Morphological and Genetic Heterogeneity of Synchronous Multifocal Lung Adenocarcinoma in a Chinese Cohort

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Research article

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Abstract

Background: Synchronous multifocal lung cancer (SMLC) is seen with increasing frequency in clinical practice globally. Because of innate variation in clinical management and outcome, it is vital to distinguish properly between synchronous multifocal primary lung cancer (SMPLC) and intrapulmonary metastasis (IM). The pathologic features and principal classification criteria of multifocal lung cancer remain unclear.

Methods: We have collected a unique cohort of Chinese patients with SMLC, and fully explored the morphologic, immunohistochemical, and molecular features of the disease. Twenty-one SMLC patients with a total of 50 tumors were included in our study. The pathological features presented by these cases were analyzed, including tumor location, tumor size, pathological types, predominant pattern of adenocarcinoma, and immunohistochemical staining. We undertook molecular testing of nine driver oncogenes associated with lung cancer, including EGER, KRAS, BRAF, NRAS, ALK, ROS1, RET, HER2, and PIK3CA.

Results: According to Martini-Melamed classification and refined standard, 8 and 17 cases were considered as SMPLC respectively. Gene mutations were identified in 18 tumors (36%). There were 12 patients had different gene mutations.

Conclusions: We demonstrate that conventional morphological assessment is not sufficient to establish clearly the clonal relationship of SMPLC. Instead the evaluation of histological subtypes, including non-mucinous adherent components, is required. Multiplex genotypic analysis may also prove a useful additional tool.

Background

With the greater use of high-resolution computed tomography (CT) in lung cancer screening, there has been a considerable rise in the apparent incidence of pulmonary nodules, particularly synchronous multifocal lung cancers (SMLCs)[1, 2]. The presence of more than one pulmonary nodule raises a critical clinical question: do such nodules arise from the same clone or do they represent multiple lung cancers with independent lineages? As it guides therapeutic management, distinguishing properly between synchronous multifocal primary lung cancers (SMPLCs) and intrapulmonary metastasis (IM) is vital. Yet this remains challenging[3].

The original diagnostic criteria for SMLCs was defined by Martini and Melamed in 1975. They mainly use clinical and pathological characteristics, such as histological classification, tumor location, presence/absence of adenocarcinoma in situ (AIS), and lymph node metastases. A prime limitation of their approach is that only the major histological tumor type, such as adenocarcinoma or squamous cell carcinoma, are considered. However, the morphology of the primary and metastatic foci should be consistent, without considering the histological subtypes and molecular features of the tumor[4].

Revisions to the histologic classification published by the World Health Organization (WHO) in 2015 described five main morphologically-distinct subtypes of invasive adenocarcinoma: lepidic, acinar, papillary, micropapillary, and solid[5]. Solid and micropapillary subtypes usually have a poor prognosis, while the lepidic subtype usually has a more favorable outcome[6, 7].

Molecular typing has also become much more prevalent in pathological diagnosis. Various oncogene mutations are implicated in lung cancer. They play an often decisive role in targeted therapy, and are likewise important in determining tumor origin.

It is still widely thought that while comprehensive histological assessment (CHA) has defects, it can nonetheless largely address the issue of cancer classification satisfactorily. However, there is still no consensus on proper classification of SMLC. The overall landscape of SMLC lesions remains poorly defined.

To address these various problems, a cohort of 21 patients with SMLCs were reviewed retrospectively. The histological subtypes, immunohistochemical phenotypes, and molecular characteristics were determined. Exploring pathological and genetic features in each individual tumor lesion seemingly provides important additional information relevant to distinguishing properly between SMPLC and IM.

Material And Methods

Patients

Patients included in this study underwent pulmonary resection at the Affiliated Suzhou Hospital of Nanjing Medical University between January 2018 and December 2019. A total of 50 distinct lung tumors removed from 21 patients with at least two lesions were selected for histopathologic examination. No patient had received treatment before surgery. Clinical demographic characteristics (age, sex, smoking history, tumor size, pathologic classification, histological subtypes, clinical stages, and lymphatic metastase) were obtained from electronic medical records.

Histologic assessment

Specimens were fixed using 10% neutral buffered formalin, paraffin embedded, and then stained with hematoxylin and eosin (HE). Evaluation of histological subtypes of lung adenocarcinomas was undertaken using 2015 WHO classification criteria[5]. Each histological component was recorded in 5% increments. Tumors were categorized by their main pattern: lepidic predominant adenocarcinoma (LP), acinar predominant adenocarcinoma (AP), papillary predominant adenocarcinoma (PP), micropapillary predominant adenocarcinoma (MP), and solid predominant adenocarcinoma (SP).

CHA and nonmucinous lepidic components were used as complementary approaches. SMLC can thus be considered as SMPLC if the following four criteria are met: (1) different in major histology subtype; (2) at least one tumor diagnosed as AIS; (3) low grade lepidic componentpresent in all tumors; (4) similar major histology subtype, but different in other histology subtypes.

Genomic DNA extraction and Gene Mutation Analysis

DNA was extracted from sections of the FFPE. Target tumor lesions and control tissues were evaluated by pathologists. A DNeasy Blood and Tissue Kit was used to isolate the genomic DNA (Qiagen, Hilden, Germany). Gene mutations (EGER, KRAS, BRAF, NRAS, ALK, ROS1, RET, HER2, PIK3CA) were analyzed using an amplification refractory mutation system (ARMS) with a gene mutations detection Kit (Amoy Diagnostics Co, Xiamen, China).

Statistical analysis

Statistical analyses and data visualization were performed using IBM SPSS Statistics version 22.0 (IBM SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics of SMLC patients

Clinical characteristics of the 21 patients are summarized in Table 1. There were 10 men and 11 women. The mean age was 60.5 years, ranging from 32 to 87 years. Among the 21 patients, 16 had two lesions, two had three lesions, and three patients had four lesions. Tumors occurred in different lobes in 10 patients. Lymph node metastases were present in four patients. 50 tumors were used in this study: nine located in the left lung, and 41 in the right lung. There was one adenosquamous carcinoma and 49 adenocarcinomas, including one mucinous adenocarcinoma. Tumor diameter ranged from 0.5 to 4 centimeters.

Morphological and immunohistochemical assessment

According to the Martini-Melamed classification, 8 cases were considered SMPLC. In case 1, the histological types of the two lesions were different: adenocarcinoma and adenosquamous carcinoma (Figure 1). In case 8, the histological types were adenocarcinoma and mucinous adenocarcinoma respectively (Figure 2). In case 2, both lesions were AIS (Figure 3). In the other 5 patients, lesions were located in different lung lobes, and no lymph node or distant metastasis was present.

According to the refined standard, nine of 13 cases, originally classified as IM, were SMPLC. Three of these cases (cases 9 to 11) contained two lesions, one of which was AIS. There were three and four lesions in cases 12 and 13; each lesion contained either a nonmucinous lepidic component or was just AIS. The histological subtypes of multiple lesions were all different in the remaining four cases (Figure 4).

The other four cases were classified as IM due to lymph node metastasis. Overall, of the 21 tumor pair comparisons, 17 (81%) were independent primaries and four (19%) related metastases.

Immunohistochemical testing showed the adenocarcinoma components were positive in TTF-1, NapsinA, and CK7 and negative in CK5/6 and p40. The Ki-67 index was different; it was only related to the histological subtypes of a lesion, and was not related to the specific case or whether the lesion was primary.

Mutational profiling

Fifty lung carcinomas from 21 patients were investigated. They were screened for mutations in EGFR, KRAS, BRAF, NRAS, ALK, ROS1, RET, HER2, and PIK3CA using ARMS. Thirteen patients with at least one sample had a point mutation or rearrangement. Of the 50 successfully tested carcinomas, EGFR mutations were identified in 16 cases (32%; two exon 18, four exon 19, two exon 20, eight exon 21) and KRAS mutations in two (4%; two exon 2). No BRAF, NRAS, ALK, ROS1, RET, HER2 and PIK3CA mutations were found. No mutation was identified in 32 of the 50 (64%) screened tumors.

Eight patients were diagnosed as SMPLC according to the Martini-Melamed criteria (cases 1-8). Among these, three oncogenes were wild-type (case1-3), and the lesions were the same. The remaining 5 patients had different gene mutations.

According to CHA, cases 9 to 17 can be interpreted as SMPLC. Cases 9 to 11 all contained two lesions, one of which is AIS. Both lesions of case 9 were wild-type. The other two cases had different molecular characteristics. In

case 10, the AIS had a KRAS mutation, while no mutation was identified in the solid lesions. In case 11, EGFR mutations were found in both lesions but their specific sites were different.

In cases 12 and 13, the multiple foci were either *in situ* carcinoma or contained non-mucinous lepidic components. Case 12 had three lesions, including two mutations in EGFR 21 exon (L858R), while the AIS lesion was wild-type EGFR. Case 13 had four lesions, of which three lesions had mutations at different sites within the EGFR gene, and one lesion was wild type, suggesting different origins for the multi-lesion clones.

Not all lesions in cases 14-17 had lepidic components, but those without were of different histological subtypes. Only two lesions of case14 had incongruent molecular characteristics. One of the two lesions was wild-type and the other had mutations in EGFR exon 18 (G719X). In cases 15-17, as no driver gene mutations were detected, the clonal origin of the multiple lesions could not be determined.

Cases 18-21 were diagnosed as IM due to lymph node metastasis. In case 18, both lesions were wild-type. Both lesions of case 19 had a L858R mutation in exon EGFR21. The right middle lesion of Case 20 was an EGFR exon 18 (G719X) mutation. The lower lesion was wild-type. Case 21 had a KRAS (G12DS) mutation in the right middle lesion, while the lower nodule was wild-type.

In seven of the 17 SMPLC cases, there were no mutations in any lesion. Seven patients had a different mutation status in different lesions. In the other 3 cases, mutations in driver genes were observed in some lesions, but not all. See Figure 5.

Discussion

Pathological assessment of multiple pulmonary nodules is crucial when distinguishing SMPLC from IM. Differentiation of biologically-unrelated SMPLC from IM leads to accurate prognosis and helps guide treatment. The Martini-Melamed criteria were of limited use as they considered only the major histologic tumor types: adenocarcinoma versus squamous cell carcinoma. Accurate histological evaluation should also include the relative proportion of each histologic subtype.

In our previous study, 164 patients with multifocal lung cancer were grouped and their outcomes analyzed. We found no significant difference in overall survival or in disease-free survival between patients grouped according to Martini-Melamed criteria. Survival was only related significantly to tumor size, suggesting this classification system is of limited prognostic value in SMLC[8]. Andrew *et al*. had an international panel of lung pathologists conduct a detailed assessment of histological characteristics using the criteria of Martini and Melamed. They reported an agreed set of specific features: histological subtypes (predominant and minor histologic patterns), size and pleomorphism of the nucleus, acinar structure formation, nucleoli size, and pathological mitosis rates[9]. They did not compare their results to molecular cloning or immune indicators. Studies indicate the whole spectrum of adenocarcinoma with lepidic components (AIS or lepidic predominant adenocarcinoma) gives a favorable prognosis[10]. Thus, except for AIS, minimally invasive adenocarcinoma (MIA) and lepidic growth-dominated invasive adenocarcinoma should be considered as unrelated primary tumors. According to Ning *et al*., nonmucinous lepidic components with mild nuclear atypia (NLCMA) are signs suggesting primary lesions. They sought to confirm this conjecture by analyzing retrospectively 116 lesions from 54 patients using a combination of the CHA and NLCMA criteria, with statistical results indicating a significant difference in disease-free survival after grouping[11].

Pathologists view similar histological patterns as only relative arguments in favor of homologous tumor sources. In evaluating multifocal lung cancer, pathologists should seek an appropriate balance between molecular detection and histological features of the tumor. Recently, several studies have emerged which use different molecular biology techniques to analyze SMLC. These include comparative genomic hybridization (CGH), DNA microsatellite analysis, and next-generation sequencing (NGS)[12-14]. Generally, tumors with similar molecular characteristics are believed to be IM with a monoclonal origin. Discordant tumors are thought to be independent primary tumors. Discrepancies between histopathological and molecular SMPLC classification in various cohorts range between 18% and 30%[15].

Many studies have defined tumors with specific driver mutations as monoclonal[16]. Driver mutations in clinical applications can determine tumor lineages as successfully as histological and clinical reviews, but may lead to misclassification in challenging cases[17-19]. We must be extremely careful in our interpretation.

First, the same genetic mutation should occur in morphologically different lesions[20]. Another problem is the heterogeneity of mutations in primary tumors and metastatic lesions, particularly EGFR and KRAS, with misclassification ranging from 0 to 45%[21-22]. Moreover, driver mutations also occur in normal-appearing lung in para-cancer tissues[23]. Second, data shows known driver mutations cannot be detected in approximately 50% of lung cancers, and thus cannot provide useful predictions[24-26]. Third, multiple driver mutations in a single NSCLC tumor occurs rarely, and it is difficult to extrapolate from a single mutation in a single case.

It is clear that the similarity of gene mutation lineages in different lesions, including the same mutation or wild-type, does not necessarily indicate a clonal relationship. Likewise, different gene mutation lineages among multiple lesions does not indicate different primary origins.

Even integrating clinical assessment, histology, and the presence of driver mutations is not sufficient to predict accurately if multifocal tumors have the same origin[27]. More objective methods and more data are required to address this problem.

We collected 21 cases of SMLC and analyzed their histological subtypes. The cohort includes explicit SMPLC cases conforming to the Martini-Melamed criteria, cases with non-mucinous adherence component as defined by the improved criteria, and explicit cases of IM. Their molecular properties were analyzed further, indicating eight cases had no driver gene mutation, and whose clonal origin could not be determined. Of the eight cases classified as SMPLC using the Martini-Melamed criteria, driver mutations were not found in three. In the other five cases, four had two lesions, and a gene mutation was identified in one foci, suggesting different clonal origins. In the remaining case, there were four lesions, in which an EGFR T790M mutation was found in one and no driver mutations detected in the other three. Using gene mutation to define clonal origin was of limited value.

In our cohort, nine cases were identified as SMPLC using our recently revised organizational standard rather than the Martini-Melamed criteria. The lesions of these cases all occurred in the same lung lobe, and not all of the lesions were AIS. However, careful analysis of histological subtypes showed all lesions to contain non-mucinous lepitic lung cancer components. Analysis of gene mutations indicated four cases had no driver mutations, and it was of limited value to determine if they were primary or not using gene mutation.

The remaining four cases were consistent with the known genetic characteristics of SMPLC: mutation sites were different or there was a mutation from wild-type in only one lesion. However, the same mutation was found in two

lesions of case 12 (EGFR 21 exon L858R). According to histomorphology, the histological subtypes of the two lesions were similar, mainly composed of lepidic and acinar type, without complex components such as papilla, micropapilla or solid pattern, making it problematic to determine the clonal origin of these lesions.

However, we note interfocal molecular characteristics may also differ in cases identified histologically as IM. The two lesions of case 18 were wild-type, and the gene mutations of both lesions of case 19 were L858R in 21 exon of EGFR. The mutation profiles of these cases suggested that the multiple lesions derived from the same clone. However, in the two other IM cases, no mutations were found in the acinar-type lesions of case 20, while EGFR gene mutations were detected in the other solid-type lesion. Only one lesion of case 21 had a KRAS mutation. Consistent with cases reported in the literature, this is indicative of clear inconsistencies between molecular and histological characteristics when studying multifocal lung cancer.

It is often difficult to determine definitively if SMLPs are independent primary or intrapulmonary metastases. The classification of difficult cases requires detailed histological analysis, molecular characterization, and often benefits from multidisciplinary discussions between oncologists, pathologists, and surgeons. The increasing popularity of NGS and more comprehensive whole exome sequencing should ultimately improve the accuracy of interpretation.

Conclusions

To conclude, our results suggest that using the presence of non-mucinous lepidic components as a sign of a primary tumor usefully complements traditional histological classification of multifocal lung cancer. Moreover, it is necessary to identify and even sequence driver mutations in each lesion. This can play a key role in staging and grading multifocal lung cancer patients, directly affecting the targeted treatment regimens. For the clinical stage assessment of patients with multifocal lung cancer and to formulate treatment plans properly, specific case-analysis and precise personalized treatment are required.

Abbreviations

AP: acinar predominant adenocarcinoma; ARMS: amplification refractory mutation system; CGH: comparative genomic hybridization; CHA: comprehensive histological assessment; CT: computed tomography; HE: hematoxylin and eosin; IM: intrapulmonary metastasis; LP: lepidic predominant adenocarcinoma; MIA: minimally invasive adenocarcinoma; MP: micropapillary predominant adenocarcinoma; NGS: next-generation sequencing; NLCMA: nonmucinous lepidic components with mild nuclear atypia; PP: papillary predominant adenocarcinoma; SMLC: synchronous multifocal lung cancer; SMPLC: synchronous multifocal primary lung cancer; SP: solid predominant adenocarcinoma

Declarations

Authors' contribution

DLZ and JHL designed the study and analyzed the data. DC analyzed the genetic mutations. MHS performed paraffin sectioning and immunohistochemical staining. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate

This publication is approved by the Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University.

Consent for publication

Consent from the patient is obtained.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Clinicopathologic characteristics of patients with SMLC

Case	Sex	Age	Tumor	Site	Size (cm)	LM	Type	Subtype(%)					Histologic type
								L	A	P	M	S	
1	F	62	01	LUL	2.5	N	AP	0	100	0	0	0	
			02	RUL	3.5		ASC	N/A	N/A	N/A	N/A	N/A	
2	F	51	01	RLL	0.6	N	AIS	100	0	0	0	0	
			02	RLL	0.4		AIS	100	0	0	0	0	
3	F	55	01	RUL	0.4	N	AIS	100	0	0	0	0	
			02	RML	0.8		LP	80	20	0	0	0	
4	F	43	01	RLL	0.3	N	AIS	100	0	0	0	0	
			02	RML	0.7		LP	60	40	0	0	0	
			03	LUL	0.5		AIS	100	0	0	0	0	
			04	LLL	0.4		AIS	100	0	0	0	0	
5	F	51	01	RML	0.6	N	AIS	100	0	0	0	0	
			02	RLL	0.5		LP	70	30	0	0	0	
			03	RLL	0.4		AIS	100	0	0	0	0	
6	F	75	01	RLL	1.5	N	AP	20	80	0	0	0	
			02	RUL	1.7		AP	0	70	20	10	0	
7	F	47	01	RLL	1.3	N	AP	0	100	0	0	0	
			02	RUL	0.5		AIS	100	0	0	0	0	
8	M	74	01	RUL	4	N	AP	0	50	0	0	50	
			02	RLL	0.7		MA	N/A	N/A	N/A	N/A	N/A	
9	F	32	01	RLL	0.8	N	AP	0	100	0	0	0	
			02	RLL	0.5		AIS	100	0	0	0	0	
10	M	78	01	RUL	2.0	N	SP	0	0	0	0	100	
			02	RUL	1.4		AIS	100	0	0	0	0	
11	M	67	01	RLL	0.9	N	AIS	100	0	0	0	0	
			02	RLL	0.8		LP	60	20	20	0	0	
12	F	61	01	RUL	1.2	N	AP	40	60	0	0	0	
			02	RUL	0.9		LP	80	20	0	0	0	
			03	RUL	0.9		AIS	100	0	0	0	0	

13	M	65	01	LUL	1.2	N	LP	40	40	10	10	0
			02	LUL	1.1		LP	80	15	5	0	0
			03	RUL	0.5		AIS	100	0	0	0	0
			04	RLL	2.0		PP	20	30	40	10	0
14	M	87	01	LLL	1	N	AP	20	50	0	0	30
			02	LLL	1.3		AP	0	90	0	0	10
15	M	50	01	LUL	0.5	N	AP	0	100	0	0	0
			02	LUL	2.0		LP	55	45	0	0	0
16	M	71	01	RUL	2	N	AP	0	60	20	0	20
			02	RUL	0.8		AIS	100	0	0	0	0
			03	RUL	0.5		AIS	100	0	0	0	0
			04	RUL	0.8		AP	0	100	0	0	0
17	M	63	01	RML	1.2	N	LP	70	30	0	0	0
			02	RML	1		SP	0	20	0	0	80
18	M	54	01	RML	1	Y	SP	0	30	30	0	40
			02	RLL	0.4		LP	90	10	0	0	0
19	F	65	01	RUL	1.2	Y	AP	0	60	40	0	0
			02	RLL	1.1		AIS	100	0	0	0	0
20	F	60	01	RLL	2.5	Y	AP	30	70	0	0	0
			02	RUL	4		SP	0	40	0	0	60
21	M	63	01	RLL	1.0	Y	SP	0	10	10	0	80
			02	RML	0.5		AP	20	80	0	0	0

Abbreviations: A, acinar; AIS, adenocarcinoma in situ; AP, acinar predominant; ASC, adenosquamous carcinoma; F, female; L, lepidic; LLL, left lower lobe; LM, Lymph node metastasis; LP, lepidic predominant; LUL, left upper lobe; M, male; M, micropapillary; MA, mucinous adenocarcinoma; N, none; P, papillary; PP, papillary predominant; RLL, right lower lobe; RML, right middle lobe; RUL, right upper lobe; S, solid; SP, solid predominant; Y, yes.

Figures

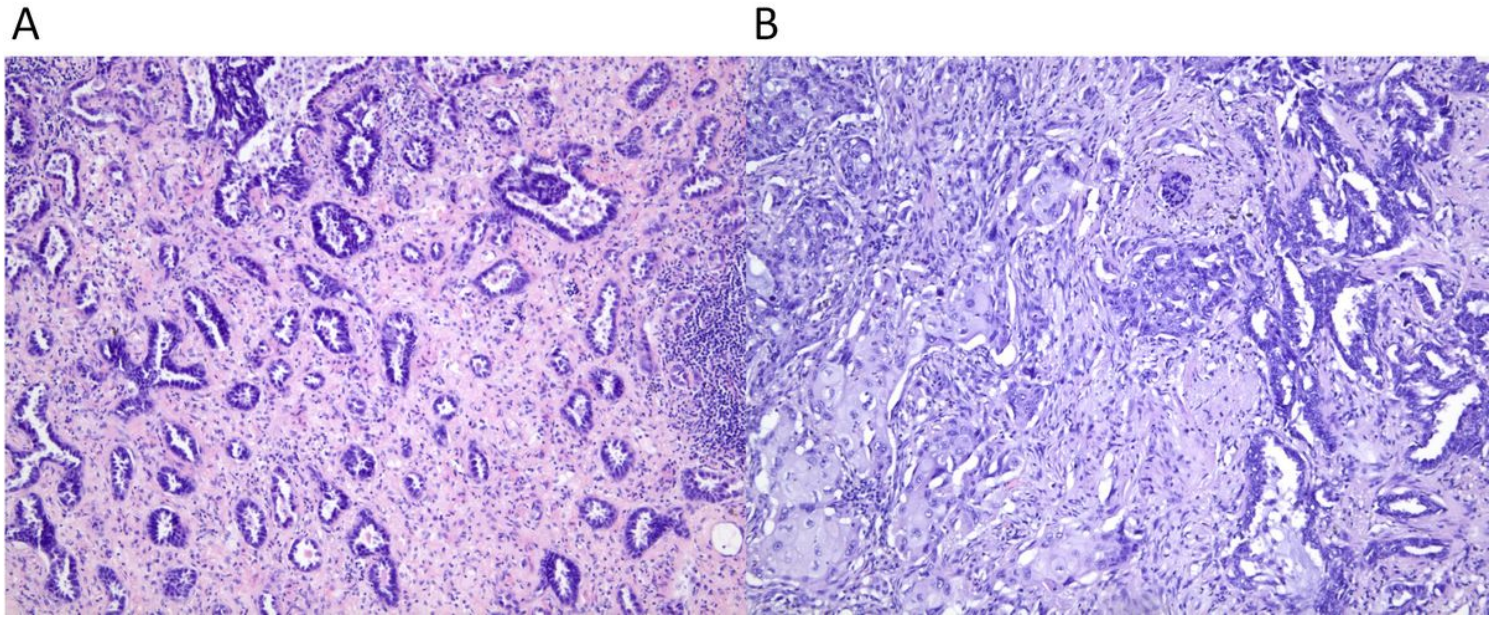

Figure 1

A case of SMPLC classified by the Martini and Melamed criteria (hematoxylin-eosin staining, 100 ×). Adenocarcinoma in the left upper lobe (A) and adenosquamous carcinoma in the right upper lobe (B) of Case 1.

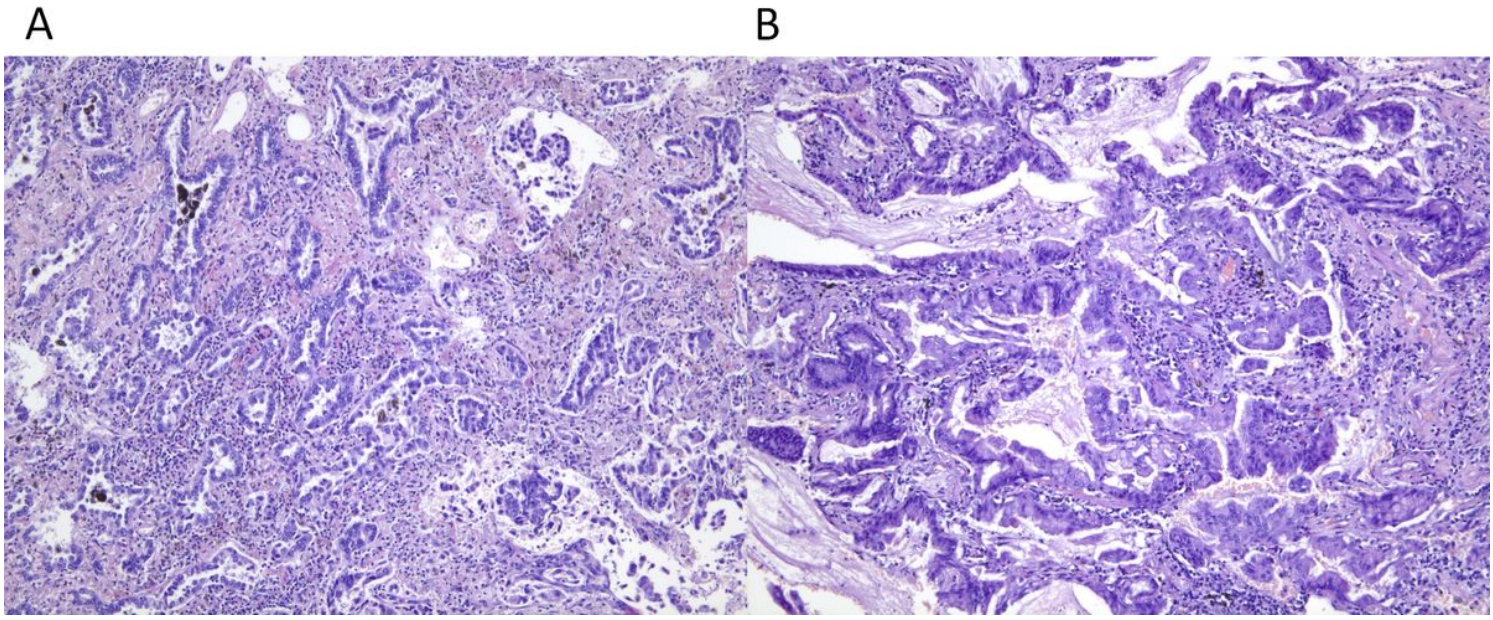

Figure 2

A case of SMPLC classified by the Martini and Melamed criteria (hematoxylin-eosin staining, 100 ×). Adenocarcinoma in the right upper lobe (A) and mucinous adenocarcinoma in the right lower lobe (B) of Case 8.

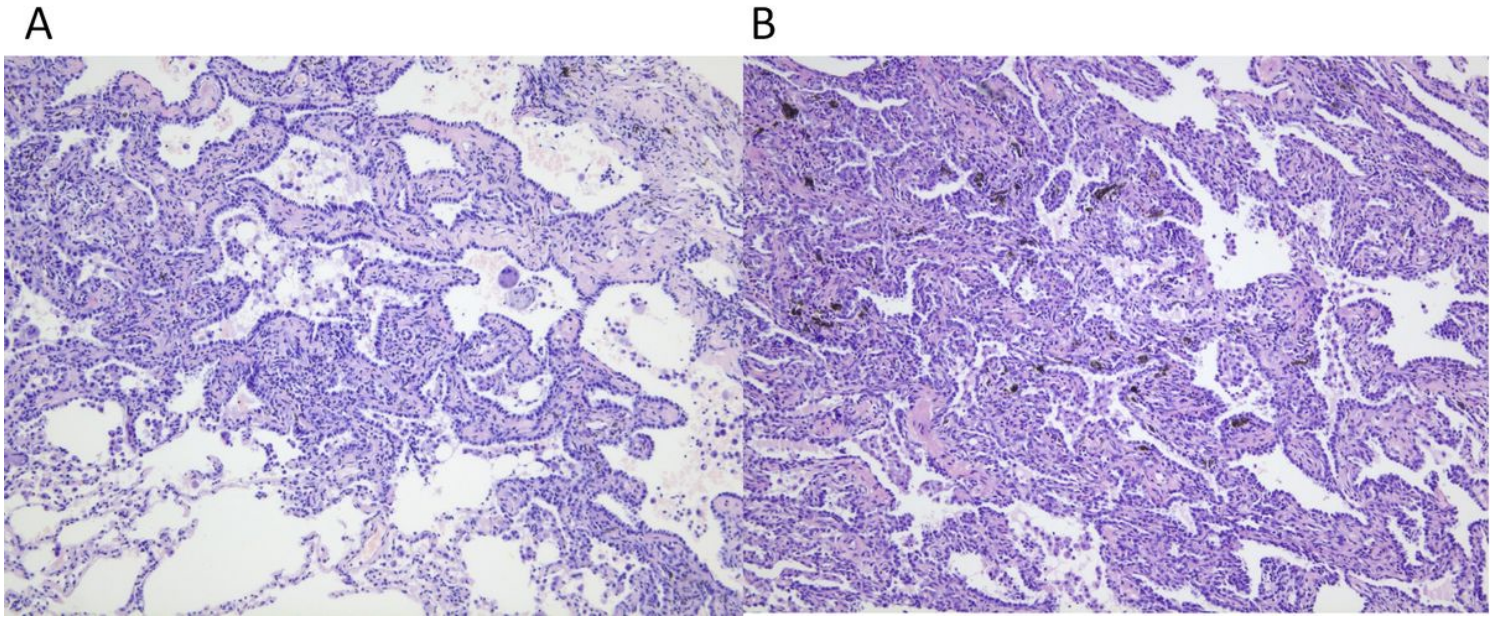

Figure 3

A case of SMPLC classified by the Martini and Melamed criteria (hematoxylin-eosin staining, 100 ×). Two separate adenocarcinoma foci in situ in the left lower lobe (A and B) of Case 2.

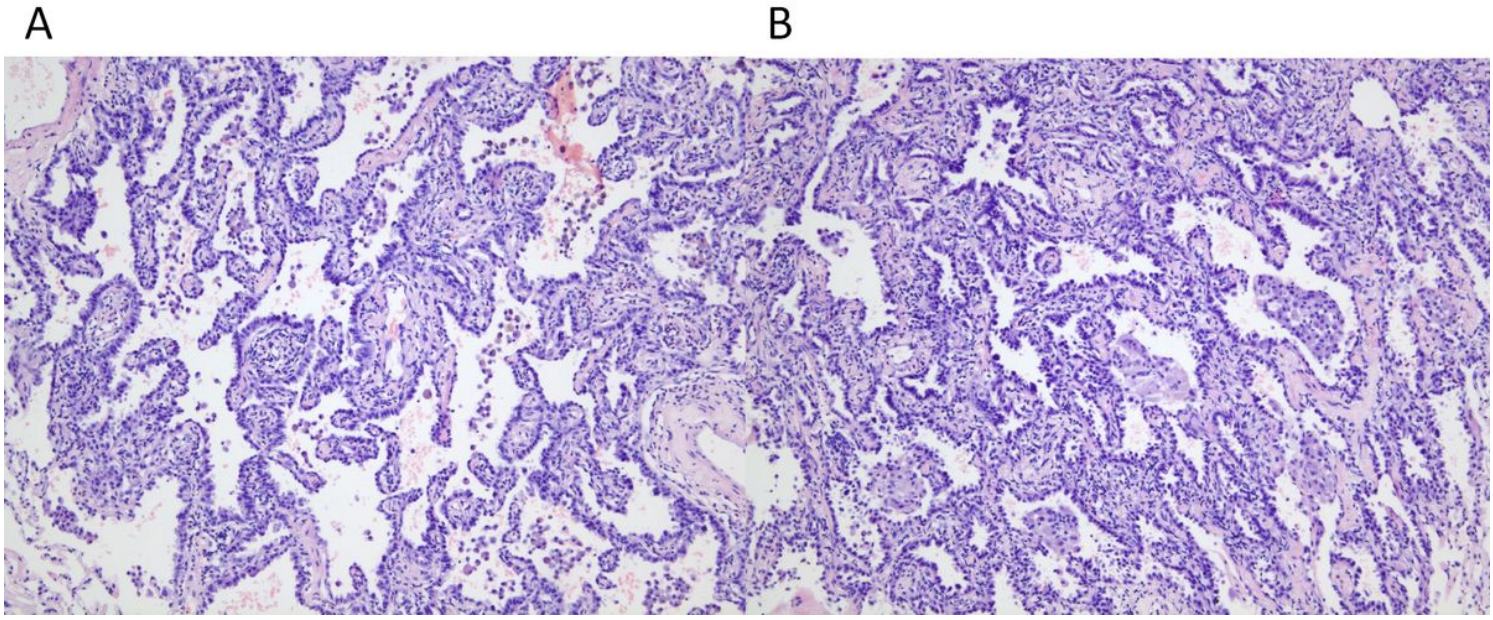

Figure 4

A case of SMPLC according to the refined standard (hematoxylin-eosin staining, 100 ×). An AIS (A) and a lepidic predominant adenocarcinoma lesion (B) in the right lower lobe of Case 11.

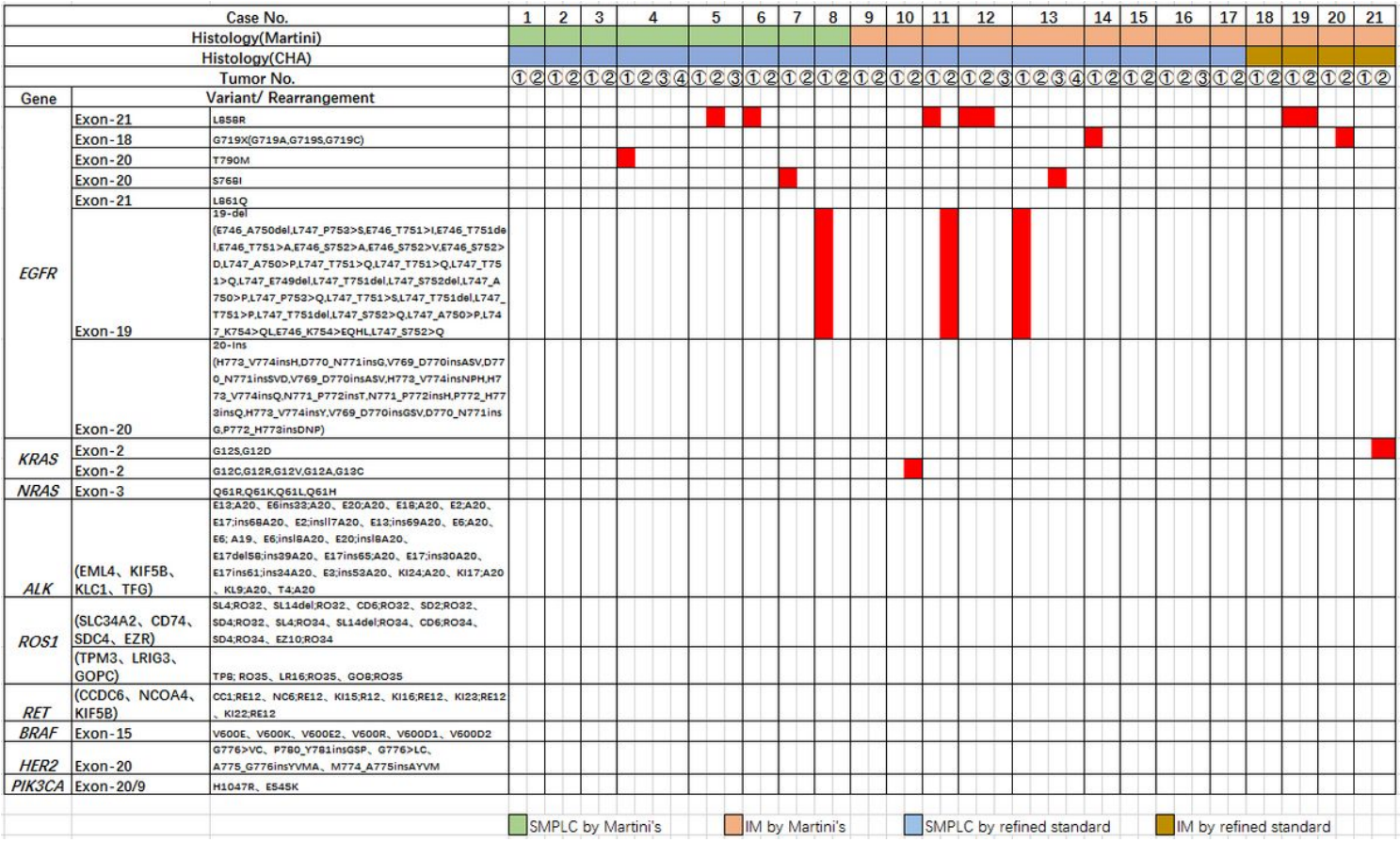

Figure 5

Mutation analyses of 50 tumors from 21 patients. The map shows the genetic mutation status of each tumor. The red columns indicate the presence of mutations. Gene mutations were identified in 18 tumors (36%).