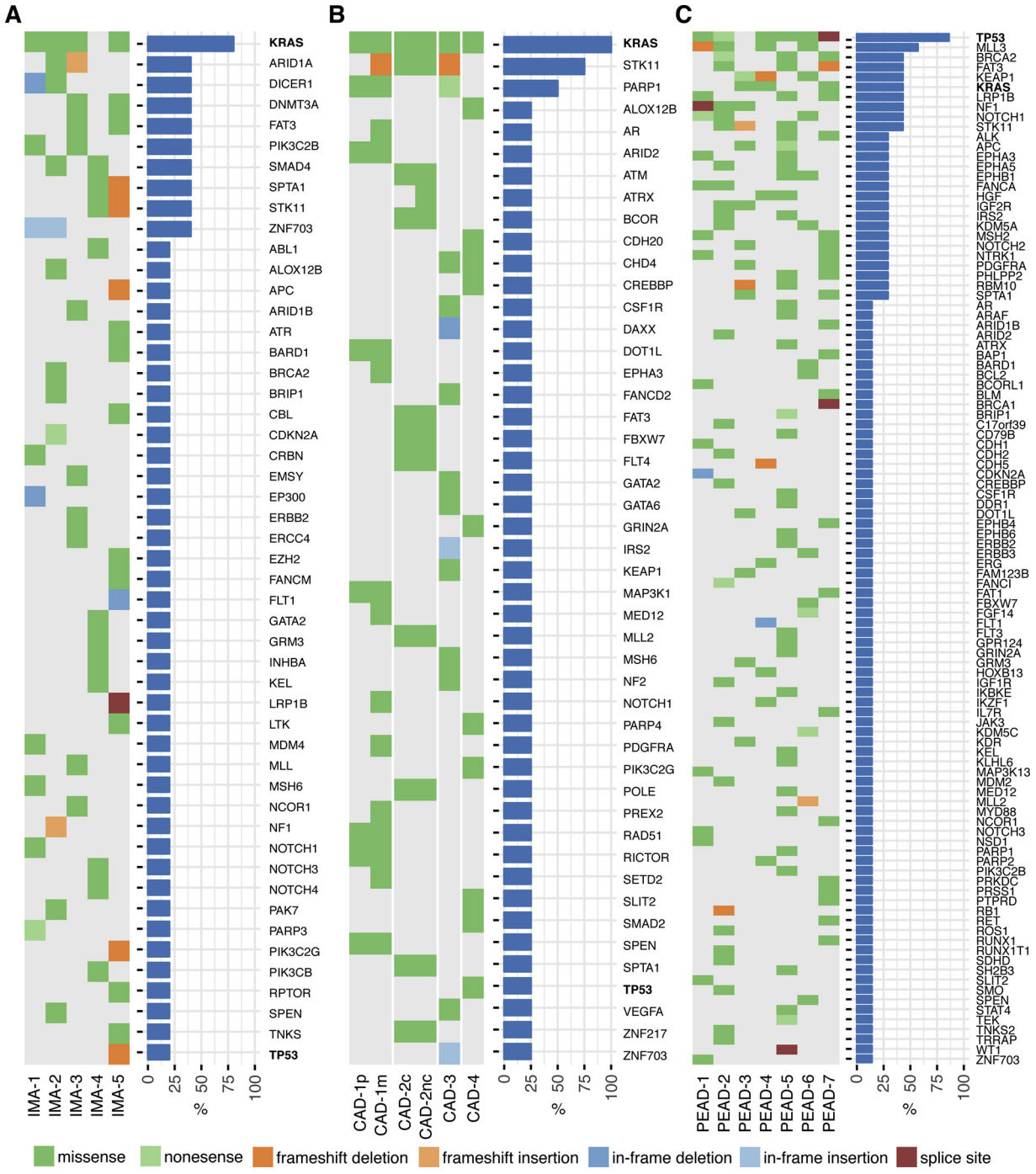

Fig. 3. Heatmap summarizing the observed mutations in invasive mucinous adenocarcinomas (IMA; **A**), colloid adenocarcinomas (CAD; **B**) and pulmonary enteric adenocarcinomas (PEAD; **C**).

Furthermore, we observed that *KRAS* mutations were more frequent than in common pulmonary adenocarcinomas, which is in line with previous reports [29]. Within our cohort of IMA, we exclusively observed *KRAS* mutations that resulted in a G12 V and G12D amino acid change whereas G12C is most common in non-mucinous lung adenocarcinomas. Interestingly, as previously reported, G12 V and G12D amino acid changes are usually seen in colorectal tumors [30]. This finding is in line with previous reports [25]. *TP53* mutations are rare in IMA. However, they might indicate a more aggressive course of disease, as the only patient within our cohort that died of this disease had a *TP53* mutation. Furthermore, we observed that C:G > G:C nucleotide substations were extraordinary rare in IMA. However, there is no data on the characteristics or causes for this transversion, so the biological significance of this observation remains unknown.

Compared to IMA, there is very little data on the mutational landscape of CAD. With this study, to the best of our knowledge, we provide the most detailed molecular characterization of this rare non-small cell lung cancer (NSCLC) subtype. In our investigation, we observed *KRAS* mutations in all investigated samples, including a variety (G12 V, G12D, G12C and G12R) of amino acid changes. To date, there is conflicting data on the frequency of *KRAS* mutations in CAD, ranging between 20% and 100% [6,13,14]. Our findings support the hypothesis that *KRAS* is recurrently altered in this adenocarcinoma subtype. Furthermore, we observed a high frequency of *MYC* amplifications (three

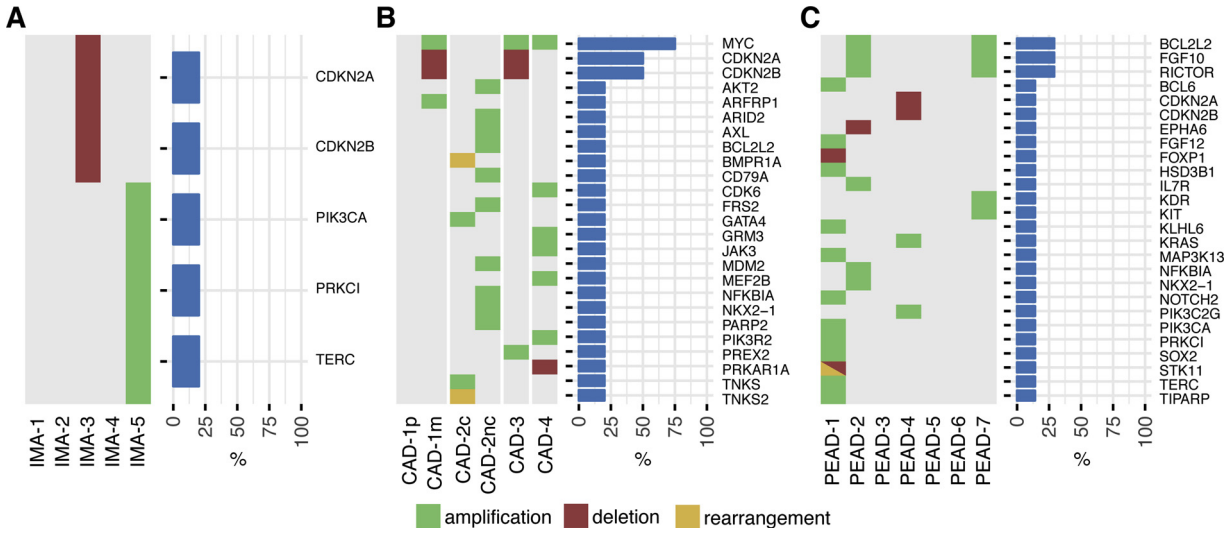

Fig. 4. Heatmap summarizing the observed chromosomal alterations in invasive mucinous adenocarcinomas (IMA; **A**), colloid adenocarcinomas (CAD; **B**) and pulmonary enteric adenocarcinomas (PEAD; **C**).

of four individual samples) compared to common lung adenocarcinomas (10%). Remarkably, these three samples were also the only specimens that harbored MYC amplification within the whole investigated cohort. However, the role of *MYC* in this tumor entity has not been investigated before and further studies are required to evaluate if *MYC* is recurrently amplified in this tumor entity.

CAD used to be regarded as benign or borderline malignant, and some authors hypothesized that the presence of a non-colloid component might be an indicator for worse prognosis [6]. Therefore, one of our goals was to investigate the molecular difference between the colloid and the non-colloid tumor compartment of the same tumor as well as the changes occurring during the development of distant metastases. Using a comparative analysis of the colloid and the acinar component of the same tumor, we observed an almost identical mutational profile. However, there were vast differences with regards to chromosomal alterations. This might indicate that the two components consist of different tumor cell clones, resulting in a distinct morphology. Although it is difficult to determine the more aggressive component based on the observed mutational profile, this hypothesis seems strengthened by our observation of an increased proliferation rate in the acinar component. In another comparative analysis evaluating the mutational profile of a primary CAD and a matched brain metastasis, we observed the acquisition of a variety of alterations in the metastasis, including both numerous mutations and several chromosomal alterations. In line with our findings from the comparison of the colloid and the non-colloid component, the brain metastasis adopted a distinct, papillary-like growth pattern. Furthermore, Ki-67 IHC revealed a significantly increased cell proliferation. Based on this data, it seems likely that the non-colloid differentiation is the result of a different tumor cell subclone or of an increased overall genetic instability. Although further investigation is required to confirm these findings, this might eventually lead to the development of distant metastases.

As described in previous studies, we observed a relatively high amount of *KRAS* mutations in PEAD, again including a variety of different amino acid changes (G12D, G12 V and G12C). In contrast to IMA and CAD, *TP53* mutations were much more common in this tumor entity. Considering the morphological similarities between this tumor subtype and colorectal cancer, we did observe *APC* mutations in two samples. This is in contrast to other studies on PEAD, which did not find any mutations frequently altered in colorectal cancer. However, none of the patients showed any primary cancer in the gastrointestinal system.

Additionally, in line with previous reports, PEAD seem to occur more frequently in heavy smokers [31]. This can also be seen on a molecular level, as we did not only observe a higher tumor mutational burden, but also a significantly higher proportion of C:G > A:T nucleotide substitutions within these tumors. As previously reported, this type of nucleotide change is consistent with smoking damage [32]. Even though the number of patients is limited in our study, it seems worth to further study the role of the tumor mutational burden in the context of overall survival and within special NSCLC subtypes. To this end, definitions of potential and reliable cut-offs are highly needed. Additionally, we observed PD-L1 expression in almost half of our cases. Although none of the investigated samples harbored microsatellite instability, these results indicate that pulmonary enteric adenocarcinoma is a subset of intestinal type lung adenocarcinomas that might particularly profit from immunotherapy. This is of certain clinical importance, as other targetable alterations such as *EGFR* mutations, *ALK* or *ROS1* translocations were reported to be extraordinary rare in this tumor subtype [17,31,33,34]. This is also in line with the results from our study, as we did not observe any of these alterations in our cohort. However, interestingly, *RICTOR* amplification was present in two PEAD. Previous studies showed that these tumors show response to treatment with mTORC1/2 inhibitors [35]. Although this finding requires further evaluation, *RICTOR* amplification might represent a new therapeutic target for patients with PEAD.

The main limitation of our study is the small sample size of adenocarcinomas with intestinal differentiation. Therefore, it is not possible to derive dependable conclusions for the different subtypes in general and additional studies on independent cohorts will be required to verify our findings. Furthermore, the NGS panel that has been used in this study is limited to known cancer-related genes. This limits the scope of our investigation, as it might miss new and currently unknown alterations that could potentially play an important role in these tumor entities.

Despite these limitations, our study still provides the most detailed molecular characterization of CAD and PEAD to date. Furthermore, this is the first investigation to directly compare IMA, CAD and PEAD. By doing so, we were able to show that these three NSCLC subtypes show huge molecular differences despite their common feature to resemble intestinal adenocarcinomas.

In summary, we shed light on the molecular differences between different subtypes of lung adenocarcinomas with intestinal differentiation as well as the changes occurring during the development of metastases. From a clinical view, our findings could contribute to a better risk stratification of patients with CAD and provide first evidence for the eligibility of patients with PEAD for treatment with immune

checkpoint inhibitors.

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Conflict of interest Statement

None.

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Appendix A. Supplementary data

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