Use of MicroRNA Expression Profile For The Discrimination of Multiple Primary Lung Cancers From Intrapulmonary Metastasis

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Research article

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Abstract

Background: The increasing number of patients with multifocal lung cancers (MFLCs) complicates tumor, node, and metastasis (TNM) staging and treatment assignments. Clinical guidelines, comprehensive histologic subtyping (CHS) and molecular analyses are available to help distinguish multiple primary lung cancers (MPLCs) from intrapulmonary metastasis (IPM). However, these guidelines and criteria are difficult to apply and often lead to conflicting results.

Methods: We enrolled 44 patients with MFLCs, 32 of whom had tumors with the same histology. We assessed their statuses using existing guidelines, which led to integrated results. Twelve patients with lymph-node metastasis (LNM) were selected for comparison.

Results: The sum of the differences of five microRNAs (miRNAs) in matched tumors was evaluated by quantitative real-time polymerase chain reaction (RT-qPCR). All 12 patients with LNM showed a sum of differences of < 9 in terms of miRNA expression between primary tumors and LNM. According to the integrated analysis, seven patients were diagnosed with definite MPLCs, which were newly classified as MPLCs according to the miRNA criteria. Four patients with “no definite conclusion” according to the integrated analysis were newly classified by the miRNA criteria. The results between the integrated analysis and the miRNA criteria were contradictory in 7 patients and consistent in 14 patients.

Conclusions: A gold standard has yet to be developed for MPLCs. However, currently, an analysis of the miRNA expression profile is a useful way to distinguish MPLCs from intrapulmonary metastasis.

Background

Treatments such as surgery have significantly prolonged the survival of patients with lung tumors in the past several decades and have led to an increase in the occurrence of multifocal lung cancers (MFLCs). MFLCs include multiple primary lung cancers (MPLCs) and primary lung tumors with intrapulmonary metastatic nodules (intrapulmonary metastasis, IPM)[1]. MPLCs refer to two or more primary cancers at different sites in one or both lungs, with either consistent or different histologies; however, no association exists between the two cancers. Depending on the time interval (occurring at the same or at a different time), MPLCs are classified as either synchronous or metachronous[2], respectively. The incidence of MPLCs in lung cancer patients ranges from 0.7% to 15%[3-6].

A crucial issue regarding MFLCs is to determine whether they represent separate primary lesions or solitary pulmonary metastases, which is typically difficult to accomplish. The determination would impact stage assessment, subsequent prognosis and treatment planning (surgery for multiple primary tumors versus nonsurgical treatment for metastases)[7]. The existing diagnostic criteria for MPLCs are mostly based on clinical criteria with or without pathology and molecular data, and consequently, a subset of MFLCs cannot be correctly classified. The Martini and Melamed (MM) guideline published in 1975 [8] and the updated version of the American College of Chest Physicians (ACCP)[1] each have different classification criteria for MFLCs. Comprehensive histologic subtyping (CHS) criteria distinguish MFLCs and consider the histologic subtypes of each lung tumor[7]. Mutations in genes that code for epidermal growth factor receptor (EGFR)
and others can be used to define molecular subsets of lung tumors and can also be used as molecular criteria to establish lineage relationships of matched tumors in patients with MFLCs [7, 9].

MicroRNAs (miRNAs), which are small non-coding RNAs (approximately 22 nucleotides in length) that are found in plants, animals and some viruses, bind to target mRNAs to suppress gene expression[10, 11]. MiRNAs regulate the expression of genes that are involved in tumor development and progression. It is estimated that miRNAs regulate 30% of human genes [12, 13]. MiRNAs are involved in a variety of biologic and pathologic processes[14] and have emerged as highly tissue-specific biomarkers[15, 16]. MiRNAs also show potential applicability for the definition of cancer origins[17]. MiRNA expression profiles have been suggested to hold superior promise in tumor classification, especially in the determination of tumor origin and lineage relationships[15, 17]. MiRNAs are less susceptible to chemical or enzymatic degradation, and due to their small size and high stability, they can be easily isolated from formalin-fixed and paraffin-embedded (FFPE) tissues [18, 19]. Since miRNAs are well preserved in FFPE tissues, they are ideal candidates to serve as molecular markers that may be obtained from routinely processed materials[20, 21].

Here, we used miRNA testing to establish lineage relationships of MFLCs and to discriminate MPLCs from intrapulmonary metastasis.

**Methods**

**Patients and Clinical Characteristics**

Among patients who underwent surgical resections between October 2003 and October 2019 in the Department of Thoracic Surgery at West China Hospital and Tongji Hospital, 196 patients had more than two pulmonary nodules. Of these patients, 152 were excluded from this study due to the lack of surgical specimens (100 patients received nonsurgical treatment for the second tumor; in 36 patients, the first lesion was resected at another hospital; for 16 patients, FFPE tissues were unavailable). Finally, 44 patients (32 patients with tumors of the same histological type, 12 patients with tumors of different histological types) with MFLCs for whom paraffin sections of all tumors were available were enrolled. Patients had no extrapulmonary metastases before surgical resection. Twelve patients with lymph-node metastasis (LNM) were selected as controls for comparison of the expression of several miRNAs between the primary tumor and the metastatic lymph node lesion.

To differentiate MPLCs from metastases at the clinical level, we used both the MM criteria [8] and the ACCP criteria (3rd edition)[1] (Table 1). A tumor, node, and metastasis (TNM) stage was assigned to each patient according to the 7th revision of the International Association for the Study of Lung Cancer (IASLC)[41].

All experiments were approved by the Ethics committee at West China Hospital and Tongji Hospital, and all patients agreed to participate in the study and signed an informed consent.

**Pathologic Review and molecular analysis**
Pathologic review was conducted using serial sections from the same FFPE blocks that were processed in a similar manner according to standard histologic procedures. All specimens were stained with hematoxylin and eosin (H&E) for histological diagnosis. Routine immunohistochemical (IHC) experiments were also performed with antibodies against thyroid transcription factor 1 (TTF-1), cytokeratin 7 (CK7), novel aspartic proteinase A (Napsin A), cytokeratin 5/6 (CK5/6), p63 and p40 for histological diagnosis.

We used the CHS criteria and molecular analysis [EGFR mutation testing/anaplastic lymphoma kinase (ALK) rearrangement testing/c-ros oncogene 1 receptor tyrosine kinase (ROS-1) rearrangement testing] to define lineage relationships among MFLCs of the same histological type. Tissue specimens from these patients were formally reviewed again in a blind manner by two pathologists for tumor histopathologic subtyping according to the 2015 World Health Organization Classification of Lung Tumors[42]. The CHS criteria of lung cancer were used to evaluate the relative area of each type of distinct morphology, including adenocarcinoma (ADC; lepidic, acinar, papillary and micropapillary, solid components) and squamous cell carcinoma (SCC; keratinizing, non-keratinizing, basaloid).

Patients with the same subtype according to CHS underwent further molecular testing (EGFR/ALK/ROS-1 status). EGFR status was tested by DNA sequencing, while ALK or EML4-ALK and ROS1 rearrangements were screened by IHC and were confirmed using the break-apart fluorescence in situ hybridization assay (FISH).

The bigger tumor (synchronous MFLCs, sMFLCs) or the first tumor (metachronous MFLCs, mMFLCs) was designated Tumor 1 (T1), while the subsequent tumor was designated Tumor 2 (T2).

**RNA extraction**

Total RNA fractions including miRNAs were extracted from the FFPE tissues. A representative section from each sample was stained with H&E and reviewed to identify regions that contained more than 70% malignant epithelial cells for macrodissection. Five 8-μm-thick sections from FFPE tumor tissues were collected by dissecting away the tumor stroma using a sterile scalpel for each case. Total RNA (including miRNAs) from FFPE was deparaffinized with 100% xylene, washed with 100% ethanol, then extracted using a miRNAprep pure FFPE Kit (Tiangen Biotech Co. Ltd., Beijing, China); finally, RNA was treated with DNase I according to the manufacturer's instructions.

Total RNA quantity and quality were determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). The optical density (OD) 260/280 and OD 260/230 ratios were used for quality control.

**Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)**

Five miRNAs (hsa-miR-21-5p, hsa-miR-30a-5p, hsa-miR-126-3p, hsa-miR-129-5p, and hsa-miR-182-5p) were selected and used to evaluate the differential diagnosis of MPLCs and IPM. The levels of the five miRNAs and hsa-U6 were detected by reverse transcription and RT-qPCR. The expressions of the five miRNAs were normalized to the levels of the internal control, U6.
The first-strand cDNA was synthesized using an *Escherichia coli* Poly(A) polymerase for Poly(A) addition and oligo(dT)-universal as the primer; the reaction was performed with a miRcute miRNA first-strand cDNA synthesis kit (Tiangen Biotech).

The mature sequences of human miRNAs were as follows: 5’-UAGCUUAUCAGACUGAGUUGA-3’ (miR-21-5p); 5’-UGUAAACAUCUCUCGACUGGAAG-3’ (miR-30a-5p); 5’-UCGUACCGUGAGUAUUAAUGCG-3’ (miR-126-3p); 5’-CUUUUUGGGGUCUGGGCUUGC-3’ (miR-129-5p); 5’-UUUGGCAAUGGUAGAUCACACU-3’ (miR-182-5p). All primers were chemically synthesized by Tiangen Biotech.

RT-qPCR was performed to detect the levels of miRNAs using an SYBR Premix miRcute miRNA qPCR Detection kit (Tiangen Biotech) in a CFX96™ RT-PCR system (Bio-Rad, Hercules, CA, USA) using the following thermal cycle protocol: (1) 94 °C for 120 s; (2) 94 °C for 20 s, 60 °C for 34 s, for a total of 45 cycles.

The melting curve data were collected to verify the specificity of the PCR. All samples were analyzed in triplicate. The same reaction for U6 was set up in parallel. Negative control reactions were performed without reverse transcription and without template cDNA. The cycle threshold (Ct, the PCR cycle at which the probe signal reaches the threshold) was determined for each well. All samples presented qRT-PCR repeats within 1Ct. If the AvgCt$_{\text{miR-U6}}$ was not between 20 and 32 Ct values, the assay was repeated.

**Data analysis and statistics**

Data were analyzed by the comparative Ct method or the $\Delta \Delta$Ct method. Sample scores were obtained using $\text{Avg} (\text{Ct}_{\text{miR-X}} - \text{Ct}_{\text{miR-U6}})$, $|\Delta \Delta \text{Ct}| = |\text{Avg} (\text{Ct}_{\text{miR-X}} - \text{Ct}_{\text{miR-U6}})_{T1} - \text{Avg} (\text{Ct}_{\text{miR-X}} - \text{Ct}_{\text{miR-U6}})_{T2}|$. The primary tumor was designated Tumor 1 (T1), while the metastatic tumor in the lymph node was designated Tumor 2 (T2). With MFLCs, the bigger tumor (sMFLCs) or the first tumor (mMFLCs) was designated Tumor 1 (T1), while a subsequent tumor was designated as Tumor 2 (T2).

Data-plotting was conducted using Prism 6.0 (GraphPad, La Jolla, CA, USA) unless noted otherwise.

**Results**

**Patient Characteristics**

We identified 44 patients with MFLCs for which individual FFPE tumor samples were available. The characteristics of these 44 patients and the 12 patients with LNM are shown in Table 2. These 44 patients included 32 patients with tumors of the same histological type and 12 patients with tumors of different histological types; 31 patients had synchronous multiple lung cancers (SMLCs), while and 13 had metachronous multiple lung cancers (MMLCs). The 32 patients with tumors of the same histological type included 24 patients with SMLCs (10 men and 14 women; 47 to 80 years of age) and 8 patients with MMLCs (4 men and 4 women; 52 to 71 years of age). The time interval ranged from 15 to 76 months. The 12 patients with LNM included 7 men and 5 women who ranged in age from 50 to 80 years.

**Histopathologic Characteristics and Molecular Analysis**
All 44 patients were evaluated according to the CHS criteria in order to determine the differential diagnosis of MPLCs and IPM. Patients with the same CHS result were further analyzed according to molecular criteria. The results of the patients with tumors of the same histological type are shown in Table 3. The patient numbers are ranked by miRNA criteria results in the next section. Among the 32 patients with tumors of the same histological type, 25 patients were diagnosed with adenocarcinoma (ADC), 4 were diagnosed with squamous cell carcinoma (SCC), 2 were diagnosed with adenosquamous carcinoma (ADC-SCC) and 1 was diagnosed with small cell lung carcinoma (SCLC). In regards to ADC, 44 specimens exhibited mixed morphologies and 6 specimens exhibited a unique morphology; the subtypes of these matched tumors were the same in 21 patients but were different in 4 patients. For SCC, 8 specimens were of a unique subtype; the tumor subtypes were the same in the matched tumors from all 4 patients. For ADC-SCC, 4 specimens exhibited characteristics unique to the ADC and SCC subtypes; the characteristics of the tumor subtypes were the same in the matched tumors from both patients. In regards to SCLC, 2 specimens were of the same histologic subtype but were not characteristic of combined small-cell lung cancer (CSCLC); the characteristics of the tumor subtypes were the same in the matched tumors from this patient.

The proportions of the matched tumors with specific tumor subtypes might differ for all patients, especially in those with ADC. Different proportions of tumor subtypes were not considered in the criteria for MPLCs because a unique clone might exhibit different proportions of histologies in matched tumors from one patient.

The results of the molecular analysis (EGFR/ALK/ROS-1 status) are shown in Table 3. Of the 24 patients tested for EGFR status, 21 patients had MFLCs with the same EGFR mutations in matched tumors (11 patients showed the same EGFR mutation; 10 patients were negative for mutations). Three patients had matched tumors with different EGFR mutations. Patient 24 exhibited metachronous bilateral nodules with an L858R mutation in the first tumor and a T790M mutation in the second tumor. Patient 27 exhibited two synchronous nodules in the right lung, in which the upper lobe harbored an L858R mutation and the lower lobe was negative. Patient 31 had metachronous nodules in the right lung, in which the upper lobe contained an exon 19 deletion and the middle lobe was negative. In all 32 patients tested for ALK/ROS-1 by IHC, positive specimens were confirmed by FISH. Of the 32 patients, patient 21 had two synchronous nodules in the right lung (upper and lower lobes), which were ALK-positive; patient 11 had two synchronous nodules in the left lung (upper and lower lobes), which were ROS1-positive.

MiRNA analysis

First, the difference in the expression of the five miRNAs between the primary lung tumor (T1) and the metastatic tumor in the 12 patients with lymph node involvement (T2) was compared using the \( \Delta \Delta \text{Ct} \) method (\( |\Delta \Delta \text{Ct}| = |\text{Avg} (\text{Ct}_{\text{miR-X}} - \text{Ct}_{\text{miR-U6}})_{T1} - \text{Avg} (\text{Ct}_{\text{miR-X}} - \text{Ct}_{\text{miR-U6}})_{T2}| \)) and is shown in Table 4. The sum of the difference in the Ct values of the 5 miRNAs was the sum of \( |\Delta \Delta \text{Ct}| \). The maximum sum of \( |\Delta \Delta \text{Ct}| \) was 8.15 (patient 11) and the minimum was 1.51 (patient 1). The limit of the sum of \( |\Delta \Delta \text{Ct}| \) of tumors diagnosed as identical was < 9 (Figure 1). Therefore, if the sum of \( |\Delta \Delta \text{Ct}| \) in matched tumors is < 9, the tumors are considered identical (i.e., the primary tumor and its IPM nodule). When the sum of \( |\Delta \Delta \text{Ct}| \) in
matched tumors exceeds 9, the 2 tumors are considered to be different from each other (i.e., they are MPLCs).

All 32 patients with tumors of the same histological type were evaluated by the miRNA criteria to ensure that a second primary or IPM nodule was present. The \( \Delta \Delta Ct \) method was used, and the bigger tumor (sMFLCs) or the first tumor (mMFLCs) was designated Tumor 1 (T1), while subsequent tumors were designated Tumor 2 (T2). Patients were ranked by the sum|\( \Delta \Delta Ct \)| of the five miRNAs (Figure 2). The maximum sum of the differences in the Ct values of the 5 miRNAs was 24.98 (patient 32) and the minimum was 1.63 (patient 1). According to the miRNA criteria, 20 of 32 (62.5%) patients were diagnosed with newly classified IPM, and 12 (37.5%) patients were diagnosed with newly classified MPLCs.

**Characterization as MPLCs or IPMs**

MM criteria and ACCP criteria (3rd edition) were used to evaluate the differential diagnosis of MPLCs and IPM. Assuming that components of different histologic subtypes arise in independent clones and that the components of the same subtype would be found in metastases, we found that all 44 patients with multiple resected lung lesions were evaluated according to the CHS criteria. We assume that different mutations arise in independent clones and that the same mutation would be found in metastases. All 32 patients with tumors of the same histological type were evaluated according to molecular criteria. These patients were also diagnosed according to the miRNA criteria. Twelve patients with tumors of different histological types received a definite diagnosis of MPLCs.

According to different criteria, patients with MFLCs were determined to have MPLC or IPM (Table 5). For sMFLCs, use of the two criteria led to the same result for 20 out of 24 patients (83.3%): 7 patients were diagnosed with MPLCs, and 13 patients were diagnosed with IPM. As for the other 4 patients, 3 of them (patients 16, 19, 20) had tumors in the same lobe, which were classified as IPM by the MM criteria and as satellite lesions by the ACCP criteria. One patient (patient 27) had matched tumors with N1 lymph node involvement, which were classified as IPM by the MM criteria and as MPLCs by the ACCP criteria. For mMFLCs, use of the two criteria led to the same result for 2/8 patients (patients 13 and 31 with MPLCs). Tumors in patients 07 and 08, for whom the time intervals were within 2 years and in whom no lymph node or systemic metastasis was detected, were classified as MPLCs by the MM criteria and as IPM by the ACCP criteria. Tumors in patients 06, 24, 26, and 29, for whom the time intervals were between 2 and 4 years and in whom no lymph node or systemic metastasis was detected, were classified as MPLCs by MM criteria and as an undefined cluster by the ACCP criteria. Out of 32 patients, 17 patients were diagnosed with IPM and 15 patients were diagnosed with MPLCs (46.9%) by the MM criteria. Eighteen patients were diagnosed with IPM (satellite), 10 patients were diagnosed with MPLCs (31.3%) and 4 patients had tumors that were “not classified” by the ACCP criteria. According to the CHS criteria, 5 patients had matched tumors with different histologic subtypes, and 27 patients had matched tumors with the same histologic subtype, and patients whose tumors were of different histologic subtypes (15.6%) were diagnosed with MPLCs. According to the molecular criteria, different molecular testing results were obtained in 3 patients with matched tumors, the same result was obtained in 13 patients, negative results were obtained in 8 patients, and a molecular
analysis was not performed in 8 patients. Patients with different molecular results (9.4%) were diagnosed with MPLCs.

An integrated analysis of 32 patients was performed according to the MM, ACCP, CHS and molecular criteria (Table 5). The CHS and molecular criteria led to a definite diagnosis of MPLCs if differences were apparent between the matched tumors (7 patients: patients 24, 25, 27, 28, 29, 31, 32). If the CHS and molecular criteria achieved the same result in the matched tumors, a conclusion was made simply by the combination of the MM and ACCP criteria. A conflict between these two criteria was resolved by an integrated analysis result of no definite conclusion. Of the 32 patients, 7 were diagnosed with definite MPLCs, 16 were diagnosed with IPM, and 5 were diagnosed with MPLCs; no definite conclusion was reached in 4 patients.

With the miRNA criteria, 20 of 32 (62.5%) patients were diagnosed with newly classified IPM, and 12 (37.5%) patients were diagnosed with newly classified MPLCs (Table 5). Tumors in seven patients who were diagnosed with definite MPLCs by integrated analysis were newly classified as MPLCs. Tumors in four patients who were diagnosed with “no definite conclusion” by integrated analysis were newly classified with MPLCs (patient 26) or IPM (patients 06, 07, 08). Tumors in four patients who were diagnosed with MPLCs by integrated analysis were newly classified as IPM (patients 01, 03, 11, 13). Tumors in three patients who were diagnosed with IPM by integrated analysis were newly classified as MPLCs (patients 21, 22, 30). The same conclusion was reached in other patients using integrated analysis and miRNA criteria: 13 with IPM, 1 with MPLCs.

Discussion

The classification of additional nodules according to the seventh edition[22] of the lung cancer staging system is focused on these tumor nodules in a way that may be potentially misleading. Cohorts in the IASLC database and in other databases that contain patients with additional nodules excluded those with synchronous primary cancers as well as those with systemic spread[1]. This means that a large number of patients with MPLCs were not diagnosed appropriately.

On the basis of analyses of overall survival among pathologically staged cases, additional nodules in the same lobe are categorized as T3, nodules in the ipsilateral lobe are categorized as T4 and those in the contralateral lobe are categorized as M1 by the TNM Classification for Lung Cancer (7th edition)[23]. The IASLC Lung Cancer Staging Project (8th edition)[24] recommends that separate tumor nodule(s) in the same lobe as the primary tumor be categorized as T3, that separate tumor nodule(s) in an ipsilateral lobe different from the primary tumor be categorized as T4 and that separate tumor nodule(s) in a contralateral lobe be categorized as M1a. The new edition of the TNM Classification should discriminate MPLCs from IPM. Following resection, this kind of information could help to guide treatment decisions in terms of the need for adjuvant chemotherapy and/or closer surveillance of patients.

Patients with a primary lung cancer and additional nodules or lesions are categorized on the basis of their clinical presentations, but it is often a challenge to manage this diagnosis clinically; clinicians may be easily confused by these lesions because the terms and definitions are ambiguous[1, 25]. The distinction of
MPLCs from IPM may be easy when the matched tumors in one patient are of different histologic types, but it becomes quite difficult when the tumors are of the same histologic type. This is because the determination of whether the neoplasms are clonally related (which reflects metastatic disease) or clonally unrelated (which indicates multiple primary cancers) is impossible[1].

The MM and ACCP criteria are used to classify the matched tumors as MPLCs or IPM in a clinical setting[1, 8, 22]. As for patients with histologically matched tumors, diagnostic uncertainty occurs quite often due to the inherent limitations of the criteria: neither the MM nor the ACCP criterion incorporates information that definitively indicates MPLCs. These clinical criteria are faced with four problems, as follows: (1) Clinical criteria cannot achieve a definite diagnosis for MPLCs. With MFLCs, no matter the clinical characteristics involved, the presence of different histologic characteristics, different molecular genetic characteristics and characteristics that arise from a separate focus within a carcinoma in situ, indicates MPLCs. (2) Some conflicts have arisen between the MM and ACCP criteria, and no strong supporting evidence exists for the endorsement of either criterion. For patients with sMFLCs, matched tumors in the same lobe are classified as IPM by the MM criteria and as satellite lesions by the ACCP criteria. Matched tumors with N1 lymph node involvement are classified as IPM by the MM criteria and as MPLCs by the ACCP criteria. In patients with mMFLCs, for whom the time interval is within 2 years and in whom no lymph nodes or systemic metastasis are detected, tumors are classified as MPLCs by the MM criteria and as IPM by the ACCP criteria. Tumors in patients with a time interval between 2 and 4 years are classified as MPLCs by the MM criteria but are unable to be classified when no N2-N3 or systemic metastasis is observed; tumors are classified as IPM by the ACCP criteria when N2-N3 or systemic metastasis involvement is noted. According to the ACCP criteria, tumors in patients with a time interval above 4 years are classified as MPLCs by the MM criteria, as MPLCs when no systemic metastasis is observed and as IPM when systemic metastasis is observed. (3) The MM and ACCP criteria are highly dependent on the involvement of lymph nodes, which increases the possibility that the second nodule is a metastatic nodule, although this is not inevitable. The presence of metastatic nodes, hematogenous spread and direct dissemination are not included. (4) Some patients with MPLCs are diagnosed with advanced lung cancer according to the presence of a “metastatic nodule” by clinical criteria based on radiology. As a result, these patients are not recognized as fewer specimens are obtained.

ADCs display mixed histologic characteristics in more than 80% of cases[26], and thus, in some instances, components of each morphologic area may help to determine whether multiple tumors are clonally related[7]. It has been suggested that ADCs can be distinguished on the basis of the components of different histologic subtypes[27-29]. However, CHS is associated with four limitations: (1) CHS cannot be used on specimens obtained by fine-needle biopsy. (2) Only tumors of different subtypes will be recognized as MPLCs, and out of these, the percentage is low. (3) The lineage relationships determined may be subtype-specific, but that is not a definite result. (4) Even when histology or histology subtypes of matched tumors are different, the possibility of metastasis cannot be excluded given the heterogeneity.

Assuming that different mutations found in separate lung tumors reflect independent clones[30], we used the molecular criteria. It has been found that EGFR/KRAS mutation testing and CHS can help to differentiate multiple primary lung ADCs from metastatic lesions[7], but that study included only seven patients. The molecular criteria have three limitations: (1) In our study, tumors were analyzed for only EGFR,
ALK and ROS-1 alterations. Since EGFR/ALK/ROS-1 alterations are found in only approximately 30%-50%, 3%-5%/ and 1% of resected lung tumors, respectively, there is a significant probability that a given tumor harbors no mutations, which means that molecular testing will be uninformative for the determination of lineage relationships. Therefore, the molecular criteria are highly specific despite the variable sensitivity. (2) Even though all mutations and gene fusions were tested (i.e., EGFR, KRAS, HER2, MEK1, BRAF, PIK3CA, ALK fusion, ROS fusion, PDGFR amp), there may be other types of mutations, which will lead to classification of tumors as MPLCs, but they may not be tested. (3) The possibility of metastasis or recurrent tumors cannot be excluded in patients with MPLC with different molecular results given the heterogeneity of tumors.

The importance of histologic type and genetic fingerprinting has been questioned by recent data on tumor heterogeneity, which states that secondary tumors can either acquire de novo mutations or they can acquire mutations from primary tumors. The parallel progression model posits that metastasis occurs early in cancer development and that primary and secondary tumors evolve independently[31]. As a result, the molecular results of these matched tumors will indicate a high degree of divergence, while the divergences in the molecular results are used as “definite” criteria for MPLCs. A high degree of divergence, therefore, may lead to different results: MPLCs or metastasis as in the “parallel progression model”. Moreover, a high degree of divergence is not easily defined[32]. With a higher baseline mutational burden (the number of all mutations detected between matched tumors), a certain number of mutations that are discordant between matched tumors leads to lower degree of divergence, and vice versa. The definition of the baseline mutational burden is complicated by the presence of a large number of mutations in tumors. Moreover, the dividing line between low-degree and high-degree divergences is difficult to determine. At this point, data regarding histopathologic characteristics and molecular genetic characteristics of cancers should be considered. However, these data alone should not be regarded as definitive criteria, and the consideration of other clinical and radiographic characteristics should not be obviated. In patients with two foci typical of a primary lung cancer (either proven or suspected), the identification of these foci as second primary lung cancers (either synchronous or metachronous) should be based on the judgment of a multidisciplinary team. This team should discuss the clinical, radiologic, and (if available) tumor cytologic/histologic characteristics [1], since no gold standard exists that can discriminate MPLCs from IPM.

MiRNAs are non-protein-coding molecules with important regulatory functions, many of which are tissue- and lineage-specific. We selected five miRNAs that interacted with cancer-related genes as the miRNA criteria for the discrimination of MFLCs. To our knowledge, this is the first report to integrate miRNA expression profiling with other criteria to distinguish these types of lesions. MiRNA-21, which has been studied extensively and is overexpressed in lung cancer[33, 34], predicts poor survival[35]. MiRNA-30 is associated with poor prognosis in patients with lung cancer[36], whereas miRNA-126 might be a tumor suppressor and a potential prognostic biomarker in patients with NSCLC[37]. The miRNA-129 level is decreased in patients with NSCLC and may be related to the metastasis of NSCLC[38]. MiRNA-182 plays an oncogenic role [39] in lung cancer cell lines, and its expression in lung tumors may be a potential novel diagnostic and prognostic biomarker[40].

The difference in miRNA expression profiles is hypothesized to be large in MPLCs because the tumors have different clonal origins, but is thought to be small in IPMs because the tumors have the same clonal origin.
In practice, the sum of the differences in the Ct values of 5 miRNAs did not exceed 9 between the primary tumor and the metastatic tumors in the lymph node. This result indicates that when the sum of the differences exceeds 9, the matched tumors should be newly classified as MPLCs according to the miRNA criteria. With the miRNA criteria, 7 patients diagnosed with definite MPLCs by integrated analysis were newly diagnosed with MPLCs, which means that a divergence in the miRNA expression profile probably indicates MPLCs. Tumors in patients who were diagnosed with MPLCs by integrated analysis were newly classified as metastatic (patients 01, 03, 11, 13), which indicates similar miRNA expression profiles. Several possibilities may explain this result: the matched tumors are (1) MPLCs with similar miRNA expression profiles or (2) metastases as indicated by the miRNA criteria, while the integrated analysis result is the opposite for heterogeneous tumors. Patients diagnosed with metastasis by integrated analysis were newly diagnosed with MPLCs (patients 21, 22, 30), which indicates different miRNA expression profiles. The possibilities are as follows: the matched tumors (1) are MPLCs with different miRNA expression profiles as indicated by the miRNA criteria, while the integrated analysis result is the opposite because insufficient molecular markers were included; (2) alternatively, the matched tumors are metastases with different miRNA expression profiles because the limits of the miRNAs expression profile were generated from only twelve patients with LNM. Four patients diagnosed with “no definite conclusion” by integrated analysis were newly classified. Metastasis is defined as similar molecular results, including results from the miRNA criteria, between matched tumors. Since no gold standard exists, patients for whom inconsistent conclusions were reached with respect to the miRNA criteria and integrated analysis cannot be assigned any final diagnosis or classification.

In conclusion, miRNA expression profiling is helpful for the discrimination of MPLCs from IPM. However, because of the small sample size in this study, these findings should be validated by further studies of larger cohorts.

This study has some limitations. Our patient samples were limited since all patients were recruited from a single institution. Most patients with two foci were excluded from this study because they were considered metastases, and therefore, they did not undergo surgery for the second focus; this is also the primary problem in other studies and databases. The miRNA expression profiles in the plasma will be more meaningful as non-invasive test biomarkers for MPLCs, which will be demonstrated in future research if the role of miRNA expression profiles in tissues in a larger cohort of patients is confirmed.

(In addition, the cut-off value of the sum ΔΔCt was based on a small number of patients, and only five cancer-related miRNAs were included. Patients with SMPLCs had a better overall survival than those with intrapulmonary metastases, and with the new miRNA evaluation system, the outcomes are similar. Considering the different TNM stages of patients with SMPLCs and intrapulmonary metastases in our study, and the number of cases evaluated in this study was relatively small. Therefore, further studies with larger cohorts are necessary to validate our results.)

Conclusions
The results of this study suggest that an analysis of miRNA expression profiles is helpful in the discrimination of multiple primary lung cancers from intrapulmonary metastases. There is still no gold standard for this diagnosis, which is based on the judgment of a multidisciplinary team.

**Abbreviations**

MFLCs: multifocal lung cancers; TNM: complicates tumor, node, and metastasis; CHS: comprehensive histologic subtyping and; MPLCs: multiple primary lung cancers; RT-qPCR: real-time polymerase chain reaction; MM: Martini and Melamed; ACCP: American College of Chest Physicians; FFPE: formalin-fixed and paraffin-embedded; IASLC: International Association for the Study of Lung Cancer; ADC: adenocarcinoma; SCC: squamous cell carcinoma; FISH: fluorescence in situ hybridization assay

**Declarations**

**Acknowledgements**

All authors contributed to the data analysis, drafting, and revision of the paper and agreed to be accountable for all aspects of the work. We greatly appreciate the assistance of the staff of the Department of Thoracic Surgery, West China Hospital and Tongji Hospital and thank them for their efforts.

**Authors’ contributions**

XZ, JH, JX were involved in study conception and in the development and illustration of the methods. XZ, JH, JX were involved in the development of protocol. XZ, JX were involved in manuscript drafting. All authors have reviewed and approve the final version.

**Competing interest**

The authors declare no conflicts of interest.

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**Availability of data and materials**

Unpublished data will be available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

All experiments were approved by the Ethics committee at West China Hospital and Tongji Hospital, and all patients agreed to participate in the study and signed an informed consent.

**Consent for publication**
All participants will be informed that the result of this study will be disseminated through presentations at national and international scientific meetings, publications in peer-reviewed journals and public events involving the local administrations of the cities where the study participants are resident. And written consent will be obtained. All data related to specific patients will be de-identified.

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Tables
Table 1. Clinical criteria to define multiple primary lung cancers

### Synchronous multiple tumors, Martini and Melamed criteria

| Location                  | Same histologic characteristics | Different histologic characteristics |
|---------------------------|---------------------------------|--------------------------------------|
| Same segment              | IPM                             | MPLCs                                |
| Different segment         | Origin from carcinoma in situ and no carcinoma in lymphatics common to both, and no systemic metastasis: MPLCs | MPLCs                                |
| Different segment         | No carcinoma in situ or carcinoma in lymphatics common to both, or systemic metastasis: IPM | MPLCs                                |

### Metachronous multiple tumors, Martini and Melamed criteria

| Interval between cancers | Location              | Same histologic characteristics | Different histologic characteristics or arising separately from focus of carcinoma in situ |
|-------------------------|-----------------------|---------------------------------|-------------------------------------------------------------------------------------|
| ≥2y                     | Same lobe             | MPLCs                           | MPLCs                                                                               |
| 12y                     | Same lobe             | IPM                             | MPLCs                                                                               |
| 12y                     | Different lobe        | No carcinoma in lymphatics common to both and no systemic metastasis: MPLCs | MPLCs                                                                               |
| 12y                     | Different lobe        | Carcinoma in lymphatics common to both or systemic metastasis: IPM | MPLCs                                                                               |

### Synchronous multiple tumors, ACCP criteria

| Location                  | Same histologic characteristics | Different histologic characteristics, or different molecular genetic characteristics, or arising separately from focus of carcinoma in situ |
|---------------------------|---------------------------------|--------------------------------------------------------------------------------|
| Same lobe                 | No systemic metastases: IPM (satellite nodule) | MPLCs                                                                |
| Same lobe                 | Systemic metastases: IPM        | MPLCs                                                                |
| Different lobe            | No N2,N3 involvement and no systemic metastases: MPLCs | MPLCs                                                                |
| Different lobe            | N2,N3 involvement or systemic metastases: IPM | MPLCs                                                                |

### Metachronous multiple tumors, ACCP criteria

| Interval between cancers | Same histology | Different histologic characteristics, or different molecular genetic characteristics, or arising from a separate focus of carcinoma in situ |
|-------------------------|----------------|--------------------------------------------------------------------------------|
| ≥4y                     | No systemic metastases: MPLCs | MPLCs                                                                |
| ≥4y                     | multiple systemic metastasis: IPM | MPLCs                                                                |
| ≥2y and ≤4y             | No N2-N3 and no systemic metastases: not classified | MPLCs                                                                |
| ≥2y and ≤4y             | different lobes and present of N2,N3 involvement or multiple systemic metastasis: IPM | MPLCs                                                                |
| 12y                     | IPM             | MPLCs                                                                |

Abbreviations: ACCP, American College of Chest Physicians; MPLCs, multiple primary lung cancers; IPM, intrapulmonary metastasis
| Table 2. Characteristics of multiple lung cancers of the same histological type |
|-----------------------------|-------------------------------|-------------------------------|-----------------------------|
| Characteristics             | Synchronous multiple lung cancers | Metachronous multiple lung cancers | Lymph node metastasis |
| Patients on study, n       | 24                             | 8                             | 12                          |
| Gender, n                  |                                |                               |                             |
| Men                        | 10                             | 4                             | 7                           |
| Women                      | 14                             | 4                             | 5                           |
| Age at initial diagnosis, mean year [range] | 61.7[47-80] | 59[52-71] | 61.3[50-80] |
| Time interval, mean month [range] | - | 38.5[15-76] | - |
| Year of initial diagnosis, mean year [range] | 2011.4[2006-2015] | 2010.5[2003-2014] | 2014.6[2014-2015] |
| Tumors, n (%)              | 2                             | 23(95.8)                      | -                           |
| Histological type, n (%)   | 3                             | 1(4.2)                        | -                           |
| Adenocarcinoma             | 18(75)                         | 7(87.5)                       | 6(50)                       |
| Squamous cell carcinoma    | 4(16.7)                        | 0(0)                          | 4(33.3)                     |
| Others                     | 2(8.3)                         | 1(12.5)                       | 2(16.7)                     |
| Stage, n                   |                                |                               |                             |
| IA                         | 1                              | 3                             | 7                           |
| IB                         | 2                              | 0                             | 1                           |
| IIA                        | 2                              | 0                             | 0                           |
| IIB                        | 0                              | 0                             | 0                           |
| IIIA                       | 5                              | 3                             | 0                           |
| IIIIB                      | 5                              | 1                             | 0                           |
| IV                         | 9                              | 1                             | 0                           |

Table 3. Histopathologic Characteristics and Molecular analysis of multifocal lung cancers (MFLCs) with the Same Histological Type.
T1: The bigger tumor (synchronous multiple lung cancers) or the first tumor (metachronous multiple lung cancers), T2: subsequent tumors except T1

Abbreviations: ADC, adenocarcinoma; SSC, squamous cell carcinoma; ASC, adenosquamous carcinoma; SCLC, small cell lung cancer; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS-1, c-ros oncogene 1 receptor tyrosine kinase
| Case No. | Age, y | Sex | Histology | p-Stage | \( \Delta\Delta C_t \) (Primary tumor/Metastatic lymph node)\# | Sum of the differences* |
|----------|-------|-----|-----------|---------|-------------------------------------------------|------------------------|
|          |       |     |           |         | miR-21-5p miR-30a-5p miR-126-3p miR-129-5p miR-182-5p |                        |
| 1        | 61    | M   | SCC       | IIIa    | 0.16  0.07  0.54  0.08  0.66                        | 1.51                   |
| 2        | 62    | M   | SCC       | IIIb    | 1.17  0.07  0.07  1.17  0.12                        | 2.60                   |
| 3        | 59    | M   | SCLC      | IIIb    | 1.07  0.70  0.10  1.12  0.32                        | 3.31                   |
| 4        | 52    | F   | ASC       | IIib    | 0.70  1.19  1.15  3.32  0.48                        | 6.84                   |
| 5        | 53    | F   | ADC       | IV      | 3.10  0.22  0.65  1.18  0.82                        | 5.97                   |
| 6        | 80    | M   | ADC       | IIIb    | 2.06  0.82  0.31  1.47  0.33                        | 4.99                   |
| 7        | 56    | M   | ADC       | IIIa    | 1.95  0.36  2.02  1.95  1.48                        | 7.76                   |
| 8        | 57    | M   | ADC       | IIIa    | 2.31  0.42  1.66  1.74  0.89                        | 7.22                   |
| 9        | 72    | F   | ADC       | IIIa    | 3.31  0.38  0.31  2.45  1.29                        | 7.74                   |
| 10       | 63    | M   | SCC       | IIIa    | 0.32  0.21  0.81  0.74  0.56                        | 2.64                   |
| 11       | 71    | F   | ADC       | IIIa    | 2.58  0.73  1.78  2.01  1.05                        | 8.15                   |
| 12       | 50    | F   | SCC       | IV      | 1.02  0.16  0.32  0.86  0.51                        | 2.87                   |

\( \Delta\Delta C_t = \text{Avg}(C_{\text{miR-X}} - C_{\text{miR-U6}})_{T1} - \text{Avg}(C_{\text{miR-X}} - C_{\text{miR-U6}})_{T2} \). Differences in miRNAs expression between primary tumor (T1) and metastatic tumor in the lymph node (T2).

*Sum of the differences is the value used to add up differences of miRNAs expression among 5 miRNAs.

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; ASC, adenosquamous carcinoma; SCLC, small cell lung cancer; miR, microRNA
| Patient No | Martini and Melamed criteria | ACCP criteria | Histologic subtyping | Molecular analysis | Integrated analysis | miRNAs criterion |
|------------|-----------------------------|---------------|-----------------------|--------------------|---------------------|------------------|
| Patient01  | MPLCs                       | MPLCs         | Same                  | Not done           | MPLCs              | IPM              |
| Patient02  | IPM                         | IPM           | Same                  | Not done           | IPM                | IPM              |
| Patient03  | MPLCs                       | MPLCs         | Same                  | Not done           | MPLCs              | IPM              |
| Patient04  | IPM                         | IPM           | Same                  | Not done           | IPM                | IPM              |
| Patient05  | IPM                         | IPM           | Same                  | Not done           | IPM                | IPM              |
| Patient06  | MPLCs                       | Not classified| Same                  | Negative           | No definite conclusion | IPM              |
| Patient07  | MPLCs                       | IPM           | Same                  | Same               | No definite conclusion | IPM              |
| Patient08  | MPLCs                       | IPM           | Same                  | Not done           | No definite conclusion | IPM              |
| Patient09  | IPM                         | IPM           | Same                  | Negative           | IPM                | IPM              |
| Patient10  | IPM                         | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient11  | MPLCs                       | MPLCs         | Same                  | Same               | MPLCs              | IPM              |
| Patient12  | IPM                         | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient13  | MPLCs                       | MPLCs         | Same                  | Same               | MPLCs              | IPM              |
| Patient14  | IPM                         | IPM           | Same                  | Not done           | IPM                | IPM              |
| Patient15  | IPM                         | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient16  | IPM (satellite)             | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient17  | IPM                         | IPM           | Same                  | Negative           | IPM                | IPM              |
| Patient18  | IPM                         | IPM           | Same                  | Not done           | IPM                | IPM              |
| Patient19  | IPM (satellite)             | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient20  | IPM (satellite)             | IPM           | Same                  | Same               | IPM                | IPM              |
| Patient21  | IPM                         | IPM           | Same                  | Same               | IPM                | MPLCs            |
| Patient22  | IPM                         | IPM           | Same                  | Negative           | IPM                | MPLCs            |
| Patient23  | MPLCs                       | MPLCs         | Same                  | Same               | MPLCs              | MPLCs            |
| Patient24  | MPLCs                       | Not classified| Same                  | Different          | MPLCs; definitely | MPLCs            |
| Patient25  | MPLCs                       | MPLCs         | Different             | Same               | MPLCs; definitely | MPLCs            |
| Patient | MPLCs | Not classified | Same | Negative | no definite conclusion | MPLCs |
|---------|-------|---------------|------|----------|------------------------|-------|
| Patient26 | MPLCs | Not classified | Same | Negative | no definite conclusion | MPLCs |
| Patient27 | IPM | MPLCs | Same | Different | MPLCs | MPLCs |
| Patient28 | MPLCs | MPLCs | Different | Negative | MPLCs | MPLCs |
| Patient29 | MPLCs | Not classified | Different | Negative | MPLCs | MPLCs |
| Patient30 | IPM | IPM | Same | Same | IPM | MPLCs |
| Patient31 | MPLCs | MPLCs | Different | Different | MPLCs | MPLCs |
| Patient32 | MPLCs | MPLCs | Different | Negative | MPLCs | MPLCs |

Abbreviations: MPLCs, multiple primary lung cancers; IPM, intrapulmonary metastasis

**Figures**
Figure 1

The sum of the differences in miRNA expression in patients with primary tumors and metastatic lymph nodes.
Figure 2

The sum of the differences in miRNA expression in patients with multifocal lung cancers of the same histological type. A: newly classified intrapulmonary metastasis by the miRNA criteria, B: newly classified multiple primary lung cancers by the miRNA criteria