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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Differential diagnosis of pulmonary enteric adenocarcinoma and metastatic colorectal carcinoma with the assistance of next-generation sequencing and immunohistochemistry

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Abstract

Purpose Pulmonary enteric adenocarcinoma (PEAC), defined as tumors with an enteric component exceeding 50% and a histological morphology similar to colorectal cancer (CRC) and metastatic colorectal carcinoma (MCC), is an extremely rare primary lung adenocarcinoma, which was recently recognized by World Health Organization (WHO). Adenocarcinomas with intestinal differentiation have also been described in other anatomic sites, including paranasal sinuses, extrahepatic biliary tree, uterine and cervix, ovary. The morphologic spectrum and immunohistochemical profiles of PEAC overlap with those of colonic adenocarcinomas, the diagnosis of PEAC remains challenging. Currently, colonoscopy has to be performed to confirm the diagnosis, resulting in low compliance due to its invasiveness. Due to the rareness of PEAC, its molecular signature has not been comprehensively examined.

Methods In this study, we investigated the molecular signatures associated with PEAC and its histological counterparts, CRC and MCC using capture-based targeted sequencing.

Results We revealed that 12/13 (92.31%) PEAC patients harbored mutations in well-established driver genes for non-small cell lung cancer and none of them had mutations unique to CRC. Furthermore, 13/15 (86.7%) of MCC harbored mutations that are frequently seen in CRC.

Conclusion Collectively, our study showed that PEAC, exhibiting a similar mutational profile with NSCLC, showed a distinctive signature from CRC and MCC. Furthermore, we derived a classification model, intergrading both IHC markers and genetic signature, to accurately diagnose PEAC.

Keywords Primary pulmonary enteric adenocarcinoma (PEAC) · Pulmonary metastases from colorectal carcinoma (MCC) · Next-generation sequencing (NGS) · Mutation profile

Abbreviations

PEAC	Primary pulmonary enteric adenocarcinoma
MCC	Pulmonary metastases from colorectal carcinoma
CRC	Colorectal cancer
NGS	Next-generation sequencing
IHC	Immunohistochemistry
FFPE	Formalin-fixed paraffin-embedded
H&E	Hematoxylin & eosin
CK7	Cytokeratin 7
TTF-1 (nkx2-1)	Thyroid transcription factor-1
CK20	Cytokeratin 20
CDX2	Caudal type homeobox 2
LOF	Loss of function
CNV	Copy number variation

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NSCLC	Non-small cell lung carcinoma
LUAD	Lung adenocarcinoma
APC	Adenomatous polyposis coli protein
MMR	Mismatch repair
ALK	Anaplastic lymphoma kinase
EGFR	Epidermal growth factor receptor
KRAS	Kirsten rat sarcoma viral oncogene homolog
BRAF	Serine/threonine-protein kinase B-raf

Introduction

Primary pulmonary enteric adenocarcinoma (PEAC),a rare histologic type of lung adenocarcinoma, was first described in 1991 by Tsao and Fraser (1991). It was introduced in 2011 for the first time in the international multidisciplinary classification of lung adenocarcinoma, sponsored by International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) (Travis et al. 2011), and was subsequently recognized by World Health Organization (WHO) in 2015 (Travis et al. 2015). PEAC, an adenocarcinoma that resembles adenocarcinomas arising in the colorectum (Travis et al. 2015), is defined as tumors with an enteric component exceeding 50%, and a histological morphology similar to CRC (Travis et al. 2015). Some tumors have enteric differentiation, evident by positive for CDX2 and CK20, and negative for CK7; while others have only enteric morphology and lack the typical immunoprofile. Hence, distinguishing PEAC from pulmonary metastases of colorectal carcinoma (MCC) is further complicated by the rare expression of TTF1 in MCC. TTF-1 is expressed consistently throughout the life stages and uniformly in the terminal respiratory unit. Therefore, it is difficult to make a final diagnosis solely based on histological features and IHC markers recommended by the WHO in 2015. To date, seven cases of PEACs resembling the immunohistological properties of MCCs were reported (Hatanaka et al. 2011; Stojsic et al. 2014; Li et al. 2009; Lin et al. 2016; Laszlo et al. 2014; Garajova et al. 2015). Currently, enteric adenocarcinoma can be regarded as a lung primary only after the clinical exclusion of metastases from colorectal carcinoma by colonoscopy (Travis et al. 2015). Approximately 75% of primary pulmonary invasive adenocarcinomas are positive for TTF1. Primary pulmonary invasive adenocarcinomas (excluding the mucinous adenocarcinomas) usually express CK7 diffusely and express CK20 rarely, if CK20 is positive, the positivity may be weak or focal. The expression of CDX2 in primary pulmonary invasive adenocarcinomas is similar to that of CK20.

With advancements in sequencing technologies in the past decade, NGS has significantly facilitated molecular profiling of cancer subtypes. Numerous studies have reported diagnosis of carcinomas with uncertain origin based on molecular signatures (Chevrier et al. 2014; Kunze et al. 2014; Crobach et al. 2016). Due to the rareness of PEAC, its molecular signature has not been comprehensively examined. Therefore, elucidating molecular profiles of PEAC is critically important, which can potentially supplement its diagnosis. Here, we conducted a retrospective study to investigate the mutational signatures associated with PEAC and its counterparts, MCC and CRC by performing capture-based targeted deep sequencing using a panel consisting of all exons and critical introns of 295 genes, spanning 1.44 MB of human genomic regions. Our cohort consisted of 33 patients, 13 PECAS, 15 MCCs and 5 CRCs. Our data suggest that PEAC exhibits a distinct molecular profile, compared to MCC and CRC, despite the histological similarity and to some extent immunohistochemical similarity. To the best of our knowledge, it is the first time that molecular signatures have been investigated for PEAC and MCC from a genome-wide perspective.

Materials and methods

Patient demographics

The retrospective study enrolled 33 patients including 13 patients with PEACs, 15 with MCCs, and 5 with CRCs. The 13 PEAC cases and 15 MCCs were diagnosed based on surgical specimen obtained between February 2013 and January 2016 in Shanghai Chest Hospital. The 5 CRCs were diagnosed in January 2016 at the Fifth People's Hospital of Shanghai, Fudan University based on colonoscopy result. The additional 135 NSCLC patients were diagnosed at Shanghai Chest hospital between February 2013 and January 2016. Each sample was histologically assessed by three independent pathologists based on the 2015 WHO classification guidelines. Of the PEAC cases, 7 patients had colonoscopy examinations that revealed no evidence of gastrointestinal tumor. Due to various reasons, colonoscopy was not performed on the remaining six patients; however, they did not have clinical history of gastrointestinal carcinoma and lack clinical symptoms of CRC during follow-ups scheduled at a regular interval. Informed consent was obtained from each patient at recruitment, and the present study was approved by the Ethics Committee of Shanghai Chest Hospital and the Fifth People's Hospital of Shanghai.

DNA extraction

For genetic analyses, we collected 5–8 of 5 μm tissue sections from FFPE tumor samples. Of note, the neoplastic area was micro-dissected using a parallel hematoxylin and eosin (H&E) slide as a reference, with subsequent DNA isolation

from samples with > 80% tumor infiltration. Genomic DNA was extracted with the QIAamp DNA FFPE Tissue Kit (QIAGEN, Heidelberg, Germany) following the manufacturer's instructions. The DNA quality was assessed by NanoDrop™ 8000 (ThermoFisher Scientific, MA, US) and agarose electrophoresis, and the quantity was measured by Qubit®dsDNA HS Assay Kit on Qubit®3.0 Fluorometer (Invitrogen, CA, US).

NGS library preparation and capture-based targeted sequencing

DNA was profiled using a commercially available capture-based targeted sequencing panel (Burning Rock Biotech Ltd, Guangzhou, China), targeting 295 genes and spanning 1.44 MB of human genomic regions. DNA shearing was performed using Covaris M220 (Covaris, Inc., MA, US), followed by end repair, phosphorylation and adaptor ligation. Fragment sizes ranging from 200 to 400 bp were selected using Agencourt AMPure beads (Beckman Coulter, CA, US) followed by hybridization with capture probes baits, hybrid selection with magnetic beads and PCR amplification. Subsequently, Qubit® 3.0 and Agilent 2100 bioanalyzer (Agilent Technologies Inc., CA, US) was performed to assess the quality and size of the fragments. Indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., CA, US) with pair-end reads.

Sequencing data analysis

Sequencing data were mapped to the human genome (hg19) using BWA aligner 0.7.10. Local alignment optimization, variant calling and annotation were performed using GATK 3.2, MuTect, and VarScan. Variants were filtered using the VarScan fpfilter pipeline, with loci with depth less than 100 filtered out. Minimum of five supporting reads were needed for INDELs and eight supporting reads were needed for SNV calling. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using both Tophat2 and Factera 1.4.3.

Immunohistochemistry

Briefly, 3-μm-thick FFPE sections were subjected to routine immunohistochemistry (IHC) for CK7 (OV-TL12/30, Dako), TTF-1 (SPT24, Leicabiosystems), CK20 (SP33, Longislandbiotec), CDX2 (DAK-CDX2,Dako), at dilutions of 1:200, 1:200, 1:100, 1:200, respectively, using Leica BOND-III (LeicaBiosystems, Wetzlar, Germany) automated immunohistochemistry platforms. Cells were scored on the basis of cytoplasmic and/or nuclear staining intensity as 3 (positive), 2 (partial positive), 1 (focal positive), 0 (negative).Appropriate negative and positive controls were included.

Decision tree construction

Four genetic variant groups clustered from 12 genes (NSCLC driver mutations, RAS family, APC and MMR genes) were utilized as input variables for model construction. In each round, the best model was selected by tenfold cross-validation, where all samples were randomly partitioned into ten sub-groups, and each sub-group was retained as the validation group while the other nine sub-groups were used as the training group. In the end, ten models were averaged to generate the parameters for the final model.

Statistical analysis

The data were analyzed using SPSS 16.0 software. Pearson's Chi-square test was used for comparisons between groups. The differences in pathology in terms of gender, age, smoking status, tumor size, TNM stage, and IHC results were calculated by Fisher's exact tests. Kaplan–Meier assays were used for the progression-free survival (PFS) curves and the statistical difference was calculated by the log-rank Mantel-Cox test. $p < 0.05$ was considered as statistically significant.

Results

Clinicopathological characteristics

Collectively, our cohort included 13 PEACs, 15 MCCs and 5 primary CRCs. The average age for patients with PEACs was 61.2 years, ranging from 45 to 69, with 7 males and 6 females. Among them, 3 were smokers and 10 were non-smokers. The average tumor size was 38.5 mm, ranging from 10 to 80 mm. In the 15 MCCs cases, the average age was 57.3 years, ranging from 44 to 71, with 10 males and 5 females, 2 smokers and 13 non-smokers. The average tumor size was 25.9 mm, ranging from 11 to 64 mm. In the 5 CRCs cases, the average age was 59.6 years, ranging from 33 to 78, with 3 males and 2 females, and the average tumor size was 54 mm, ranging from 30 to 100 mm. A comparison of clinicopathologic data for the PEAC, MCC and CRC groups revealed no statistically significant difference in age, gender, smoking history, or tumor size (Table 1). The detailed clinicopathologic profiles are shown in Supplemental Table 1.

Immunohistochemistry

Expression of certain immunohistochemistry (IHC) markers has been reported to partially distinguish PEACs from

Table 1 Distributions of select characteristics by PEACs, MCCs, and CRCs

Variables	PEAC ($N=13$)	MCC ($N=15$)	CRC ($N=5$)	p value
Mean age in years (S.D.)[a]	61.2 (9.1)	57.7 (8.2)	59.6 (17.3)	0.353
Gender (%)[b]				0.395
Male	7 (53.8)	10 (66.7)	3 (60.0)	
Female	6 (46.2)	5 (33.3)	2 (40.0)	
Smoking status (%)[b]				0.75
Never	10 (76.9)	13 (86.7)		
Ever	3 (23.1)	2 (13.3)		
Mean tumor size mm (S.D.)[a]	38.5 (20.0)	25.9 (16.8)	54 (27.0)	0.476
Stage (%)[c]				
I	7 (53.8)	0 (0.0)		28.000
II	2 (15.4)	0 (0.0)		
III	4 (30.8)	0 (0.0)		
IV	0 (0.0)	15 (100.0)		
Site(%)[c]				
LUL	5 (38.5)	1 (5.9)		5.498
LLL	3 (23.1)	7 (41.2)		
RUL	2 (15.4)	4 (23.5)		
RML	2 (15.4)	2 (11.8)		
RLL	1 (7.6)	3 (17.6)		

RLL right lower lobe, *RUL* right upper lober, *RML* right middle lobe, *LLL* left lower lobe, *LUL* left upper lobe

[a]Student *t*-test and

[b]Fisher's exact test

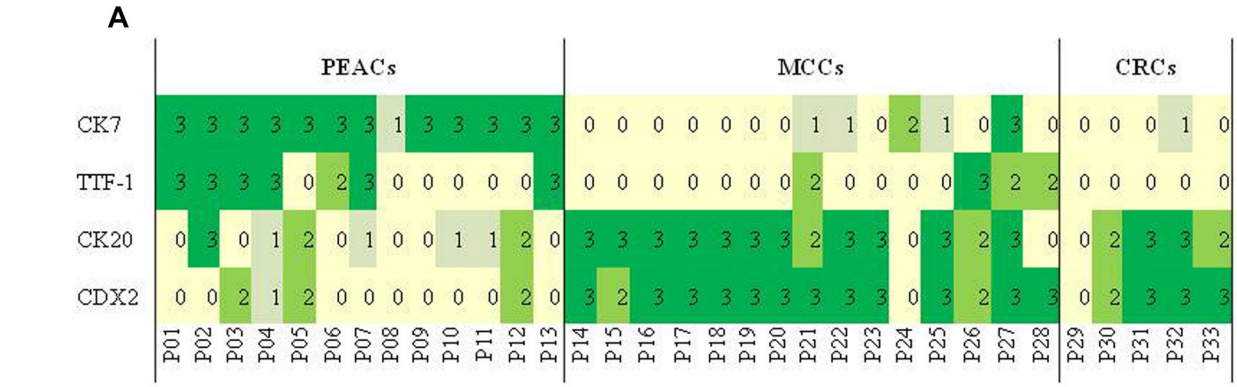
[c]Pearson Chi-Square test for the differences between PEACs and MCCs/CRCs

MCCs (Inamura et al. 2005; Yousem 2005; Wang et al. 2014). In 2015, WHO has recommended to interrogate the expression of CK7, TTF-1, CDX2, and CK20 and PEAC has to show positivity in at least one of the above-four markers. We assessed the expression of the above-four IHC markers and results were summarized in Fig. 1.Our IHC results revealed that all PEAC samples strongly expressed CK7 with the exception of 1 (P08); 7/13 (53.8%) of them expressed TTF1 and 7/13 (53.8%) expressed CK20. Among them, 3 patients expressed both TTF1 and CK20. CDX2 were expressed in 4/13 (30.8%) of cases. In contrast, 17/20 (88%) patients diagnosed with MCC and CRC co-expressed CK20 and CDX2. Only 6/20 (30%) patients expressed CK7; and 4/20 (20%) expressed TTF-1. Collectively, we observed a similar expression pattern of these markers between MCCs and CRCs, with the expression of CK20, CDX2 and CK7 in most cases. In contrast, PEACs exhibited a different expression pattern, but we have difficulty in distinguishing PEACs and MCCs using only four IHC markers.

When comparing the 3 groups, PEACs showed significantly higher positive rates on CK7, TTF1 and CK20. Only CK7 has significant difference between PEACs and MCCs/CRCs ($p<0.001$), yet no significant difference on CK20 ($p=0.063$) and TTF-1 ($p=0.08$) compared to MCCs and CRCs. PEACs showed lower positive rates on CDX2 ($p=0.005$) (supplement Table 2).It has been reported that the co-expression of CK7 and TTF1 can help to distinguish PEACs from MCCs (Travis et al. 2011). However, only about half of the PEACs can be distinguished from MCCs in our cohort based on this rule, and four MCC cases would be mis-classified as PEAC, necessitating more accurate diagnosis criteria.

Mutation spectrum

To investigate the molecular profiles of primary PEACs, MCCs of lung, and primary CRCs, we performed capture-based targeted deep sequencing on 33 tumor samples, using a panel consisting of all exons and critical introns of 295 genes, to detect and quantify genetic alterations. We achieved a mean coverage depth of 1000× across all targeted regions. Among all samples, the mapped reads percentage was over 99%. All detected mutations are depicted in Fig. 2a. All patients with PEAC harbored at least one well-established driver mutation for NSCLC, except for one (P09), who had no mutation detected from this panel. None of them carried mutations that are unique to colorectal cancer, such as APC. Three of them had concurrent mutations in the



B

	Positive rate (PEACs vs. MCCs/CRCs)	
CK7	100.0%	30.0%
TTF-1	53.8%	20.0%
CK20	53.8%	85.0%
CDX2	30.8%	90.0%

C

	Diagnosis		
	correct	missed	
PEACs	7(53.8%)	6(46.2%)	χ^2=0.459
MCCs	11(73.3%)	4(26.7%)	p=0.498

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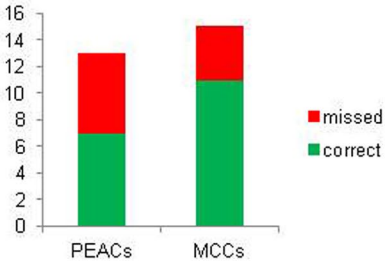

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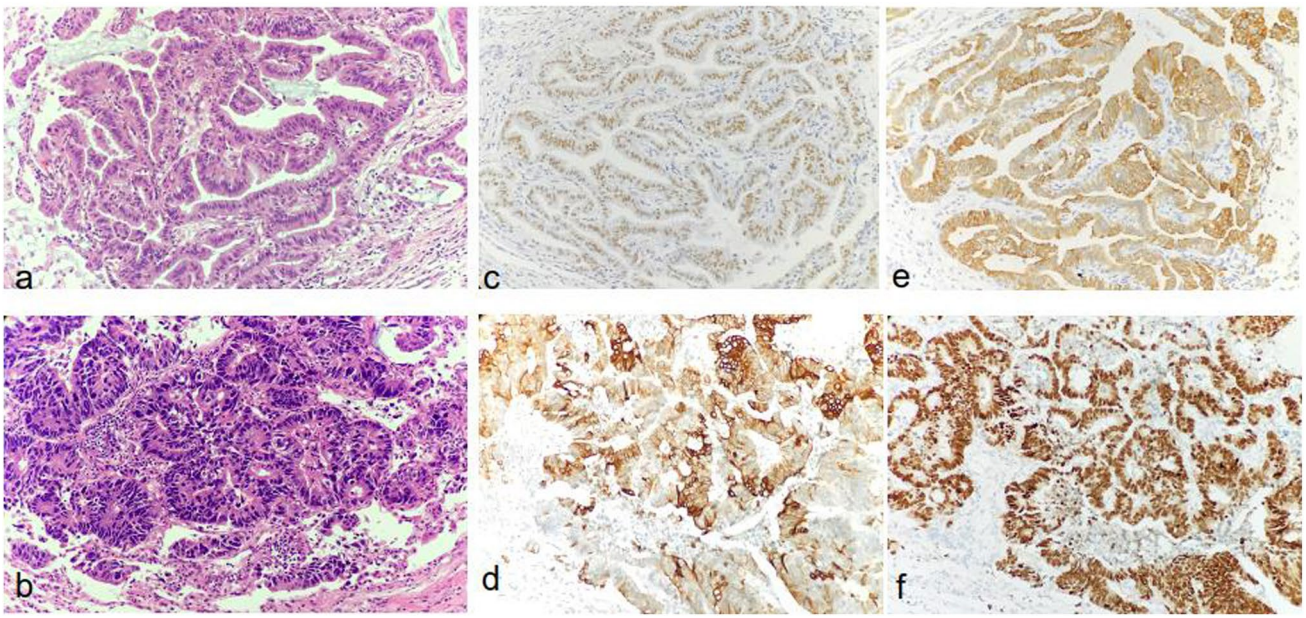

Fig. 1 **a** The immunostaining staining of PEAC, MCC, and CRC. Results were scored on the basis of cytoplasmic or nuclear staining intensity, ranged from 0 to 3 as 3 being positive, 2 being partial positive, 1 being focal positive, and 0 being negative. **b** The positive rates of each marker in PEACs versus MCCs/CRCs are shown. **c** The diagnostic accuracy of the combined four IHC markers. **d** The histogram of the diagnostic accuracy of the combined four IHC markers. **e** The histologic and IHC results of PEAC (up) and MCC (down). (a) Hematoxylin and eosin staining of PEAC. (b) Hematoxylin and eosin staining of MCC (H&E,). Immunohistochemistry for TTF-1 (c) and CK7 (e) show positive staining in PEAC. Immunohistochemistry for CK20 (d) and CDX2 (f) show positive staining in MCC. Original magnification × 200

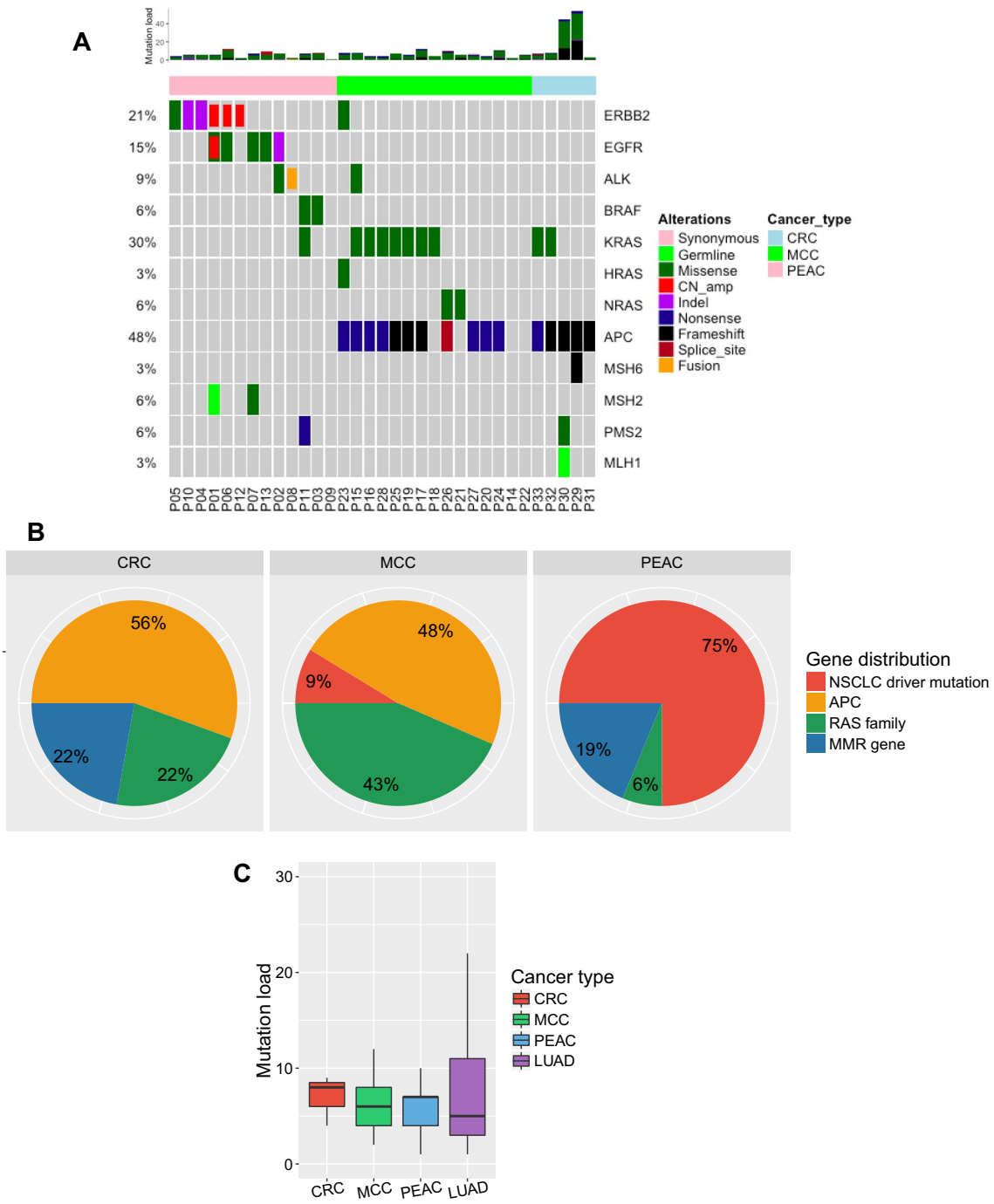

Fig. 2 **a** Mutation spectrum of PEAC, MCC and CRC. Patients were grouped according to their histopathological diagnosis. Different types of mutations are denoted in different colors. The frequency of each mutation in this cohort was shown on the left of the oncoprint. The total number of mutations a patient carried is summarized on top of the oncoprint. **b** Mutation type distribution in PEAC, MCC, and CRC. Different types of mutations are denoted in different colors. **c** Mutation load in PEAC, MCC, CRC, and LUAD

mismatch repair genes, such as MSH2 and PMS2. Interestingly, one of them (P 01) carried germline MSH2 mutation. All CRC patients (5/5) harbored APC mutation. In addition, two patients had concurrent KRAS mutation and another two had concurrent mutations in mismatch repair genes. No CRC patient had mutations in driver genes for NSCLC. Ten out of 12 MCC patients harbored mutations that are frequently mutated in CRC, such as APC and multiple types of RAS mutations. Interestingly, two patients carried concurrent driver mutations, P23 and P15, who harbored mutations

in *ERBB2* and *ALK*, respectively. Two MCC patients (P14/P22) had no mutations detected from this panel.

Next, we examined what major mutations associated with each cancer type. Classic colon cancer mutations, such as APC and KRAS mutations, were the predominant mutations detected in CRC and MCC, accounting for 78% and 91% of mutations listed in the mutation spectrum, respectively. In contrast, the predominant mutations associated with PEAC were driver mutations in NSCLC, such as *EGFR, ALK* and *ERBB2*, accounting for 75% of mutations. No classic CRC mutation was detected in PEAC (Fig. 2b). Collectively, our data revealed that the molecular profile of patients with PEAC, distinctive to CRC and MCC, resembles that of NSCLC; in contrast, the mutation spectrum of patients with MCC resembles that of CRC.

Previous studies have indicated differential tumor mutation load between CRC and NSCLC. We performed mutation load analysis on the 3 groups as well as NSCLC by including 135 NSCLC patients because PEACs and NSCLC share a similar mutation profile. Our mutation load analysis revealed that four cancer types had different mutation load

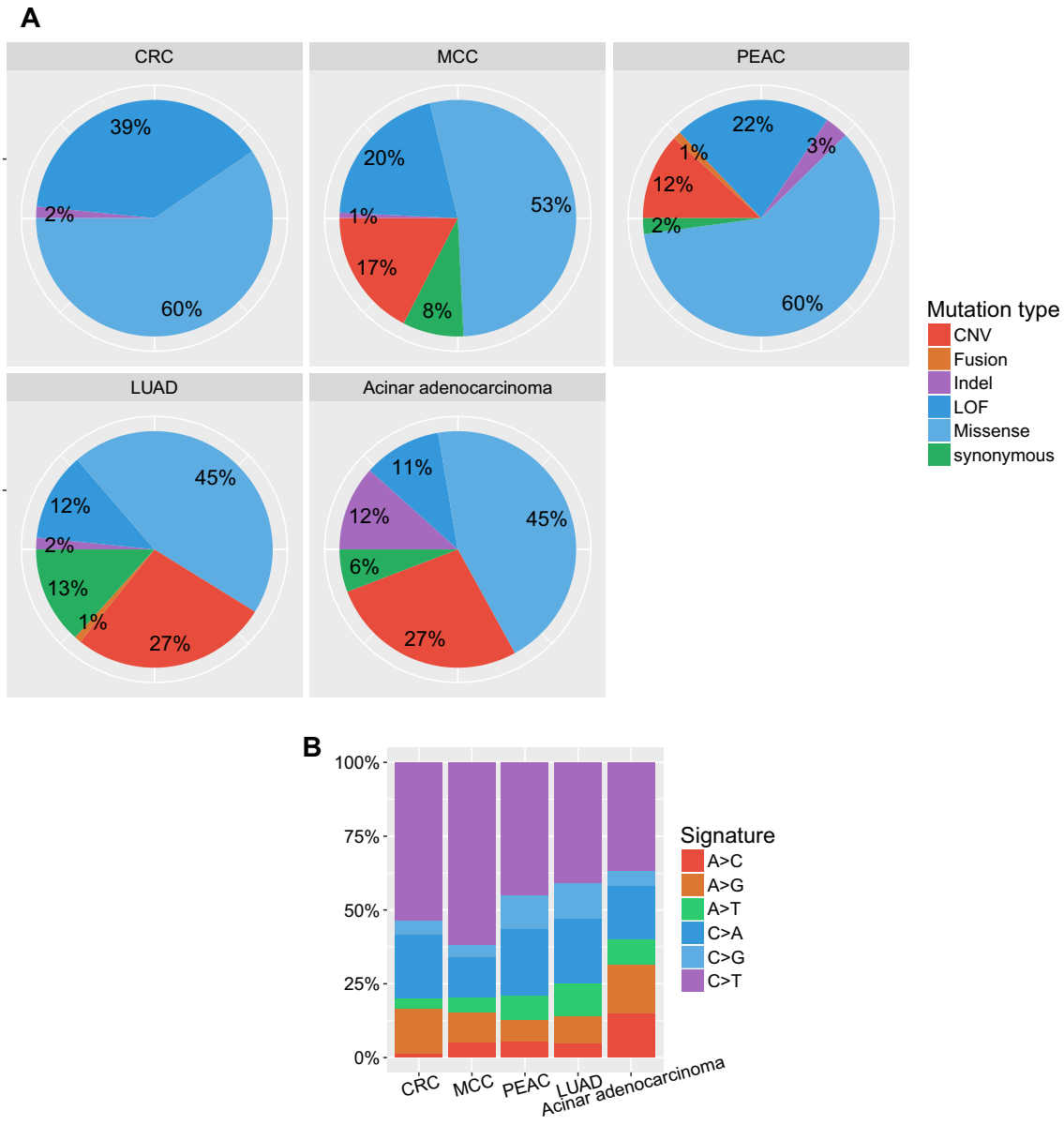

Fig. 3 Mutation type distribution (**a**) and nucleotide changes (**b**) in CRC, MCC, PEAC, LUAD and acinar adenocarcinoma are shown. **a** Percentages of various mutation types constituting each cancer type were shown. Different color denotes different mutation type. Acinar carcinoma is derived from a subpopulation of lung adenocarcinoma. **b** Percentages of different types of nucleotide change for each cancer type were shown. Different color denotes different types of nucleotide change

with CRC being the highest and NSCLC being the lowest among them ($p = 0.097$) (Fig. 2c).

Furthermore, we performed analysis on types of mutations involved in each cancer type (Fig. 3a).The mutation profiles of lung adenocarcinoma (LUAD), MCC and PEACs shared some degree of similarity, consisting of a variety of mutation types, primarily missense mutations, copy number variation (CNV) and loss of function (LOF) mutations. In contrast, CRC has its own unique mutation type pattern, only consisting of missense mutations (60%), LOF (39%) and 2% indel. Furthermore, fusions were only detected in PEAC and NSCLC. Adenocarcinomas are extremely heterogeneous, with acinar adenocarcinoma being the predominant architecture in Chinese adenocarcinoma patients. We also investigated the mutation composition of acinar, which was very similar to lung adenocarcinoma with missense mutations and CNVs being the top two mutation types (Fig. 3a). Next, we investigated the composition of nucleotide change in each group (Fig. 3b). Consistent with the results from our mutation type analysis, CRC has its own distinctive signature, with more A > G and less A > C compared to the other three types. Taken together, in addition to mutation spectrum, CRC has its own distinctive signature in terms of mutation type and composition of nucleotide change.

Genetic signature can distinguish PEAC from its histological counterparts

Next, we compared genetic features of PEAC against its histological counterparts CRC/MCC using both TCGA data and sequencing data from our cohort. Genetic features included were typical features for NSCLC and CRC, such as classic drivers for NSCLC, APC, genes in mismatch repair pathways etc. Both our data and TCGA data revealed a combination of driver gene for NSCLC, APC, and RAS family genes can be used to distinguish PEAC from CRC (Fig. 4a). Furthermore, none of the listed genetic features can distinguish PEAC from lung adenocarcinoma, in agreement with our sequencing results that PEAC shared a similar genetic profile as NSCLC. To accurately distinguish PEAC from its counterparts, we constructed a decision tree model to elucidate the best rule based on the molecular profile to distinguish PEAC from other histological types. The decision tree was constructed based on 4 genetic variant groups clustered from 12 genes (NSCLC driver mutations, RAS family, APC and MMR genes) for model construction, resulting in a two-level model. Firstly, classic NSCLC driver genes, including *ERBB2, EGFR, ALK*, and *BRAF*, were utilized as input variables. Patients with NSCLC driver mutation were classified as PEAC; in contrast, patients without such mutation were classified as CRC/MCC. Patients classified as PEAC were further grouped according to the status of APC. Patients with APC mutation were classified as having CRC/MCC and patients with wild-type APC remained in the PEAC category, resulting in an accuracy of 97% (Fig. 4b). This model accurately classified every patient except for one, who had no mutation detected from this panel.

Differential DFS was observed between PEAC and MCC patients

We next performed a disease-free survival (DFS) analysis of PEAC and MCC. The median survival of PEAC and MCC were 44 and 21 months, respectively. We observed a trend of PECA patients with longer DFS ($p = 0.059$, Log-rank Mantel–Cox test, $n = 28$) (Fig. 5).

Discussion

The emerging interest on PEAC was primarily aroused by the challenges associated with the differential diagnosis with pulmonary metastases from primary CRC due to similar morphological and histological features. PEACs composed of medium-to-large glandular (or garland-like) structures and cribriform patterns, with illumina filled with 'dirty necrosis'. Furthermore, PEACs share some immunohistochemical features with MCC, such as markers of enteric differentiation (CK20, CDX2). Although it has been reported that the expressions of CK7 and TTF-1 aim in differentiating PEAC from MCC (Li et al. 2009; Inamura et al. 2005), exceptions do exist. It has been reported that some PEACs lack CK7 and TTF-1 expression (Hatanaka et al. 2011; Stojsic et al. 2014; Li et al. 2009; Lin et al. 2016; Laszlo et al. 2014; Garajova et al. 2015); in contrast, MCC patients with positive expression for both markers. In our cohort, we also observed two MCC (P21 and P28, who have no history of colon cancer) patients with positive expression of above markers. Currently, clinical exclusion of gastrointestinal tract origin by colonoscopy is necessary to establish accurate diagnosis (Travis et al. 2015). Yet colonoscopy, the "gold standard" and the most prevalent procedure for primary colorectal cancer screening (Winawer 2007; Sovich et al. 2015), has been frequently accompanied with unfavorable patient compliance. Furthermore, exceptional cases such as PEAC patients with a history of metachronous CRC, or with synchronous CRC have been described (Handa et al. 2015; Canney et al. 2009; El Hammoumi et al. 2016). In such scenarios, colonoscopy might not be definitive. Therefore, there is an urgent need to develop new markers to differentiate PEAC and MCC.

Due to its extreme rareness, only single case or small series have been published and the molecular signature of PEAC has never been comprehensively explored. Only very few studies investigated molecular profile of PEAC, primarily from single-gene or a panel of few genes point of

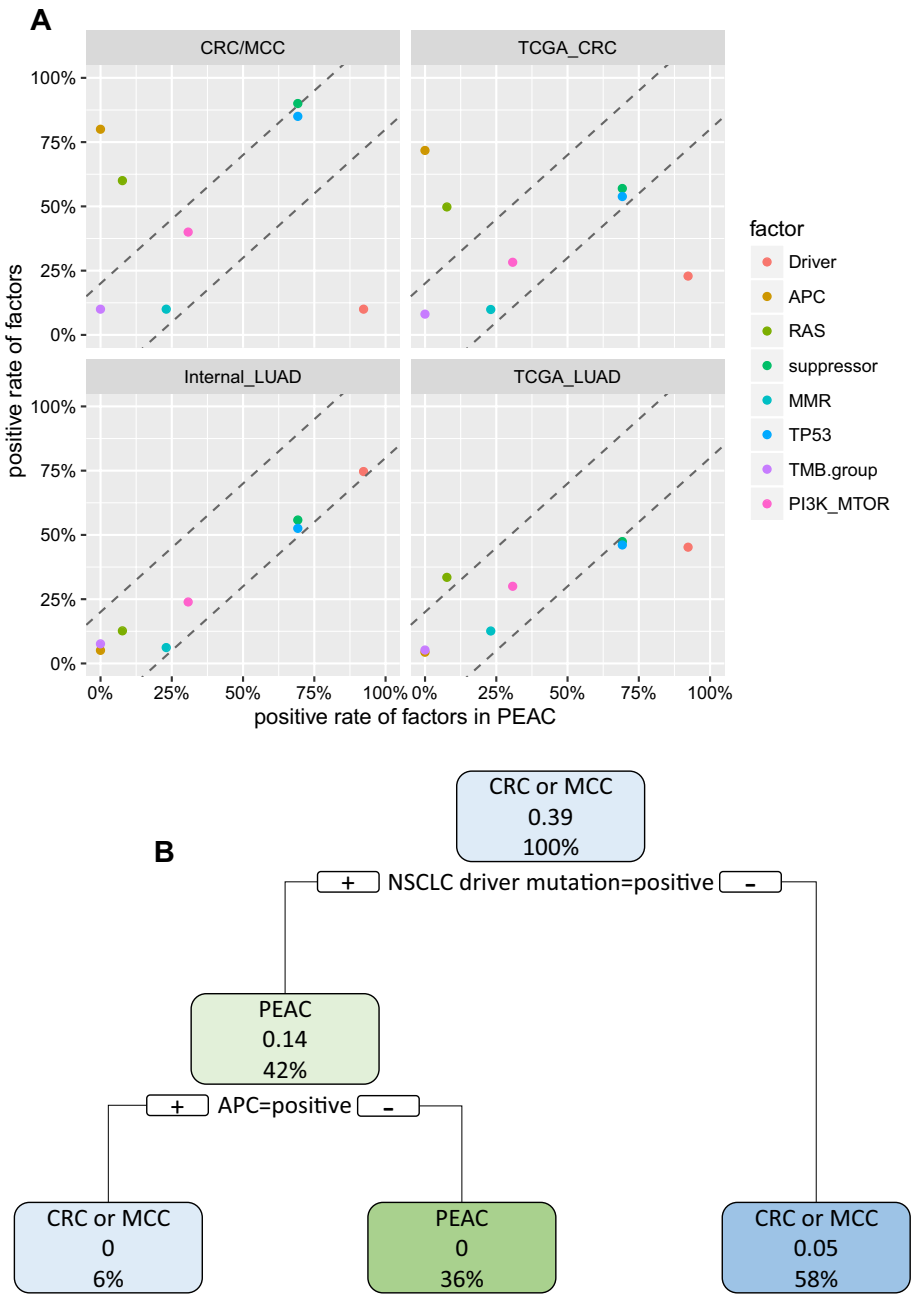

Fig. 4 Classifications of PEAC, MCC, and CRC. **a** Various genetic features were used individually to classify PEAC, MCC, and CRC. Different genetic features were denoted in different colors. The occurrence rate of a certain genetic feature in PEAC is shown on x-axis; the occurrence rate of a certain genetic feature in its counterparts is shown on y-axis. **b** A two-level decision tree. The first-level consisted of four NSCLC driver mutations, including *ERBB2, EGFR, ALK*, and *BRAF*. The second-level is based on the status of APC. The percentage in each square denotes the percentage of patients belonging to each square; the number in each square denotes the fraction of population that is mis-classified

view, focusing only on major driver mutations of NSCLC (Nottegar et al. 2017). In this study, we compared and contrasted molecular signatures associated with PEAC and its histological counterparts MCC and CRC by performing capture-based ultra-deep targeted sequencing on a cohort of 33 patients. Our data revealed that PEAC harbors a molecular profile distinctive to CRC/MCC and similar to lung adenocarcinoma. MCC and CRC shared a very similar mutation profile, with APC and RAS family genes being the most commonly mutated genes. Furthermore, we derived a two-level model using only molecular signature, consisting of four classic NSCLC driver genes and APC to distinguish PEAC from CRC/MCC. We only misclassified one patient, who had no mutation detected from this panel, achieving an accuracy of 97%. And the prognosis was altered by pathological characteristics. Our study served as foundation for the application of such diagnostic method in clinical settings. The future model of diagnosis will potentially move away from the traditional histopathological diagnosis and towards NGS-based histopathological diagnosis, in which molecular features will play a critical role in clinical decision-making.

There are a few limitations associated with this molecular profile-based diagnostic method as well as this study. Diagnosis cannot be performed on patients with no mutation

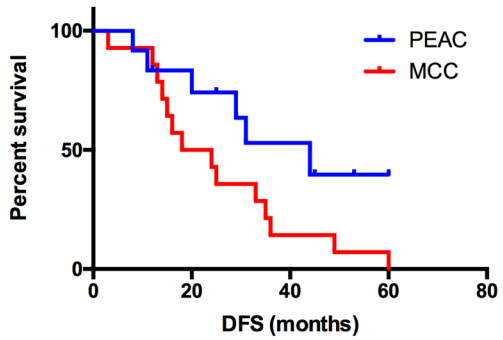

Fig. 5 Differential DFS was observed between PEAC and MCC patients. Disease-free survival (DFS) analysis of PEAC and MCC (Log-rank Mantel–Cox test, $p = 0.0588$, $n = 28$)

detected from this panel or from the five genes which were utilized to construct the model. Supplemental diagnosis is needed for such patients. Larger cohort studies are needed to validate our signature and model. In summary, our study demonstrated that molecular profiling in combination with our two-level decision model can effectively distinguish PEAC from MCC/CRC, paving its way as routine diagnostic method in clinical settings. To the best of our knowledge, this is the first study interrogating the molecular profile of PEAC in a relatively large cohort from a genome-wide perspective.

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Author contributions JZ, YH, and LZ designed research, KY, CX, HT, JL, and JS performed experiments. CX, HZ, JY and KY analyzed data; CX, HZ and KY wrote the paper.

Compliance with ethical standards

Conflict of interest The authors disclose no potential conflicts of interest.

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