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Distinguishing MPLCs from IPMs using NGS-based molecular algorithms and histological assessment

A systematic review and validation study

Desong Yang, MD[a,b,*], Xiaolong Sheng, MD[a,b], Lianghui Gong, MD[a,b], Xun Wu, MD[a,b], Jinming Tang, MD[a,b], Wenxiang Wang, MD[a,b]

Abstract

Distinguishing between multiple primary lung cancers and intrapulmonary metastases is crucial for staging, therapeutic planning, and prognosis. Traditional histological assessment provides a foundation for diagnosis, which can be limited when tumors showed identical or similar histological types. This systematic review and independent validation study aimed to evaluate the performance of next-generation sequencing (NGS)-based molecular algorithms alongside histological methods for the classification of multiple lung adenocarcinomas (MLAs). We conducted a literature search to identify relevant studies and selected algorithms for validation using a cohort of patients with MLAs. Our analysis included 27 patients with MLAs and compared histological assessment using Martini and Melamed criteria and comprehensive histologic assessment combined with a low-grade lepidic component (CHA & lepidic) with NGS data. We found a high consistency between CHA & lepidic and NGS-based diagnoses, although some discrepancies remained, particularly in cases with no somatic mutations or distant metastases. NGS-based molecular algorithms offer a high degree of accuracy in determining the origin of MLAs, supporting or challenging histological diagnoses. However, histological methods remain valuable, especially when NGS data are inconclusive. This study underscores the complementary nature of histology and molecular diagnostics in the precise classification of MLAs.

Abbreviations: CHA & Lepidic = CHA combined with a low-grade lepidic component, CHA = comprehensive histologic assessment, IPM = intrapulmonary metastases, MLA = multiple lung adenocarcinoma, M–M = Martini and Melamed, MPLC = multiple primary lung cancer, NGS = next generation sequencing.

Keywords: histological assessment, independent validation, molecular algorithms, multiple lung cancers, next-generation sequencing

1.Introduction

As radiological technology advances, the prevalence of diagnosing multiple lung adenocarcinomas (MLAs) has increased, accounting for about 10% of all lung cancers.[1,2] MLAs may originate either from local spread of a primary tumor through bronchoalveolar air spaces or pulmonary vasculature, or present as distinct individual primary cancers. The tumor staging, therapeutic approaches and prognosis notably differ between multiple primary lung cancers (MPLCs) and intrapulmonary metastases (IPMs).[3–5] MPLCs are potentially cured by surgical resection, while intrapulmonary metastases (IPM), indicative of an advanced, are generally managed with palliative treatment.[6–9] Therefore, distinguishing between MPLCs and IPMs is of significant clinical importance.

The differential diagnosis of MPLC and IPM mainly relies on the comprehensive histological assessment (CHA), which evaluates the predominant and minor histological subtypes along with cytological and stromal features.[10–12] If

DY and WW contributed to this article equally.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

This study was approved by the Ethics Committee of Hunan Cancer Hospital (KYJJ-2021-121). All the research procedures involving human subjects met the ethical standards of the Ethics Committee of Hunan Cancer Hospital, Changsha, China.

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[a] *The Second Department of Thoracic Surgery, The Affiliated Cancer Hospital of Xiangya School of Medicine, Hunan Cancer Hospital, Central South University, Changsha, Hunan Province, China,* [b] *Hunan Clinical Medical Research Center of Accurate Diagnosis and Treatment for Esophageal Carcinoma, Central South University, Changsha, Hunan Province, China.*

** Correspondence: Desong Yang, The Second Department of Thoracic Surgery, The Affiliated Cancer Hospital of Xiangya School of Medicine, Hunan Cancer Hospital, Central South University, 283 Tongzipo Road, Yuelu District, Changsha, Hunan Province, 410013, China (e-mail: yangdesong@hnca.org.cn).*

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the histological types of the multiple tumors differ, they are classified as MPLCs. However, when these multiple tumors are histologically identical or similar, discriminating between MPLC and IPM is challenging.[13] What's more, the workload for pathologists is substantial, and the quality of assessment varies.[14]

With the development of the gene research methods, studies have utilized molecular analysis for discrimination between MPLCs from IPMs.[2,13,15–22] Techniques such as DNA microsatellite analysis, array comparative genomic hybridization, the examination of major oncogenic driver genes (like TP53, EGFR, and KRAS) and the high-throughput next-generation sequencing (NGS) of multiple genes, have been utilized. Although these methods demonstrated the utility of molecular profiling in establishing genetic relatedness among tumors, the number of genes analyzed can impact the accuracy of differential diagnosis of MLAs. Focused sequencing is beneficial for patients in whom at least 1 abnormality is detected, but offers little for patients that are entirely wildtype or have only a common driver mutation, such as EGFR L858R or 19 del, and KRAS Q12X.[13,23]

The advent of NGS technologies has proven to be even more useful by incorporating more comprehensive sequencing to profile a broader array of genes. A study by Chang et al[13] conducted a comparison using the MSK-IMPACT panel on tumors from 60 patients with multiple tumors. Their findings supported that NGS results were discordant with histological predictions in 22% of cases, particularly in predicting IPMs, where the misdiagnosis rate reached up to 44%. Other studies using different gene panels have similarly shown that molecular analyses can determine whether pathologically similar lesions are related or genetically distinct.[21,22,24,25] Unfortunately, the classification criteria for MPLCs and IPMs are highly variable, ranging from the small-scale NGS panels to large-scale panels with more than 300 genes. In addition, patient inclusion criteria differ substantially across studies. To date, no molecular classification algorithms have been widely recommended for clinical use. Notably, none of these studies have included third-party external validation or comparisons among algorithms.

In this study, we aimed to systematically identify published NGS classification criteria for MPLCs and IPMs and to externally validate the performance and generalizability of these algorithms using a representative cohort of MLAs patients.

2.Materials and methods

2.1.Patients and samples

In this study, we collected a total of 59 tumor tissues from 27 patients diagnosed with multiple lung adenocarcinomas (MLA). Each patient presented with 2 to 4 distinct pulmonary lesions. These patients were diagnosed with MLA through surgical resection at Hunan Cancer Hospital between February 2021 and September 2022. This study was approved by the Ethics Committee of Hunan Cancer Hospital (KYJJ-2021-121). All patients provided written informed consent. Clinical features, including gender, age, and smoking history were obtained by reviewing the electronic medical records and listed in Table 1.

2.2.Histologic assessment

All lesions were reviewed by 2 experienced pulmonary pathologists according to the World Health Organization's 2015 classification guidelines,[26] depending on predominant architectural pattern. Each paired tumor was classified into MPLC or IPM according to the criteria established by Martini and Melamed (M–M)[11,27] and CHA combined with a low-grade lepidic component (CHA & lepidic).[10,12,28]

The M–M criteria for the MPLC diagnosis is as follows: lesions occur in different lobes or in different segments of the same lobe, lesions originate respectively from different kinds of carcinoma in situ and show different histological types, andno metastasis is detected in the lymphatic systems and other organs. Based on the M–M criteria, only tumors with lymphatic and/or other organs metastasis can be diagnosed as IPM.

The CHA criteria included evaluation of the percentages of histologic subtypes and histologic features such as grade, cytologic features, and stromal characteristics like collagen, inflammation, lymphoid hyperplasia and/or necrosis (Fig. 1). Specifically, tumors with low-grade lepidic component were also defined as MPLC.

2.3.DNA extraction and targeted NGS

Genomic DNA was extracted from 5 to 10 μm formalin-fixed paraffin-embedded tissue sections (FFPE) containing tumor content more than 20% using the QIAamp DNA FFPE Kit (QIAGEN, Valencia) following the manufacturer's instructions. Then DNA was quantified with the Qubit 3.0 Fluorometer and the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Inc., Waltham).

Libraries were made with the KAPA Hyper Prep kit (Roche) according to the manufacturer's protocol. Briefly, 50 to 200 ng fragmented genomic DNA underwent end-repairing, A-tailing and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads KAPA Hyper Prep kit (Roche). Hybridization-based target enrichment was carried out with customized xGen lockdown probes (808 tumor-relevant genes). The capture reaction was performed with Dynabeads M-270 (Life Technologies, Carlsbad) and xGen lockdown hybridization and wash kit (Integrated DNA Technologies, Coralville) in line with the manufacturers' protocols. Captured libraries were PCR amplified with KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington). The libraries were then sequenced on Novaseq 6000 NGS platforms (Illumina, San Diego) according to the manufacturer's instructions at an average depth of 1000× for tissue.

2.4.NGS data processing

After FASTQ file quality control with Fastp software (https://anaconda.org/bioconda/fastp), qualified reads were then mapped to reference human genome (hg38) using

Table 1

Clinicopathological characteristics of the 27 MLA patients.

Patient features	Number	%
Age (yr)		
Median	60	
Range	41–73	
Sex		
Male	12	44.4
Female	15	55.6
Smoking history		
Current/former	11	40.7
Never	16	59.3
Number of lesions		
2	23	85.1
3	3	11.1
4	1	3.7
Location of lesions		
Right upper	21	35.6
Right middle	4	67.8
Right lower	12	20.3
Left upper	8	13.6
Left lower	14	23.7

MLAs = multiple lung adenocarcinomas.

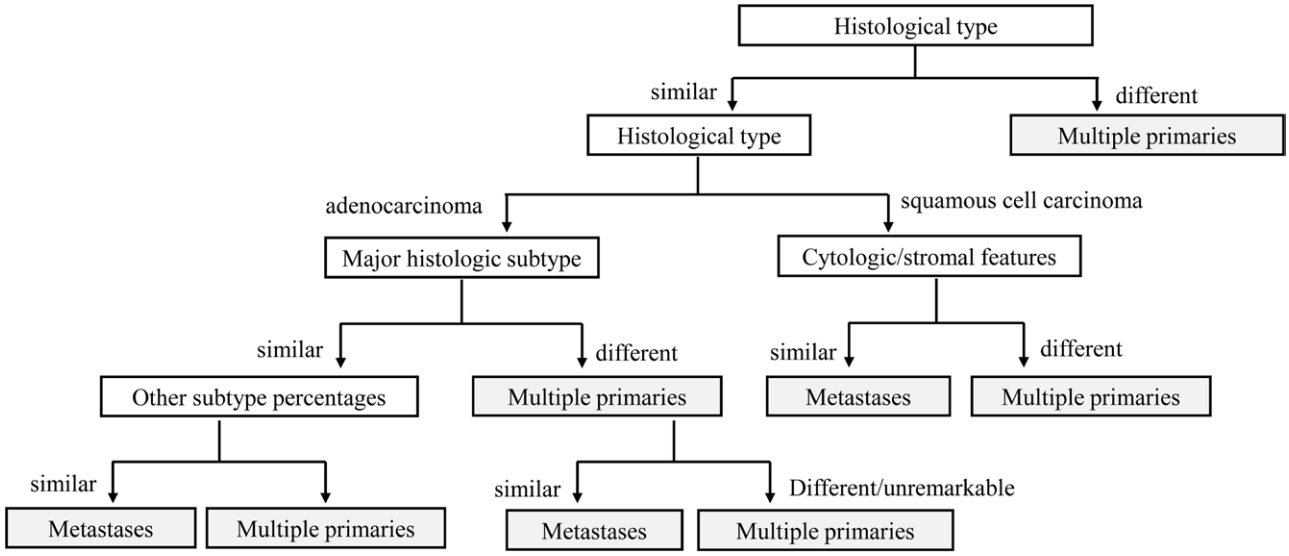

Figure 1. Comprehensive histologic assessment (CHA) methodology for NSCLC patients with multiple tumors. CHA = comprehensive histologic assessment, NSCLC = non-small cell lung cancer.

Burrows–Wheeler aligner[29] with the default parameters. Somatic mutations including single-nucleotide variations and insertion/deletion were detected using VarScan2[30] and ANOVA software using default settings. For mutations, minimum variant allele frequency was 1% and minimum variant supporting reads of 6. Single-nucleotide variations in the 1000 Genomes Project with frequency more than 1% were excluded.

2.5.Literature search strategy

Literature search via PubMed, Embase, and Web of Science databases was performed to identify studies between January 2015 and October 2023 that focused on the differential diagnostic methods of MPLC and IPM. The search strategy involved the following terms: ("multiple lung cancers" OR "multiple lung cancer" OR "multiple lung carcinoma" OR "multiple lung carcinomas" OR "multifocal lung cancers" OR "multifocal lung cancer" OR "multifocal lung carcinoma" OR "multifocal lung carcinomas") AND ("molecular" OR "next-generation sequencing" OR "genomic"). Apart from database retrieval, we manually checked reference list for all relevant papers to identify any additional studies.

The molecular algorithm inclusion criteria, all necessarily met by the selected individual studies, were subjects had pathologically confirmed MLA; the study included histology and NGS molecular algorithms as the differential diagnostic methods of MPLC and IPM, and histology assessment was the gold standard in each of the studies. Exclusion criteria were subjects did not the definitions of MPLC and IPM criteria; only difference analysis was performed rather than the construction of an integrated molecular algorithm; and case reports, meta-analysis, reviews, or comments.

Data extracted from studies included author, year of publication, number of gene panel, and classification criteria of molecular algorithms.

2.6.Statistical analysis

All analyses were performed using R version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

3.Results

3.1.Classification criteria of included studies for validation

We selected algorithms from 4 articles and reproduced their analysis methods to assess their feasibility. The criteria for determining MPLC (or SPLC) and IPM (or PM) in these studies are presented in Table 2.

3.2.Classification results

All these tumors from the 27 patients were investigated for their histological characteristic and tumor-relevant sequencing and analyzed whether the tumors from 1 individual are of common origin according to the M–M and CHA & lepidic criteria, and molecular characteristics (Fig. 2). Tumors of 21 patients obtained the same classification results in both M–M and CHA & lepidic. classification systems. The other 6 samples can be classified as MPLC or IPM by CHA & lepidic based on the histological subtype information of these tumors (Fig. 3). But M–M method cannot accurately classify them and can only be presented as inconclusive. Whatever, no opposite classification was obtained by M–M and CHA & lepidic Criteria.

Except for a few inconclusive classifications, algorithms from the 4 studies showed almost the same classification results (Fig. 2). Patients including P15 and P20 have yielded inconsistent classification results based on molecular and pathological features. P17 could not be diagnosed as MPLC or IPM due to lack of mutation. Other patients were classified consistently between pathological and molecular characteristics.

3.3.DNA sequencing enhanced the histological diagnosis of MLAs

For patients P06, P08, P09, P14 and P21, each have 2 lesions in the same lung lobe (see Table 3 and Table S1, Supplemental Digital Content, http://links.lww.com/MD/O441), with no evidence of cancer metastasis in the lymph nodes or bronchial stumps. According to the M–M criteria, these 2 tumors in each patient cannot be diagnosed as MPLC or IPM.

The subtypes and proportions of the 2 tumors in patients P06, P08, and P09 are the same, which can be considered as IPM based on CHA & lepidic. Molecular status showed multiple identical non-synonymous and synonymous mutations between the 2 tumors of each patient (Table 3 and Table S1, Supplemental Digital Content, http://links.lww.com/MD/O441), and all 4 algorithms concluded that the 2 tumors in these 3 cases are of the same origin.

In the case of P14, the 2 tumors have inconsistent subtypes. For P21, the 2 tumors have consistent subtypes but are of

Table 2

Classification criteria of the selected algorithms.

Reference	Number of genes	Classification criteria
Mansuet-Lupo et al[2]	22	1.IPM: at least 2 common mutations (EGFR 19Del or L858R and KRAS G12X), or 1 rare TP53 mutation 2.MPLCs: with different oncogenic driver mutations or without any mutation in common 3.Inconclusive: no mutations identified 4.Other: tumors shared 1 common mutation should be histologically classified
Roepman et al[22]	50	1.IPM: the exact same TP53, or other non-hotspot mutation 2.MPLC: harbored different mutations 3.Inconclusive: with only 1 same hotspot mutation; or both were wildtype
Yang et al[31]	409	1.IPMs: with identical somatic mutations 2.MPLCs: with different oncogenic driver mutations 3.Probable MPLCs: with different TP53 mutations 4.Inconclusive: could not be classified according to the above
Li et al[32]	450	1.IPM: 2 common mutations (1 driver and 1 rare), or more than 3 common mutations 2.MPLC: no common mutations and at least 1 sample with mutations among 2 samples; only 1 common mutation, or 2 common mutations (both driver or 1 driver + 1 p53) 3.Inconclusive: no mutation in all tumors; 2 common rare mutations only

IPM = intrapulmonary metastases, MPLC = multiple primary lung cancers.

the lepidic type. According to the CHA & lepidic criteria, the tumors in these 2 cases are MPLC. Molecular status showed no common mutations between the 2 tumors of each patient (Table 3 and Table S1, Supplemental Digital Content, http://links.lww.com/MD/O441), which does not support a common origin, consistent with the histological findings.

3.4.Discrepancy between molecular and histological classification

Except for patient P17, who did not have any mutations detected, all tumors histologically identified as MPLC were supported by NGS data. However, in patients with multiple tumors identified as IPM by histological assessment, there were 2 cases where the NGS sequencing results were inconsistent with this diagnosis.

Patient P15 has 2 tumors originating from the right upper lung, and this patient was found to have brain metastasis. Therefore, neither the M–M nor the CHA & lepidic criteria support a diagnosis of MPLC. However, molecular testing revealed no common mutations between the 2 lesions, with many non-common mutations present (Table 3 and Table S1, Supplemental Digital Content, http://links.lww.com/MD/O441). One tumor had 28 non-synonymous mutations, including driver mutations such as KRAS(Q61L) and TP53(R148I), while the other tumor had 48 mutations, including driver mutations like KRAS(G13C) and TP53(H47R). All 4 NGS-based algorithms support the classification of these tumors as MPLC.

Patient P20 has 2 tumors in the right lower lung and 1 tumor in the right upper lung, with no metastasis in the bronchial stump, lymphatic tissue, or other tissues. The M–M criteria determined that the tumor in the right upper lung is independently originated from the 2 tumors in the right lower lung but could not provide a diagnosis on whether the 2 tumors in the right lower lung are the same origin. All 3 tumors are adenocarcinomas primarily of the acinar type, and CHA & lepidic considered them to be IPM of the same origin. Gene sequencing found no common mutations among the 3 tumors, with some completely different non-synonymous mutations respectively (Table S1, Supplemental Digital Content, http://links.lww.com/MD/O441). One of the tumors in the right lower lung had the EGFR (E746_A750del) mutation, while the tumor in the right upper lung had the EGFR (L858R) mutation, 2 mutations that are almost never present simultaneously. Therefore, all 4 NGS-based algorithms support the classification of the tumors as MPLC.

4.Discussion

This study reviewed 2 histological classification methods, M–M[11,27] and CHA & lepidic,[10,12,28] as well as 4 molecular algorithms based on NGS data[2,22,31,32] for determining tumor origins in multiple lung cancers. M–M criteria is a simple way to determine between MPLC and IPM. However, for tumors histological features are similar, M–M criteria cannot make a judgment, and often provides conclusive results.[33–36] CHA & lepidic have increased the comparison of tumor subtypes and the use of lepidic types to help determine the correlation between multiple tumors.[33,34] This method can indeed identify more MPLCs or IPMs than the M–M criteria, at least all the multiple lung cancers mentioned in this article have been diagnosed, and most of the diagnostic results are supported by sequencing results. Especially for the multiple tumors mentioned in the article, those involving lepidic adenocarcinoma were all diagnosed with MPLC, consistent with the molecular diagnostic results, confirming the role of low-level lepidic components in MPLC diagnosis.

In some cases of multiple lung cancers located in the same lobe and with consistent subtypes, CHA & lepidic determined that they are IPM. Particularly, in 1 case, the patient even had brain metastasis. For patients with distant metastases, the multiple tumors in the same lung lobe are often considered to be metastatic cancers of the same origin from a histological perspective.[33,34] However, these histological diagnosis of IPM was not supported by sequencing data. In our cohort, 2 patients were found inconsistent of histological and molecular diagnoses. Different driver mutations, like EGFR (E746A750del) and EGFR (L858), were detected in different tumors of each patient. Multiple differences were demonstrated between tumors with such different EGFR mutations,[37,38] indicating that the MPLC results diagnosed by molecular status are more reliable. Therefore, in practical clinical situation, when determining the origin of multiple tumors of the same histological subtype occurring in the same lung lobe, it is recommended to use other methods such as gene sequencing in conjunction with the diagnosis. At the same time, even in cases with distant metastatic lesions, there is also the possibility of having multiple tumors of independent origin in the same lung lobe.

The accuracy of multiple tumor origin analysis based on NGS data is very high, which can solve many scenarios where accurate results cannot be obtained based on histology-based classification.[22] The diagnosis based on NGS data in this study is highly consistent with the diagnosis based on histology. However, it cannot be concluded that multiple tumor origin analysis based on NGS data can replace histological diagnosis. Firstly, histological analysis, especially the CHA & lepidic classification method, is convenient to operate, cost-effective, and has strong clinical application with high accuracy, which can be verified with the molecular characterization.[10] Secondly, sometimes tumor-related mutations cannot be identified from some patients. There was also a case in this study where no effective mutation was detected. This situation is mainly depending on the histological diagnosis.

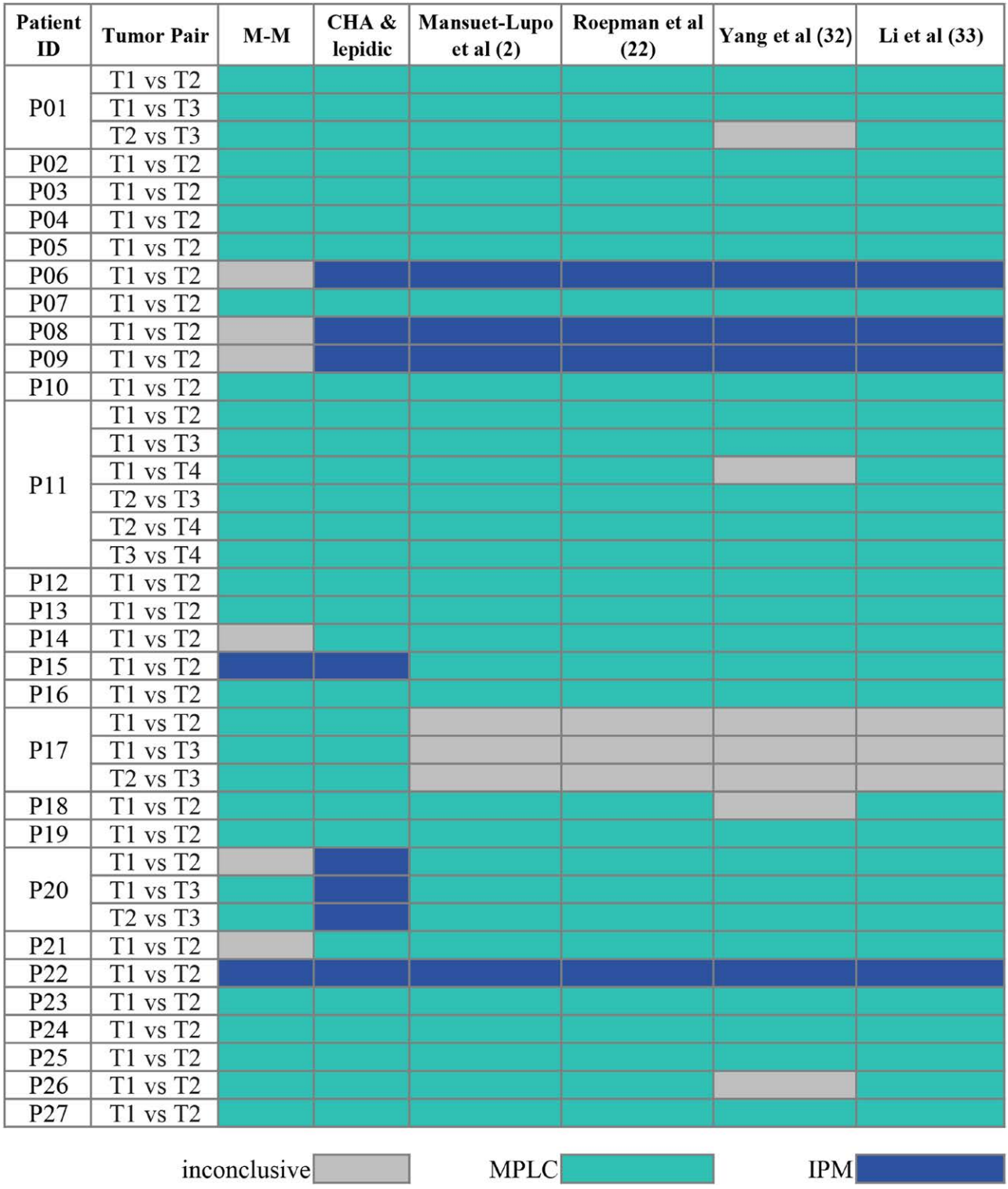

Figure 2. In our cohort, the origins of the multiple tumors within each patient were diagnosed based on pathological morphology and NGS data, respectively. Pathological morphology is based on M–M criteria and CHA & lepidic, and NGS data are based on 4 different algorithms from references.[2,22,31,32] CHA & lepidic = CHA combined with a low-grade lepidic component, IPM = intrapulmonary metastases, M–M = Martini and Melamed, MPLC = multiple primary lung cancers, NGS = next-generation sequencing.

5.Conclusion

This study reviewed diagnostic methods for multiple lung adenocarcinomas, including the histological-based M–M and CHA & lepidic methods, as well as algorithms from various literature based on NGS data. Then included an independent cohort of patients with multiple lung cancers to diagnose using these methods. The results showed that the consistency between CHA & lepidic and the diagnosis based on NGS data is very high, but both methods had their own shortcomings. The histological-based classification for the judgment of metastatic foci, as well as the classification based on NGS data for tumors without mutations, cannot achieve satisfactory results. The mutual verification and application of histology and sequencing methods can benefit more clinical patients and obtain accurate diagnosis.

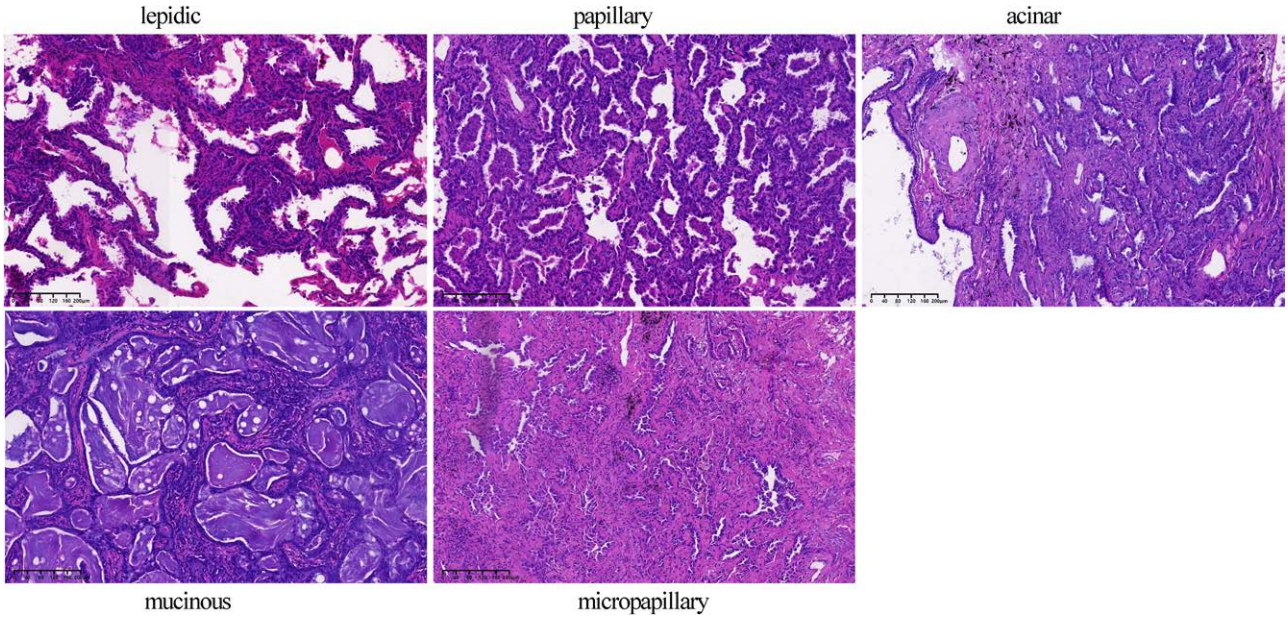

Figure 3. The 5 pathological subtypes observed in our cohort: lepidic, papillary, acinar, mucinous, and micropapillary subtypes. Scale bars are presented in the images.

Table 3

Histological and molecular characteristics of the MLAs with disputed diagnosis.

Patient	Tumor ID	Tumor location	Histological subtype	Number of common mutation	Different driver mutations
P06	T1	Right upper	Acinar	3	None
	T2	Right upper	Acinar		
P08	T1	Left upper	Acinar	21	None
	T2	Left upper	Acinar		
P09	T1	Left upper	Acinar	5	None
	T2	Left upper	Acinar		
P14	T1	Left lower	Lepidic	None	EGFR (L858R)
	T2	Left lower	Acinar		None
P21	T1	Right upper	Lepidic	None	EGFR (E746_A750del)
	T2	Right upper	Lepidic		None
P15	T1	Right upper	Solid	None	KRAS (Q61L), TP53 (R148I)
	T2	Right upper	Solid		KRAS (G13C), TP53 (H47R)
P20	T1	Right lower	Acinar	None	EGFR (E746_A750del)
	T2	Right lower	Acinar		None
	T3	Right upper	Acinar		EGFR (L858R)

MLAs = multiple lung adenocarcinomas.

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Author contributions

Conceptualization: Desong Yang, Wenxiang Wang.
Data curation: Xiaolong Sheng, Lianghui Gong.
Formal analysis: Xiaolong Sheng, Lianghui Gong.
Funding acquisition: Wenxiang Wang.
Investigation: Xiaolong Sheng, Xun Wu, Jinming Tang.
Methodology: Xun Wu, Jinming Tang.
Project administration: Desong Yang.
Resources: Desong Yang, Xiaolong Sheng.
Supervision: Wenxiang Wang.
Validation: Xiaolong Sheng.
Writing – original draft: Desong Yang.
Writing – review & editing: Desong Yang.

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