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Comparative mutational evaluation of multiple lung cancers by multiplex oncogene mutation analysis

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In patients presenting with synchronous or metachronous multiple lung cancer (MLC), it is important to distinguish between multiple primary lung cancer (MP) and intrapulmonary metastasis (IM). The present study was aimed at investigating the mutational profiles of synchronous/metachronous MLC and to compare the classification of paired tumors by multiplex gene mutation analysis with the histopathological evaluation. We carried out targeted sequencing of 20 lung cancer-related oncogenes using next-generation sequencing (NGS) in 82 tumors from 37 MLC patients who underwent surgical resection at our department. The patients were diagnosed as MP or IM cases based on the Martini and Melamed criteria, histopathological and gene mutational evaluations. Matching mutations between paired tumors was observed in 20 (54%) patients, who were diagnosed as IM cases by mutational evaluation. Patients who could not be clearly diagnosed by histopathological evaluation were classified as equivocal cases. Among the histopathological IM cases (n = 7), six (86%) were confirmed as IM cases also by mutational evaluation, and most of the paired tumors of these cases (n = 5) harbored multiple matching mutations. Among the histopathological MP cases (n = 17), mutational evaluation yielded a discordant diagnosis in eight (47%) cases. Of these, the paired tumors of four cases harbored multiple matching mutations, suggesting that the mutational diagnosis might be more suitable in these patients. Our findings suggest that multiplex mutational analysis could be a useful complementary tool for distinguishing between MP and IM in addition to histopathological evaluation.

KEYWORDS
classification, clonality, gene mutation, multiple lung cancer, next-generation sequencing

Abbreviations: IM, intrapulmonary metastasis; MLC, multiple lung cancer; MP, multiple primary lung cancer; NGS, next-generation sequencing.

1 | INTRODUCTION

Recently, with the increasing availability of high-resolution computed tomography (CT) and high rates of CT screening for lung cancer, in addition to improvement of patient prognosis after primary lung cancer resection, the diagnostic frequency of synchronous/metachronous MLC has been increasing.[1] Determination of whether multiple lung cancer arises from the same clone (intrapulmonary metastasis) or not (multiple primary) is of crucial importance in clinical cases, as it guides the management strategy and prediction of the prognosis.

In 1975, Martini and Melamed proposed criteria for distinguishing multiple primary lung tumors from pulmonary metastases.[2] Their criteria were, however, rather empirical, not definitive proof of clonality of multiple tumors, because they were mainly based on the histological characteristics of the tumors, tumor location, interval from resection, and presence/absence of carcinoma in situ, without consideration of the biological and/or molecular features of the tumors. In lung cancer, the histological features of multiple lesions often show overlapping, especially in the case of adenocarcinomas.[3] Particularly, in such cases, it is still a challenge to distinguish between multiple primary tumors and multiple intrapulmonary metastases without information about molecular biological features.

Intratumor heterogeneity has been explained by the trunk and branch model.[4,5] In this model, early somatic events which drive tumor growth are represented in every subclone and every tumor region as trunk mutations. With development of the disease, heterogeneous somatic events are represented in the primary lesions and/or the metastatic sites as branch mutations. These "branch" alterations may induce intratumor heterogeneity. According to this theory, multiple lesions harboring the same mutations are presumed to derive from the same clone. Recent advances in molecular biology have provided several markers that can be used for clonal analysis. Several studies have shown that mutations of some driver and suppressor genes, such as *EGFR*, *KRAS* and *TP53*, are useful as clonal markers in MLC.[6,7] However, in most of these reports, only a limited number of gene mutations were analyzed, and the usefulness of mutational analysis of targeted genes to distinguish between MP and IM in patients with MLC remains controversial.

In the present study, we investigated the gene mutations of 20 targeted lung cancer-related genes in surgically resected specimens of synchronous/metachronous MLC using NGS technology. In addition, we compared our classification of paired tumors as MP or IM based on the mutational profile with the histopathological evaluation of the tumors.

2 | MATERIALS AND METHODS

2.1 | Patient selection

Subjects were patients with multiple non-small cell lung cancer (NSCLC) who underwent surgical resection synchronously or

TABLE 1 Characteristics of patients with multiple NSCLC who underwent surgical resection synchronously or metachronously

Characteristics	Total (n = 37) n (%)	Synchronous MLC (n = 18) n (%)	Metachronous MLC (n = 19) n (%)	*P*
Median age (y)(range)[a]	67 (49-88)	68.5 (51-88)	66 (49-83)	0.44
Tumor size (cm)(range)[a]	1.7 (0.5-7.5)	1.7 (0.6-5.6)	1.6 (0.5-7.5)	0.41
Gender				
Male	21 (56.8)	9 (50.0)	12 (63.2)	0.74
Female	16 (43.2)	9 (50.0)	7 (36.8)	
Clinical stage[a]				
IA	26 (70.3)	14 (77.8)	12 (63.2)	0.12
IB	8 (21.6)	2 (11.1)	6 (31.6)	
IIA	0 (0)	0 (0)	0 (0)	
IIB	2 (5.4)	2 (11.1)	0 (0)	
IIIA	1 (2.7)	0 (0)	1 (5.3)	
Histological subtype				
Adenocarcinoma	35 (94.6)	17 (94.4)	18 (94.7)	1
Squamous cell carcinoma	2 (5.4)	1 (5.6)	1 (5.3)	
Location type of paired tumors				
Ipsilateral				
Same lobe	9 (24.3)	7 (38.9)	2 (10.5)	0.11
Different lobe(s)	15 (40.5)	7 (38.9)	8 (42.1)	
Contralateral	13 (35.1)	4 (22.2)	9 (47.4)	

MLC, multiple lung cancer; NSCLC, non-small-cell lung cancer.
[a]At primary surgery.

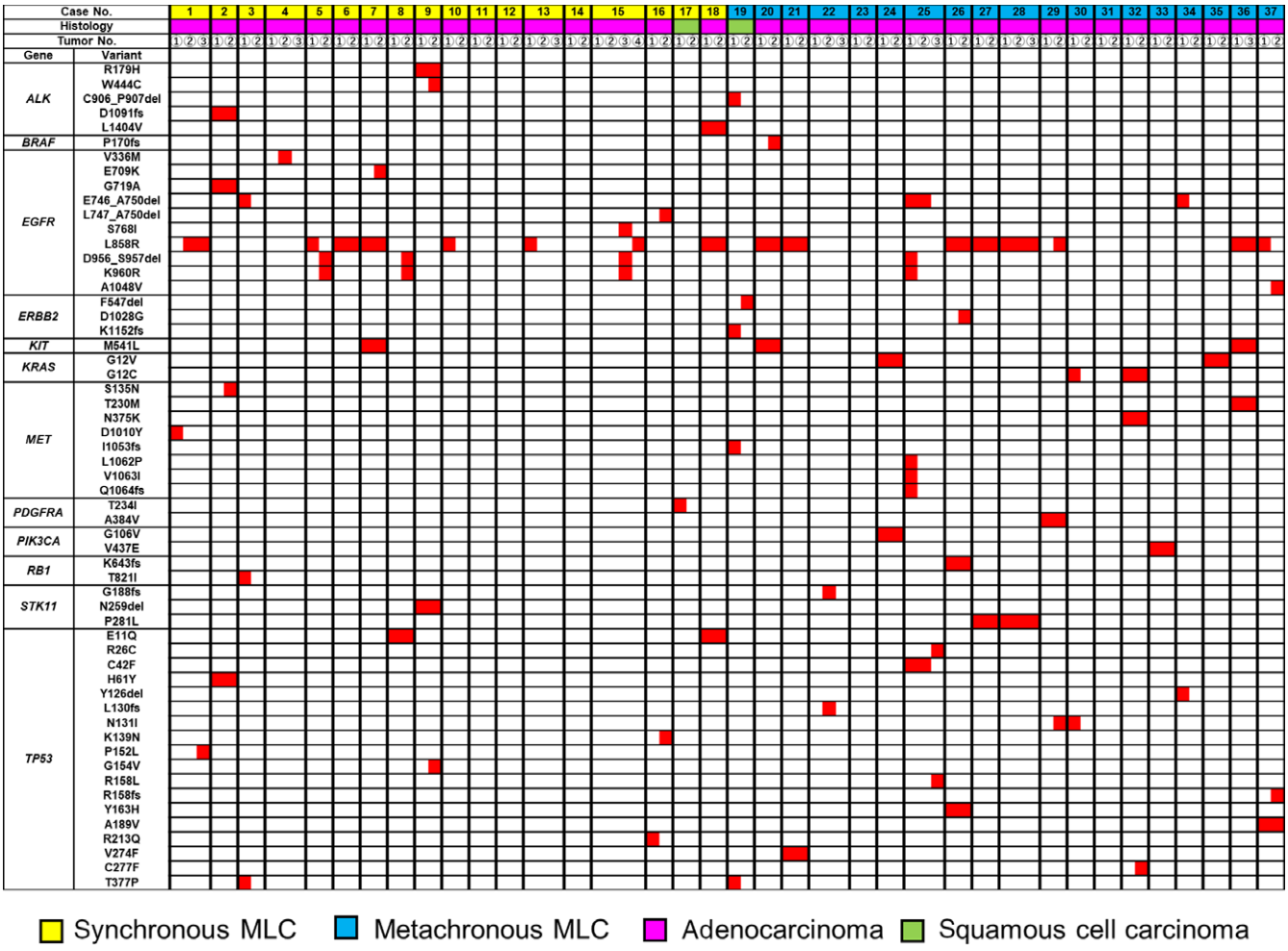

FIGURE 1 Results of mutational analyses of 82 tumors from 37 patients. Map shows the gene mutation status of each tumor. Red column indicates the presence of a mutation. Gene mutations were identified in 60 (73%) tumors, and 57 types of mutation were observed. Matching mutations between the paired tumors were observed in 20 (54%) patients

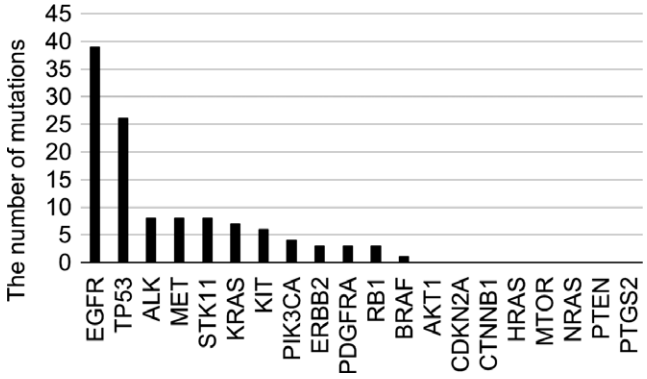

FIGURE 2 Frequencies of mutations of 20 lung cancer-related genes. *EGFR* mutations were the most frequently detected in the tumors (39 tumors; 47%), followed by *TP53* mutations (26 tumors; 32%)

metachronously at Okayama University Hospital (Okayama, Japan) between July 2002 and April 2013. According to the Martini and Melamed criteria,[2] the patients whose multiple tumors were detected at the same time or at different times were defined as synchronous MLC or metachronous MLC, respectively. Among these subjects, 37 patients in whom the paired tumors showed the same histological type were included in this study. The present study was conducted with the permission of the institutional review board and with the informed consent of each of the participants for genetic analysis of the lesions. Clinicopathological data of each patient were obtained retrospectively from the medical records. Histopathological review of the tumor specimens was conducted at Okayama University Hospital.

2.2 | Sample preparation and targeted sequencing

Genomic DNAs were isolated from fresh frozen samples using a DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands). We used a panel targeting the whole exon of 20 lung cancer-related genes (Qiagen) for NGS and carried out targeted DNA sequencing. The 20 types of lung cancer-related genes used in the panel are shown in Table S1. Sequencing libraries were prepared using GeneRead DNA Library Core/Adapter kit (Qiagen). Library samples were selected and amplified using the GeneRead Size Selection/Amp Kit

TABLE 2 Classification by Martini and Melamed criteria, histopathological and mutational evaluations

Case no.	Operation type	Mutational evaluation	Martini & Melamed criteria	Number of matching mutations	Gene
Histopathologically IM cases (n = 7)					
2	Synchronous	IM	MP	3	*ALK, EGFR, TP53*
9	Synchronous	IM	IM	2	*ALK, STK11*
17	Synchronous	MP	MP	0	–
24	Metachronous	IM	MP	2	*KRAS, PIK3CA*
25	Metachronous	IM[a]	MP[a]	2	*EGFR, TP53*
26	Metachronous	IM	MP	3	*EGFR, RB1, TP53*
33	Metachronous	IM	MP	1	*PIK3CA*
Histopathologically MP cases (n = 17)					
1	Synchronous	IM[b]	MP[b]	1	*EGFR*
3	Synchronous	MP	MP	0	–
4	Synchronous	MP	IM	0	–
6	Synchronous	IM	MP	1	*EGFR*
7	Synchronous	IM	MP	2	*EGFR, KIT*
8	Synchronous	IM	MP	1	*TP53*
10	Synchronous	MP	MP	0	–
11	Synchronous	MP	MP	0	–
12	Synchronous	MP	IM	0	–
13	Synchronous	MP	MP	0	–
14	Synchronous	MP	MP	0	–
16	Synchronous	MP	MP	0	–
20	Metachronous	IM	MP	2	*EGFR, KIT*
23	Metachronous	MP	MP	0	–
27	Metachronous	IM	MP	2	*EGFR, STK11*
28	Metachronous	IM	MP	2	*EGFR, STK11*
29	Metachronous	IM	MP	1	*PDGFRA*
Histopathologically equivocal cases (n = 13)					
5	Synchronous	MP	MP	0	–
15	Synchronous	MP	MP	0	–
18	Synchronous	IM	MP	3	*ALK, EGFR, TP53*
19	Metachronous	MP	MP	0	–
21	Metachronous	IM	MP	2	*EGFR, TP53*
22	Metachronous	MP	MP	0	–
30	Metachronous	MP	MP	0	–
31	Metachronous	MP	MP	0	–
32	Metachronous	IM	MP	2	*KRAS, MET*
34	Metachronous	MP	MP	0	–
35	Metachronous	IM	MP	1	*KRAS*
36	Metachronous	IM	MP	3	*EGFR, KIT, MET*
37	Metachronous	IM	MP	1	*TP53*

Hyphen indicates that no matching mutation was observed between the paired tumors.
IM, intrapulmonary metastasis; MP, multiple primary lung cancer.
[a]Tumor No. 1 vs No. 2.
[b]Tumor No. 2 vs No. 3.

(Qiagen) and subsequently quantified using a GeneRead Library Quant Array (Qiagen). NGS sequencing was carried out using MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA) and MiSeq Desktop Sequencer (Illumina), in accordance with the manufacturer's recommendation. Data analysis was carried out using GeneRead Targeted Exon Enrichment Panel Data Analysis (Qiagen). We excluded already

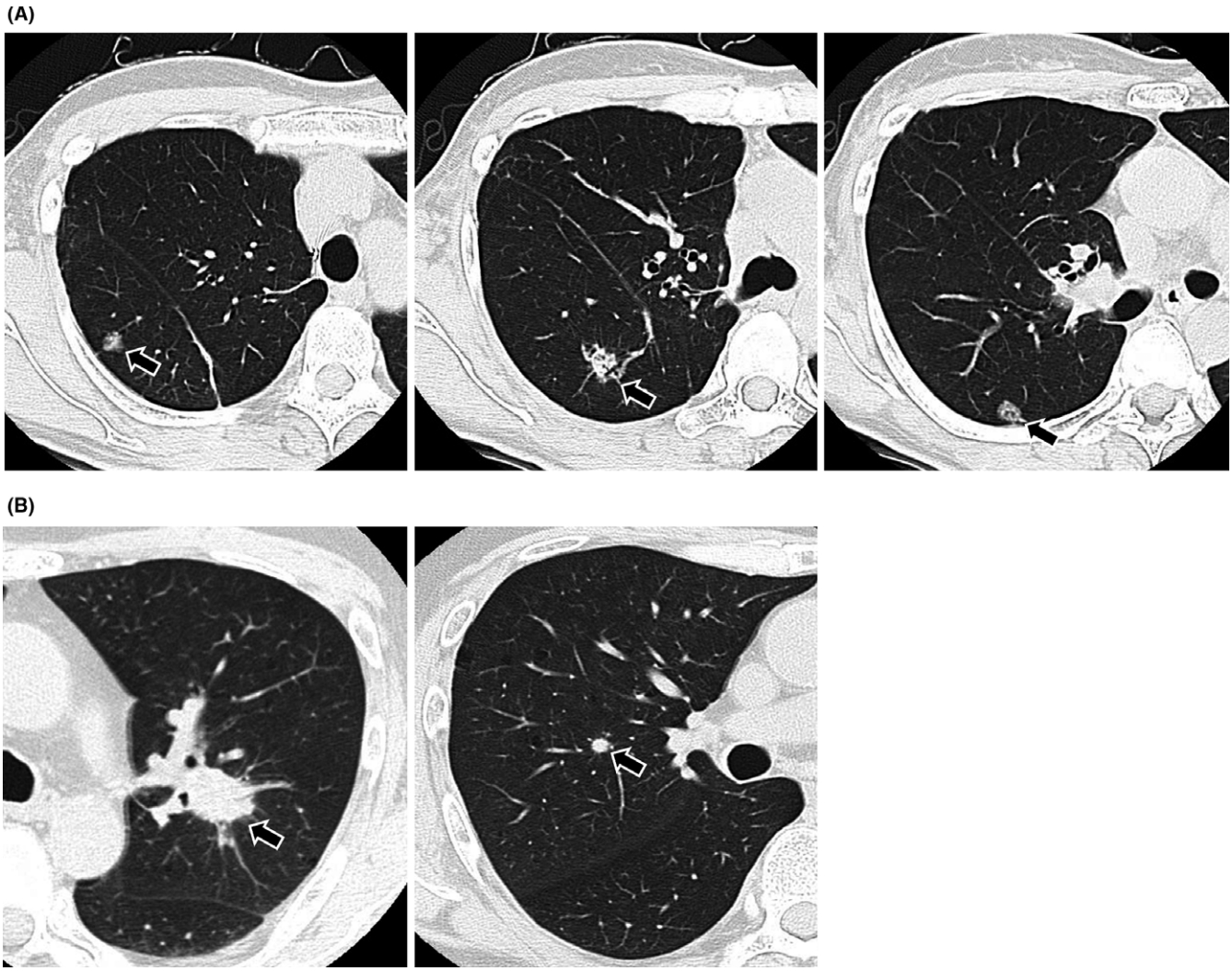

FIGURE 3 Representative computed tomography (CT) images of multiple primary lung cancer (MP) and intrapulmonary metastasis (IM) cases. Arrows indicate each tumor. A, Case 4. Part-solid nodules were observed in the superior segment of the lower lung, and segmentectomy was carried out. This case was classified as IM by Martini and Melamed criteria, whereas it was diagnosed as MP by mutational evaluation because no matching mutations were observed. B, Case 32. A solid nodule was observed in the left upper lobe, and left upper lobectomy was carried out. Three years later, a small nodule was observed in the right upper lobe, and partial resection was carried out. This case was classified as MP by Martini and Melamed criteria, whereas it was diagnosed as IM by mutational evaluation because of multiple matching mutations (*KRAS* G12C *and MET* N375K)

known single nucleotide polymorphisms (SNP) reported in the Single Nucleotide Polymorphism database (dbSNP) (https://www.ncbi.nlm.nih.gov/projects/SNP/) and only non-synonymous mutations were taken into account.

2.3 | Diagnosis by clinicopathological findings and mutational profile

We classified the paired tumors as MP or IM according to the Martini and Melamed criteria. In addition, the paired tumors were also classified as MP or IM by histopathological evaluation based on the histological subtype and the existence of in situ components by two investigators including a pathologist (S.O.). Patients in whom the diagnosis could not be made by histopathological evaluation were classified as equivocal cases. Based on the results of mutational analysis, to select the potential metastatic cases without omission, paired tumors that harbored at least one identical mutation were classified as IM, and those that did not harbor any common mutations were classified as MP.

2.4 | Statistical analysis

Overall survival (OS) was calculated from the date of primary surgery to the date of death from any cause. Survival curves were drawn using the Kaplan-Meier method, and differences between groups were compared by the log-rank test. Patients who were lost to follow up were censored on the date of last contact/follow up. Student's *t* test was applied to compare the scores between two independent groups. Fisher's exact test was applied to compare the ratios between two groups. All statistical analyses were carried out

using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander with additional statistical functions frequently used in biostatistics.[8]

3 | RESULTS

3.1 | Patient characteristics

We summarize the patient characteristics in Table 1. Thirty-seven patients with MLC, consisting of 18 patients with synchronous and 19 patients with metachronous MLC in whom paired tumors showed the same histology, were included in this study. Of the 37 patients, 30 had two lesions, six had three lesions, and one had four lesions. Therefore, a total of 82 lesions were available for this study. No significant difference in age, tumor size, gender ratio, clinical stage distribution, major histological subtype, or distribution of location of the paired tumors was observed between the two groups. None of the patients had received any treatment before the primary surgery. Detailed characteristics of each patient are described in Table S2.

3.2 | Mutational profiling

Results of the mutational analysis are shown in Figure 1. Information of allele frequency (AF) of each genetic mutation is listed in Table S3. Median AF was 32%, ranging from 8% to 62%. Average coverage of sequencing across all samples was 1677, which is supposed to provide high sensitivity to detect mutations at low allele frequencies due to heterogeneous or low-purity specimens. Gene mutations were identified in 59 (72%) tumors, and 57 types of mutation were identified. *EGFR* mutation was the most frequently detected (39 tumors; 48%), including the Leucine to Arginine substitution at amino acid position 858 (L858R) in 27 (33%) tumors, and exon 19 deletion in five (6.1%) tumors, followed by *TP53* mutation (26 tumors; 32%) (Figure 2).

Paired tumors showed matching mutations in 20 (54%) patients (including seven patients with synchronous MLC and 13 patients with metachronous MLC). The gene mutations were completely matched in nine (24%) patients and partially matched in the remaining 11 (30%) patients. The number of matching mutations varied from one to three, with seven patients showing one matching mutation, nine patients showing two matching mutations, and four patients showing three matching mutations. Discordant mutations were observed in 12 (32%) patients. Ten tumors from five (14%) patients showed no mutations of the targeted genes in this study.

3.3 | Clinicopathological and mutational evaluation

We classified the paired tumors as MP or IM according to the Martini and Melamed criteria, histopathological and gene mutational evaluations. Martini and Melamed criteria are described in Table S4. According to the Martini and Melamed criteria, three patients were classified as IM cases, whereas the remaining were classified as MP cases. Based on the results of histopathological evaluation, seven patients (including three patients with metachronous MLC and four patients with synchronous MLC) were diagnosed as IM cases and 17 (including 12 patients with metachronous MLC and five patients with synchronous MLC) as MP cases (Table 2), and the remaining (13 patients) were classified as equivocal cases (Table 2). Based on the mutational evaluation, 20 patients in whom the paired tumors harbored the same mutation(s) were determined as IM cases, and 17 patients in whom the paired tumors did not harbor any matching mutations were diagnosed as MP cases. Representative CT images of MP and IM cases are shown in Figure 3.

3.4 | Comparisons of the tumor classifications

Comparisons of the tumor classifications by the Martini and Melamed criteria, histopathological and mutational evaluations are shown in Figure 4. Diagnosis based on mutational evaluation was concordant with the diagnosis made according to the Martini and Melamed criteria in 16 (43%) cases. After exclusion of the 13 histopathologically equivocal cases, the histopathological diagnosis was concordant with the diagnosis based on the mutational evaluation in 15 (63%) patients.

Of the histopathologically IM cases (n = 7), six (86%) were diagnosed as IM cases also by mutational evaluation. Among these, the paired tumors from five patients showed multiple matching

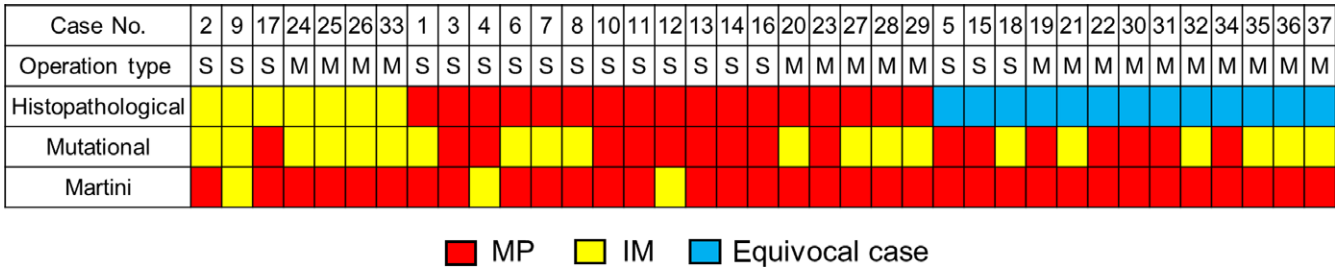

FIGURE 4 Comparisons of the tumor classifications by the Martini and Melamed criteria, histopathological and mutational evaluations. After exclusion of the 13 histopathologically equivocal cases, the histopathological diagnosis was concordant with the diagnosis based on mutational evaluation in 15 (63%) patients. IM, intrapulmonary metastasis; M, metachronous multiple lung cancer; MP, multiple primary lung cancer; S, synchronous multiple lung cancer

mutations (cases 2, 9, 24, 25, and 26). One patient (case 17) showed no mutations in this assay. Of the histopathologically MP cases (n = 17), only nine (53%) were classified as MP cases by mutational evaluation, and the remaining patients showed matching mutations. Among these, the paired tumors of four patients harbored two matching mutations (*EGFR*/*KIT* in cases 7 and 20, and *EGFR*/*STK11* in cases 27 and 28), those of two patients harbored one matching mutation in *EGFR* (cases 1 and 6), and those of two patients harbored one matching mutation in a gene other than *EGFR* (*TP53* in case 8 and *PDGFRA* in case 29).

3.5 | Survival analysis

Of the 37 patients, eight patients died by the time of the final data analysis in July 2017. Median survival time calculated for the entire study population was 103 months (range, 2-164 months). After exclusion of the histopathologically equivocal cases, there was no significant difference between the cases stratified by histopathological evaluation (Figure 5A). Similarly, no significant difference was observed between the cases stratified by mutational evaluation (Figure 5B).

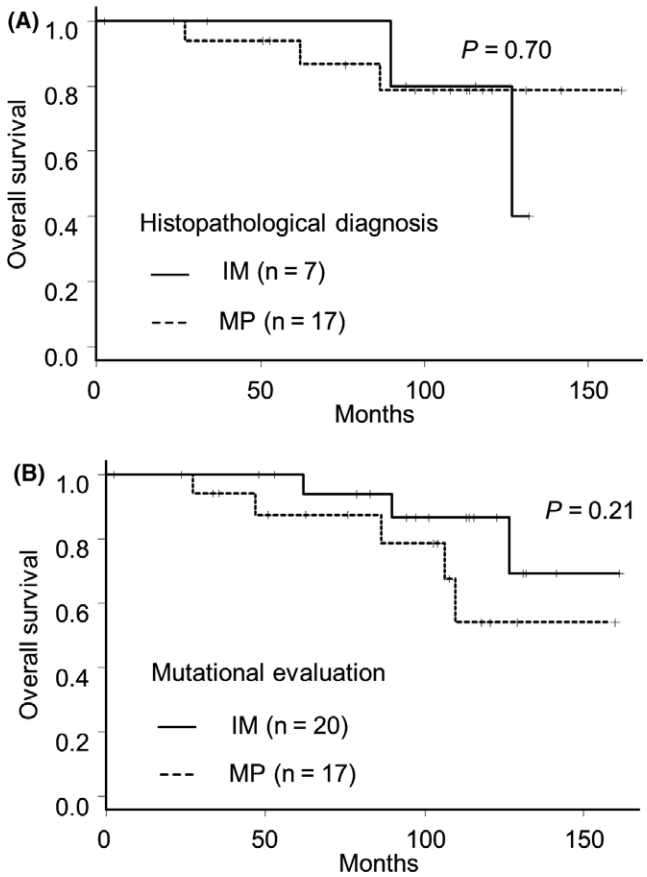

FIGURE 5 Overall survival curves for the patient groups stratified by histopathological diagnosis and mutational evaluation. No significant difference in survival was observed between the groups stratified as multiple primary lung cancer (MP) or intrapulmonary metastasis (IM) by histopathological (A) and mutational (B) evaluation ($P = 0.70$ and $P = 0.21$, respectively)

4 | DISCUSSION

In patients with synchronous/metachronous MLC, distinguishing MP from IM is of crucial importance to clinicians, to enable them to select the appropriate therapeutic strategies and predict the prognosis with accuracy. The benefits of surgical resection have been demonstrated for MP, whereas systemic chemotherapy without surgery is recommended for most cases of IM.[9,10] With the advances in high-throughput sequencing technologies, mutational analysis for specific driver and suppressor genes has been shown to be useful for distinguishing between multiple primary tumors and pulmonary metastases.[11-13] In recent years, in patients with MLC, several reports have been published of mutational analyses by NGS, which allows analysis for multiple gene mutations at once.[14,15] In this study, we investigated the mutational profiles by NGS of 20 lung cancer-related genes in surgically resected paired tumors obtained from the same patients and compared the clinicopathological diagnoses with the diagnoses based on mutational evaluation. In histopathologically IM cases, we found a good concordance rate (86%) with the diagnosis based on the mutational evaluation. Contrastingly, in histopathologically MP cases, the concordance rate with the diagnosis based on the mutational evaluation was only 53%.

Several reports have shown high concordance rates (>90%) of gene mutations between the primary lesion and metastatic lesions.[16,17] Conversely, high rates of discrepancy of driver mutations have been reported among synchronous multiple ground-grass lung nodules, which are considered to represent multiple independent lung cancer nodules.[18] In our cohort, most of the histopathologically IM cases were diagnosed as IM cases also by mutational evaluation. Among these, the paired tumors of five patients (cases 2, 9, 24, 25 and 26) harbored multiple identical alterations, representing a powerful demonstration of the clonality between paired tumors.

Girard et al have mentioned the possibility of accidental matching of gene alterations between independent tumors depending on the frequency of the mutation.[19] According to this theory, the lower the frequency of the matched mutation, the more likely that the origin of the paired tumors is the same. In addition, from the standpoint of probability, the greater the number of matching mutations, the stronger the evidence of clonality. On the contrary, matching of frequently occurring mutations requires a more cautious interpretation. *EGFR* and *TP53* mutations are the most commonly encountered mutations in NSCLC patients, especially in lung adenocarcinomas.[20,21] In our cohort, paired tumors of four patients (cases 1, 6, 8 and 37) who were diagnosed as MP cases by histopathological evaluation harbored the same mutation of only *EGFR* or *TP53*. Although we diagnosed these paired tumors as IM by mutational evaluation, careful interpretation including careful clinicopathological evaluation is needed in such cases because of the possibility of accidental matching of high-frequency mutations.

Among the histopathologically MP cases, mutational evaluation yielded a concordant diagnosis in only 53% of the cases. This result indicates that the histopathologically MP cases might also include IM cases. In general, pathologists base their diagnosis of multiple

primary lesions on such features of the lesions as the histological subtype, as described in the 2015 World Health Organization Classification of Lung Tumors,[3] the existence of in situ components, and the immunohistochemical profile. However, multiple primary cancers may not always differ completely in histopathology, because lung cancers, especially adenocarcinomas, show a spectrum of histological subtypes, and multiple lesions often show overlapping histological features. Thus, there is a limitation in histopathological diagnosis of MLC, and clonality analysis by mutational evaluation may be helpful to distinguish MP from IM. When the frequency and number of matching mutations are also considered, clonality becomes more powerful. Even in equivocal cases in which the diagnosis cannot be confirmed by histopathological evaluation, mutational evaluation could help to distinguish between MP and IM in patients with MLC.

Regarding the survival analysis, no significant difference in the overall survival was observed between the cases stratified by histopathological or mutational evaluation. This could be explained by the highly selected cohort used in our study; namely, our cohort included cases with satellite lesions in the same lobe or oligometastases, which may be expected to be associated with a better prognosis with a surgical approach.[22,23] If we had included not only operable patients, but also patients without indication for surgery, we may have obtained a worse prognosis in the IM group.

Several limitations of the present study should be acknowledged. First, the size of our study cohort was relatively small. The *EGFR* mutations were more frequently observed in Asians, and exon 19 deletion was the most frequently observed (48.2%), followed by L858R (42.7%).[24] Our data showed the relative low frequency of exon 19 deletion, but we have no reasonable explanation for this discrepancy. Further investigation regarding etiology of *EGFR* genotype in lung cancer is necessary. Second, we included operative cases only, which could have introduced a selection bias. Finally, the number of targeted genes in our NGS panel may be relatively small. Recent studies on mutational analysis using NGS have included a larger number of targeted genes than we did.[14,15] Begg et al indicated that at least 20 markers are required from a statistical perspective to distinguish between clonal and independent tumors with high accuracy.[25] In the present study, although we analyzed 20 lung cancer-related genes, paired tumors from five patients (cases 10, 11, 14, 23, and 31) failed to show any mutations. This result may imply the lack of sufficient power of our panel. Further investigation and larger studies are necessary to resolve these problems.

Translocation of genes such as *ALK/ROS1/RET* is important to characterize lung cancer.[26] However, the DNA-based panel we used in this study could detect point mutations, small insertions and deletions only, because we emphasized low cost and convenience. More comprehensive analysis including gene fusions such as *ALK/ROS1/RET* is expected to lead to more informative and interesting results for more accurate differentiation of multiple lung cancer.

In conclusion, mutational analysis of MLC for 20 targeted genes using NGS appears to be a useful complementary tool for distinguishing MP from IM in addition to histopathological evaluation. In histopathologically equivocal cases or cases with discordance between the histopathological and the mutational evaluation, consideration of the frequency and number of matching mutations may be helpful for the differentiation.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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