Method for discriminating synchronous multiple lung cancers of the same histological type
miRNA expression analysis

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Abstract
With the development of imaging technology, an increasing number of synchronous multiple lung cancers (SMLCs) have been diagnosed in recent years. Patients with >1 tumor are diagnosed with either synchronous multiple primary lung cancers (SMPLCs) or other primary tumors and metastases. Clinical guidelines, histological characteristics, and molecular diagnostics have been used to discriminate SMPLCs from other multiple lung cancers. However, there is still ambiguity in the diagnosis of SMLCs of the same histological type. We enrolled 24 patients with the same histological type of SMLCs and assessed their status using established clinical guidelines, comprehensive histologic subtyping, and molecular analysis. The sum value of the differential microRNA (miRNA) expression profiles (ΔΔCt) with matched tumors was evaluated to discriminate SMPLCs of the same histological type from metastases. Twelve patients with lymph node metastases were included for comparison, and the sum value of the ΔΔCt of 5 miRNAs between primary tumors and lymph node metastases was <9. Patients definitively diagnosed with SMPLCs by integrated analysis were also classified as SMPLCs by miRNA analysis; 6 patients showed conflicting diagnoses by integrated and miRNA analysis and 14 were given the same classification. Analysis of miRNA expression profiles is considered to be a useful tool for discriminating SMPLCs from intrapulmonary metastases.

Abbreviations: ACCP = American College of Chest Physicians, ALK = anaplastic lymphoma kinase, CHS = comprehensive histologic subtyping, CSCLC = combined small cell lung cancer, EGFR = epidermal growth factor receptor, FFPE = formalin-fixed paraffin-embedded, IASLC = International Association for the Study of Lung Cancer, KRAS = Kirsten-rat sarcoma 2 viral oncogene homolog, NSCLC = nonsmall cell lung cancer, ROS-1 = c-ros oncogene 1 receptor tyrosine kinase, SMLCs = synchronous multiple lung cancers, SMPLCs = synchronous multiple primary lung cancers, VATS = video-assisted thoracoscopic surgery

Keywords: metastases, microRNA, synchronous multiple primary lung cancers

1. Introduction
Many treatments have been developed which significantly prolong the survival of patients with lung tumors, and the number of patients presenting with multiple lung tumors has been rising. Lung cancers with multiple lesions are either synchronous (occur at the same time) or metachronous (occur at different times). The incidence of synchronous multiple lung cancers (SMLCs) in previously reported clinical studies ranges from 1% to 16%.1-4 SMLCs include synchronous multiple primary lung cancers (SMPLCs) and primary lung tumors with metastatic nodules. In particular, SMPLCs refer to 2 or more primary cancers in different sites of 1 or both lungs occurring at the same time but with no association between the 2 foci.

Whether SMLCs represent separate primary lesions impacts stage assessment and treatment planning. Nowadays, diagnosis is mostly based on clinical criteria. Martini and Melamed5 (MM) were the first to publish criteria defining multiple primary lung tumors in 1975. Since then, their criteria were updated by Antalaki et al6 in 1995 and the American College of Chest Physicians (ACCP) in 2003 (1st edition),7 2007 (2nd edition),8 and 2013 (3rd edition).9 However, these guidelines classify multiple lung tumors differently.

Girard et al10,11 proposed comprehensive histologic subtyping (CHS) criteria to classify multiple lung cancers. Studies11,12 have shown that mutations in genes encoding epidermal growth factor receptor (EGFR), among others, can be used to define clinically relevant molecular subsets of lung tumors. In other words, mutations and gene expression can be used as molecular criteria to establish lineage relationships between matched tumors. MicroRNAs (miRNAs) are small, noncoding RNAs that bind target mRNAs, thereby suppressing gene expression. MiRNAs have been shown to regulate expression of ≥30% of genes in
2. Methods

2.1. Patients and clinical guidelines

Between 2003 and 2015, 42 patients with SMLCs underwent surgical resections at the Department of the Thoracic Surgery, West China Hospital (Sichuan, China). Ten patients were excluded due to lack of FFPE tissue samples from matched tumors. Of the remaining 32 patients available for evaluation, 24 patients of the same histological type were included in our study. Twelve patients with lymph node metastases were also included as a control.

To differentiate SMPLCs from metastases at the clinical level, MM,[13,14] Antakli,[6] and ACCP criteria (3rd edition)[9] were used (Table 1). The tumor, lymph node, and metastasis (TNM) stage was assigned to each patient according to International Association for the Study of Lung Cancer guidelines (seventh revision).[23] Experiments were approved by the West China Hospital Ethics Committee, and written informed consent was obtained from all patients.

2.2. CHS criteria and molecular criteria

Serial sections from the same FFPE blocks were used for pathological review and molecular analysis. All specimens were stained with hematoxylin-eosin (HE) and subjected to routine immunohistochemical stains (TTF-1, CK7, Napsin A, CK5/6, p63, and p40) for histological diagnosis. CHS and molecular analysis of EGFR mutations, anaplastic lymphoma kinase (ALK) rearrangement, and c-ros oncogene 1 receptor tyrosine kinase (ROS1) rearrangement were used as criteria to discriminate SMPLCs from metastases. Tumor histopathological subtyping was reviewed in a blinded manner by 2 pathologists using the standard 2015 World Health Organization classification of lung tumors.[24]

CHS of lung cancer included adenocarcinoma (lepidic, acinar, papillary and micropapillary, and solid components) and squamous cell carcinoma (keratinizing, nonkeratinizing, and basaloid). For molecular analysis, EGFR status was tested using DNA sequencing, whereas an ALK fusion gene and ROS1 rearrangement were screened by immunohistochemistry and confirmed using break-apart fluorescence in situ hybridization.

2.3. MiRNA expression analysis

Total RNA, including miRNA, was extracted from FFPE tissues. A representative section from each sample was stained with HE to identify regions containing >70% malignant epithelial cells for macrodissection. Five 8-μm-thick sections of FFPE tissue samples were dissected using a sterile scalpel for each case, deparaffined with 100% xylene, and washed with 100% ethanol. Total RNA (including miRNAs) was extracted using a miRNAprep pure FFPE kit (Tiangen Biotech, Beijing, China) and treated with DNase I following the manufacturer’s instructions. RNA quantity and quality was measured by a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). The optical density ratios of 260/280 and 260/230 were used for quality control.

Five miRNAs were selected to discriminate SMPLCs from intrapulmonary metastases and were normalized to the internal

| Table 1 |
| Clinical guidelines for defining synchronous multiple primary lung cancers (SMPLCs). |
| Martini and Melamed criteria | Location | Same histologic characteristics | Different histologic characteristics |
| | Same lobe | Metastasis | SMPLCs |
| | Different lobe | Origin from carcinoma in situ, and no carcinoma in lymphatics common to both, and no systemic metastasis: SMPLCs | SMPLCs |
| | Different lobe | No carcinoma in situ, or carcinoma in lymphatics common to both, or systemic metastasis: metastasis | SMPLCs |

| Antakli criteria | Location | Same histologic characteristics | Different histologic characteristics |
| | With 2 or more (1) anatomically distinct, (2) associated premalignant lesion, (3) no systemic metastases, (4) no mediastinal spread, (5) different DNA ploidy: SMPLCs | SMPLCs |
| | With 1 or none (1) anatomically distinct, (2) associated premalignant lesion, (3) no systemic metastases, (4) no mediastinal spread, (5) different DNA ploidy: metastasis or no conclusion | SMPLCs |

| ACCP criteria | Location | Same histologic characteristics | Different histologic characteristics, or different molecular genetic characteristics, or arising separately from foci of carcinoma in situ |
| | Same lobe | No systemic metastases: satellite nodule | SMPLCs |
| | Same lobe | Systemic metastases: metastasis | SMPLCs |
| | Different lobe | No N2,N3 involvement and no systemic metastases: SMPLCs | SMPLCs |
| | Different lobe | N2,N3 involvement or systemic metastases: metastasis | SMPLCs |

ACCP = American College of Chest Physicians, SMPLCs = synchronous multiple primary lung cancers.
control, miR-U6. Mature sequences of human miRNAs were as follows:
- hsa-miR-21-5p: 5'-UAGCUUAUCAGAUCAGUUGUA-3'
- hsa-miR-30a-5p: 5'-UGUAAAACAUCCUCGACUGGAAG-3'
- hsa-miR-126-3p: 5'-UCGUACCCGUAGAUAAUUAUCCG-3'
- hsa-miR-129-5p: 5'-CUUUUUGCGUCUGGGCUUUGC-3'
- hsa-miR-182-5p: 5'-UUUGGCAAUGGUAGAACACACU-3'

First-strand cDNA was synthesized using a miRcute miRNA first-strand cDNA synthesis kit (Tiangen Biotech) with an Escherichia coli poly(A) polymerase and oligo (dT)-universal primer. All primers were chemically synthesized by Tiangen Biotech. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a SYBR Premix miRcute miRNA qPCR detection kit (Tiangen Biotech) on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA) with a thermal cycle protocol of 94°C for 120 s and 45 cycles of 94°C for 20 s and 60°C for 34 s; qRT-PCR specificity was verified by a melting curve. Samples were analyzed in triplicate, and negative control reactions without RT reaction and template were also performed. All samples represent qRT-PCR repeats within 1 threshold cycle (Ct). The assay was repeated if the average Ct of U6 was not between 20 and 32.

2.4. Statistical analysis
Data were analyzed according to the ΔΔCt method:

\[
\Delta \Delta Ct = |(Ct_{miR-X} - Ct_{miR-U6})_{T1} - (Ct_{miR-X} - Ct_{miR-U6})_{T2}| 
\]

where ΔΔCt is the fold change in expression of a given miRNA (miR-X) relative to the internal control (miR-U6) in T1 versus T2 (CtmiR-X/U6, average Ct of miR-X or U6). For lymph node metastases obtained from the 12 patients used as a control, the primary tumor was designated T1, and the metastatic tumor in the lymph node was designated T2. For SMLCs, the bigger primary tumor was designated T1, whereas subsequent tumors were designated T2. Unless noted otherwise, Prism software (version 6.0; GraphPad, La Jolla, CA) was used for data plotting.

3. Results
3.1. Patient characteristics
Twenty-four patients with SMLCs of the same histological type were included in the current study; 12 patients with lymph node metastases were included as a control. Table 2 shows the characteristics of patients with SMLCs of the same histological type and those with lymph node metastases. Patients with SMLCs included 10 men and 14 women aged 47 to 80 years old. Eighteen patients were diagnosed with adenocarcinoma, 4 with squamous cell carcinoma, 1 with adenosquamous carcinoma, and 1 with small cell carcinoma. Patients with lymph node metastases included 7 men and 5 women aged 50 to 80 years old; 6 had adenocarcinoma, 4 had squamous cell carcinoma, 1 had small cell carcinoma, and 1 had adenosquamous carcinoma. Table 2 also shows tumor location, tumor size, surgical procedures used, and the TNM stages of all patients; lobectomy was widely used with T1 of matched tumors, whereas segmentectomy and wedge resection were mostly used for T2.

| Variable | SMLCs | Lymph node metastases |
|----------|-------|-----------------------|
| Patients on study, n | 24 | 12 |
| Gender, n | | |
| Male: female | 10:14 | 7:5 |
| Age at initial diagnosis, mean year, range | 61.7 [47–80] | 61.3 [50–80] |
| Smoking, n (%) | | |
| Low | 7 (29.2) | 3 (25) |
| Histological type, n (%) | | |
| Adenocarcinoma | 18 (75) | 6 (50) |
| Squamous cell carcinoma | 4 (16.7) | 4 (33.3) |
| Others | 2 (8.3) | 2 (16.7) |
| Tumor location, n T1 T2 | | |
| Right upper lobe | 4 | 8 |
| Right middle lobe | 3 | 3 |
| Right lower lobe | 7 | 3 |
| Left upper lobe | 3 | 5 |
| Left lower lobe | 7 | 5 |
| Tumor size, cm, mean ± SD | T1 T2 | | |
| 4.7 ± 4.1 | 1.5 ± 1.1 |
| Tumor classification, n T1 T2 | | |
| T1 | 9 | 21 |
| T2 | 4 | 3 |
| T3 | 2 | 0 |
| T4 | 9 | 0 |
| N classification, n | | |
| N0 | 13 | 0 |
| N1 | 3 | 8 |
| N2 | 8 | 4 |
| N3 | 0 | 0 |
| M classification, n | | |
| M0 | 15 | 10 |
| M1a | 9 | 2 |
| M1b | 0 | 0 |
| Pleural invasion positive, n | 9 | 2 |
| Stage, n Maximum stage | | |
| IA | 1 | 0 |
| IB | 2 | 0 |
| IIa | 2 | 0 |
| IIb | 0 | 2 |
| IIIa | 5 | 6 |
| IIIb | 5 | 2 |
| IV | 9 | 2 |

SD = standard deviation, SMLCs = synchronous multiple primary lung cancers, T1 = the biggest of the matched tumors with SMLCs, T2 = the other tumor, VATS = video-assisted thoracoscopic surgery.

3.2. CHS and molecular analysis
CHS and molecular analysis results for all 24 patients are shown in Table 3. Among patients with SMLC, 18 were diagnosed with adenocarcinomas. Among the 36 specimens (2 per person), 33 had mixed subtypes, and 3 had unique subtypes. The subtypes of matched tumors were the same in 15 patients and different in 3
### Table 3

Synchronous multiple lung cancers (SMLCs) of the same histological type considered by comprehensive histological subtyping (CHS) and molecular criteria.

| Patient No | Gender | Age | Histologic characteristics | ADC | Subtypes | SCC | Molecular analysis |
|------------|--------|-----|----------------------------|-----|----------|-----|-------------------|
|            |        |     |                            |     | Lepidic  | Acinar | Papillary, micropapillary | Solid | Others | Keratinizing | Nonkeratinizing | Basaloid | EGFR | ALK | ROS-1 |
| 01         | M      | 66  | SCC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | Negative |
| 02         | M      | 62  | SCC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | Negative |
| 03         | M      | 65  | ASC                         | T1  | +        |       | +                   |       | +       | Negative | Negative | Negative |
| 04         | M      | 59  | SCC                         | T2  | +        |       | +                   |       | +       | Negative | Negative | Negative |
| 05         | M      | 62  | SCC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | Negative |
| 06         | F      | 55  | ADC                         | T1  | +        |       | +                   |       | +       | Mucinous | Negative | +           |
| 07         | F      | 58  | ADC                         | T2  | +        | +     | +                   |       | +       | Mucinous | Negative | +           |
| 08         | M      | 56  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 09         | F      | 51  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | +           |
| 10         | M      | 80  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 11         | F      | 70  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 12         | F      | 51  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 13         | F      | 58  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 14         | M      | 64  | SCC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 15         | F      | 53  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 16         | M      | 61  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 17         | F      | 53  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 18         | F      | 73  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 19         | F      | 53  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 20         | M      | 47  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 21         | F      | 72  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 22         | M      | 53  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 23         | M      | 78  | ADC                         | T2  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |
| 24         | F      | 70  | ADC                         | T1  | +        | +     | +                   |       | +       | Negative | Negative | 19-del |

**ADC** = adenocarcinoma, **ALK** = anaplastic lymphoma kinase, **ASC** = adenosquamous carcinoma, **CHS** = comprehensive histological subtyping, **EGFR** = epidermal growth factor receptor, **ROS-1** = c-ros oncogene 1 receptor tyrosine kinase, **SCC** = squamous cell carcinoma, **SCLC** = small cell lung cancer, **SMLCs** = synchronous multiple lung cancers, **T1** = the biggest of the matched tumors with SMLCs, **T2** = the other tumor.

*Patient numbers were ranked by miRNA analysis results.*
patients (patients 20, 22, and 24). Four patients were diagnosed with squamous cell carcinoma, and all had the same unique subtype of matched tumors. One patient was diagnosed with adenosquamous carcinoma and matched tumors exhibited the same subtypes. One patient had small cell carcinoma but not combined small cell lung cancer (CSCLC) exhibiting the same type.

Moreover, the EGFR status of 17 out of the 24 patients with SMLC was determined. Nine patients had the same EGFR mutation in matched tumors, 7 were mutation-negative, and 1 patient (patient 21) showed a different EGFR mutation status. All 24 patients were assayed for ALK and ROS1 status and all showed the same result (patient 8 was ROS1-positive and patient 17 was ALK-positive).

3.3. MiRNA analysis
MiRNA expression profiles between primary lung and metastatic lymph node tumors were compared and are shown in Fig. 1. The maximum sum value of the ΔCt of the 5 miRNAs was 8.15 (patient 11), and the minimum was 1.51 (patient 1). The limit of the sum value of the ΔCt of the 5 miRNAs diagnosed as identical was <9. If the sum value of the ΔCt of the 5 miRNAs in matched tumors was <9, the tumors (i.e., primary tumor and its intrapulmonary metastatic nodule) were considered to be identical. If the sum value of the ΔCt of the 5 miRNAs in matched tumors was >9, the matched tumors were different from each other (i.e., SMLCs). Figure 2 shows the maximum sum value of the ΔCt of the 5 miRNAs was 24.98 (patient 24), and the minimum was 1.63 (patient 01). According to miRNA analysis, 16 out of 24 (66.7%) patients were diagnosed with intrapulmonary metastases, and 8 (33.3%) were diagnosed with SMLCs.

3.4. Characterization of SMLCs
Patients with SMLCs were deemed to have SMLCs or metastases according to multiple clinical criteria (Table 4). MM, Antakli, and ACCP criteria (third edition) all produced the same results for 23 out of 24 patients (95.8%); 7 had SMLCs and 16 had metastases (3 with a satellite nodule). MM and Antakli criteria classified 3 patients with tumors in the same lobe (patients 12, 15, and 16) as metastases, whereas ACCP criteria classified them as satellite nodules. Matched tumors involving the N1 lymph node and no systemic metastases in 1 patient (patient 21) were classified as metastases by MM criteria and SMLCs by Antakli and ACCP criteria, leading to no definitive conclusions.

CHS showed different results for matched tumors in 3 patients (patients 20, 22, and 24), whereas 21 patients had similar CHS results. In terms of molecular criteria, 1 patient showed different results, 11 had similar results, 5 showed negative results, and 7 patients were not done. Integrated analysis of CHS and molecular results definitively diagnosed 4 patients (patients 20, 21, 22, and 24) with SMLCs.

Interestingly, 4 patients definitively diagnosed with SMLCs by integrated analysis were also classified as SMLCs by miRNA analysis. However, 3 patients (patients 01, 03, and 08) diagnosed with SMLCs by integrated analysis were also classified as metastases by miRNA analysis, whereas 3 others (patients 17, 18, and 23) diagnosed with metastases were classified as SMLCs. Tumors from the remaining 13 patients were classified as metastases by both integrated analysis and miRNA criteria.

4. Discussion
According to TNM classification (seventh edition), additional nodules in the same lobe are categorized as T3, nodules in the ipsilateral lobe are T4, and those in a contralateral lobe are M1. However, the eighth revision of the TNM classification system for lung cancer recommends that other separate tumor nodules (s) in the same lobe, different ipsilateral lobe, and different contralateral lobe be categorized as T3, T4, and M1, respectively. These updates in TNM classification make it necessary to discriminate multiple primary lung cancers from intrapulmonary metastases.

Patients with SMLCs have been shown to have better overall survival than those with intrapulmonary metastases, and the prevalence of SMLCs has increased in the past few decades. Although it may be relatively easy to diagnose multicentric
primary lung cancers in multiple lung lesions when their histological types are different, it is more difficult if they show the same histological type.

MM, Antakli, and ACCP criteria have been widely used as clinical guidelines for classifying multiple lung cancers as multiple primary lung cancers or metastases. In the current study, 7, 8, and 8 patients with SMLCs, respectively, were diagnosed with SMPLCs. Matched tumors in the same lobe were classified as metastases by MM and Antakli criteria, and as satellite nodules by ACCP criteria. Matched tumors with N1 lymph node involvement were classified as metastases by MM criteria but SMPLCs by Antakli and ACCP criteria. Based on clinical features alone, these clinical guidelines were not able to produce a definitive diagnosis. We believe this is because these clinical guidelines depend on the results of lymph node involvement and systemic metastases, which do not provide concrete evidence regarding whether multiple tumors are clonally related.

As adenocarcinomas display mixed histological characteristics in >80% of cases, it is suggested they be distinguished based on CHS. Girard et al.,[10,11] proposed that CHS may help distinguish whether multiple tumors are clonally related in some instances. Unlike molecular profiling, CHS cannot be performed preoperatively on specimens obtained from fine-needle aspirates. Assuming that different mutations found in separate lung tumors reflect independent clones,[12] molecular criteria are used. Only EGFR, ALK, and ROS1 were included in our study, whereas Kirsten-rat sarcoma 2 viral oncogene homolog (KRAS) and other mutations have been used in previously[11] EGFR, ALK, ROS1 are only found in about 30% to 50%, 3% to 5%, and 1% of lung tumors, respectively. Different results in matched tumors indicate useful information for SMPLCs, whereas with negative results, it might not make much sense: mutations indicate different results may not be tested.

Tumor heterogeneity indicates that tumors can acquire mutations from primary tumors, as well as acquire independent mutations. The “parallel progression model”[13,14] states that metastases occur early in cancer development, whereas primary and secondary tumors evolve independently, and the molecular results of these matched tumors will indicate a high degree of divergence.[15] Accordingly, a certain number of different mutations between matched tumors will have a low degree of divergence with a higher baseline mutational burden (total number of mutations detected between matched tumors) and vice versa. Baseline mutational burden is hard to define for tumors with a large number of mutations, making differentiation between low and high divergence ambiguous. Hence, there are no definitive diagnostic criteria for SMPLCs, generally speaking.

miRNAs are nonprotein coding molecules with important regulatory functions that may be tissue- and/or lineage-specific.[16,18] Herein, we chose 5 miRNAs previously shown to interact with cancer-related genes as diagnostic criteria for SMPLCs. For example, overexpression of miR-21 reportedly indicates poor survival in patients with lung cancers[14-18] and miR-30 has been associated with a poor prognosis.[19] On the other hand, miR-126 might be tumor-suppressive and is a potential prognostic biomarker for nonsmall cell lung cancer (NSCLC).[20] and a decrease in miR-129 levels in NSCLC appears to be related with metastases.[21] Furthermore, miR-182 has been shown to play an oncogenic role[22] in lung cancer cell lines, and its expression in tumors may be a potential novel biomarker for diagnosis and prognosis of lung cancer.[23]

MiRNA expression profiles are hypothesized to be highly divergent in SMPLCs because they have different clonal origins, whereas divergence would be low in intrapulmonary metastases of the same clonal origin. In the current study, the sum value of the \( \Delta \Delta Ct \) of the 5 miRNAs examined was <9 between the
primary tumor and metastatic lymph node. Thus, when the sum value of the ΔΔCt between the matched tumors is >9, they should be newly classified as SMPLCs.

Present results showed that 4 patients definitively diagnosed with SMPLCs by integrated analysis were also classified as SMPLCs by miRNA analysis. However, 3 patients (patients 01, 03, and 08) diagnosed with SMPLCs by integrated analysis were classified as metastases by miRNA analysis, meaning similar miRNA expression profiles were obtained for each patient. Such results either derive from the matched tumors being SMPLCs with similar miRNA expression profiles or metastases (as indicated by miRNA analysis) erroneously diagnosed as SMPLCs by integrated analyses. Accordingly, 3 patients diagnosed with metastases by integrated analysis (patients 17, 18, and 23) were classified as SMPLCs by miRNA analysis. SMPLCs classification by qRT-PCR means the tumors possess divergent miRNA expression profiles. This result could stem from matched tumors either being correctly diagnosed as metastases by integrated analysis due to insufficient miRNA sample numbers or correctly classified as SMPLCs by miRNA analysis due to a lack of molecular markers being included in integrated analysis.

Patients definitively diagnosed with SMPLCs by both integrated and miRNA analyses means divergence in http://www.ncbi.nlm.nih.gov/pubmed/26943588miRNA expression profiles probably indicate SMPLCs. Metastases are defined by similar molecular results between matched tumors, as opposed to divergence. As no “gold standard” for definitive diagnosis of primary SMPLCs exists, patients with inconsistent miRNA and integrated analysis results may not be accurately diagnosed. Nevertheless, analysis of miRNA expression profiles is helpful for discriminating SMPLCs from intrapulmonary metastases.

This study has some limitations. Our patient sample was limited as they were all recruited from a single institution. In addition, the cut-off value of the sum ΔΔCt was based on a small number of patients and only 5 miRNAs related to cancer were included. SMPLCs had better overall survival than intrapulmonary metastases, and with the new miRNA evaluation system, the results are similar. Considering 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, further studies with larger cohorts are necessary to validate our results.

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