Original Article

Genomic assessment distinguishes intrapulmonary metastases from synchronous primary lung cancers

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Background: Multiple synchronous lung tumors (MSLT), particularly within a single lobe, represent a diagnostic and treatment challenge. While histologic assessment was once the only method to possibly distinguish multiple primary lung cancers, there is a growing interest in identifying unique genomic features or mutations to best characterize these processes.

Methods: In order to differentiate multiple primary lung malignancies from intrapulmonary metastases in patients with MSLT, we performed whole exome sequencing (WES) on 10 tumor samples from 4 patients with MSLT.

Results: Shared mutations between tumors from the same patient varied from 0–91%. Patient 3 shared no common mutations; however, in Patients 2 and 4, identical mutations were identified among all tumors from each patient, suggesting that the three tumors identified in Patient 3 represent separate primary lung cancers, while those of Patients 1, 2 and 4 signify hematogenous and lymphatic spread.

Conclusions: A high proportion of shared mutations between different lung tumors is likely indicative of intrapulmonary metastatic disease, while tumors with distinct genomic profiles likely represent multiple primary malignancies driven by distinct molecular events. Application of genomic profiling in the clinical setting may prove to be important to precise management of patients with MSLT.

Keywords: Non-small cell lung cancer; multiple synchronous lung cancers; genomic heterogeneity; gene sequencing

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Introduction

Lung cancer is a leading cause of cancer death worldwide. Approximately, 222,500 new cases were diagnosed in the United States in 2017 (1). It has been estimated that 0.2–8% of lung cancer patients present with multiple synchronous lung tumors (MSLT) at the time of diagnosis. These tumors are distinct in that they are anatomically separate, with unclear etiologic and genomic relationships to one another (2-5). It has been noted that the incidence of MSLT has been increasing as lung cancer screening has become more widely implemented. MSLT raise a clinical dilemma, as they may represent metastatic disease from a single primary cancer, either by hematogenous spread or regional extension, or multiple primary cancers (5,6).

Determination of the etiology of MSLT is important to appropriate staging, treatment, and prognosis of these unique disease processes. Over recent years, research groups and medical communities have established guidelines to classify MSLT (5,7). Although these criteria have been widely adopted into clinical practice and patient management, they are rather empirical with little to no underwriting with molecular evidence. Thus, the classification of these tumors remains difficult.

Genome-scale sequencing studies have revealed that lung cancer is a heterogeneous disease with a complex genomic landscape. Each lung cancer may have a unique genomic profile that may be attributed to a distinct genetic background among individual patients or differing levels of carcinogen exposure (8,9). On the other hand, studies from our group and others have demonstrated that the majority of somatic mutations are universally present in different regions within a single tumor, suggesting limited intratumoral heterogeneity (10,11).

More recently, we have performed comprehensive genomic analysis from six Chinese patients with MSLT and demonstrated distinct genomic profiles in different MSLT from the same patients (12). In spite of the fact that MSLT from a single patient share identical constitutional genetic backgrounds and exposure histories, these tumors are no more similar to each other than tumors from different patients. Since metastatic lesions usually retain a significant fraction of genomic aberrations from the founding primary tumors, these data suggest that genomic profiling may be used as a complementary approach to clinicopathological analyses to accurately distinguish simultaneous primary lung cancers from intrapulmonary metastases (13,14).

In our previous study, all 6 patients were suggested to have multiple primary lung cancers by genomic analyses, and we were not able to demonstrate how close intrapulmonary metastases resemble their primary tumors (12). In addition, since all 6 patients were Asian patients, how these findings apply to Caucasian lung cancer patients is unknown. The importance of ethnicity upon the unique genomic alterations, diagnosis, and management of lung cancer are increasingly appreciated, and thus the relevance of such an investigation among an exclusively Caucasian population is understood (15-17).

Herein, in this study, we performed whole exome sequencing (WES) of ten tumors (seven adenocarcinomas, two squamous cell carcinomas and one regional lymph node metastasis) from four Caucasian patients with MSLT in order to assess the clonal relationship between different tumors from within the same patient. We aimed to determine whether an assessment of genomic heterogeneity could distinguish synchronous lung cancers from intrapulmonary metastases.

Methods

Patients

Surgical specimens were collected from four patients who had MSLT present upon initial evaluation at the University of Texas MD Anderson Cancer Center (MDACC) in Houston, Texas. Two patients (Patients 1 and 2) were found to have two tumors each; Patient 3 was observed to have three tumors; and Patient 4 had two anatomically distinct tumors with additional nodal involvement (*Table 1*). Patients 1 and 4 were never smokers, while Patients 2 and 3 were former smokers. Tumors from Patients 1, 2, and 3 were adenocarcinomas, and tumors from Patient 4 were squamous cell carcinoma. No patients had preoperative chemotherapy or radiation therapy. All patients were free of extrathoracic metastases. Upon pathologic examination, tumor sizes ranged from 0.5 to 5.1 centimeters. Tumor characteristics are shown in *Table S1* and *Figure S1*. According to the American College of Chest Physicians (ACCP) criteria, Patients 1 and 2 had satellite nodules, while Patients 3 and 4 had multiple primaries (18). The collection and analysis of patient samples was approved by the MDACC Institutional Review Board [Cancer Prevention & Research Institute of Texas Multi-Investigator Research Awards (CPRIT-MIRA), RP160668]. Informed consents were obtained from all patients.

Table 1 Clinical characteristics and sequencing information of the four patients with multiple synchronous lung cancers

Patient ID	Tumor ID	Sequencing depth	Histology	Nodal stage	ACCP category	Histopathologic analysis	Genomic profile	Adjuvant therapy	Follow-up (months)	Recurrence	Smoking status
Pa1	Pa1T1	177×	ADC	N0	Satellite nodules	Metastatic	Metastatic	CT	32	Yes	Non-smoker
	Pa1T2	188×	ADC								
Pa2	Pa2T1	30×	ADC	N0	Satellite nodules	Metastatic	Metastatic	CT	73	No	Former smoker
	Pa2T2	205×	ADC								
Pa3	Pa3T1	173×	ADC	N2	Multiple primaries	Metastatic	Primary	CRT	73	No	Former smoker
	Pa3T2	87×	ADC								
	Pa3T3	195×	ADC								
Pa4	Pa4T1	193×	SCC	N1	Multiple primaries	Metastatic	Metastatic	None	59	No	Non-smoker
	Pa4T2	161×	SCC								
	Pa4LN	210×	SCC								

ACCP, American College of Chest Physicians; Pa, patient; T, tumor; ADC, adenocarcinoma; N, node; CT, chemotherapy; CRT, chemoradiotherapy; SCC, squamous cell carcinoma; LN, lymph node.

Sample collection and processing

Patients underwent resection of all tumors, including nodal disease. After resection, ten 10-μm formalin-fixed paraffin-embedded (FFPE) sections from each tumor sample were collected. Hematoxylin-eosin (HE)-stained slides were reviewed by experienced lung cancer pathologists to determine the histomorphological subtype, as well as the proportion of malignant cells relative to nonmalignant stromal (inflammatory, vascular, and fibroblast) cells. In addition, tumor cell viability was addressed by examining the presence of necrosis in the tissues. Tumor cells were enriched by having a pathologist scrape tumor tissue from each slide. Genomic DNA was then extracted from all samples.

WES

All tumors, including the sampled lymph node, underwent WES. Genomic DNA was sheared into fragments with peaks of 150 to 200 base pairs (bp), and then adapters were ligated to both ends. The adapter-ligated templates were purified with AgencourtAMPure SPRI beads (Beckman Coulter, Inc., Brea, CA, USA), and fragments with an insert size of approximately 200 bp were excised. Extracted DNA was amplified by ligation-mediated PCR, purified, and hybridized to the Sure Select biotinylated RNA library (Agilent Technologies, Santa Clara, CA, USA) for enrichment according to the manufacturer's instructions. Paired-end multiplex sequencing of samples was performed with the Illumina HiSeq 2000 System. The average sequencing depth was 162× per sample.

Sequence alignment and variant calling

Paired-end reads in FastQ format generated by the Illumina pipeline were aligned to the reference human genome (UCSC Genome Browser, Version hg19) using the Burrows-Wheeler Aligner with default settings, except for a seed length of 40, a maximum edit distance of 3, and a maximum edit distance in the seed of 2 (19). Aligned reads were further processed according to the Genome Analysis Toolkit (GATK) for duplicate removal, indel realignment, and base recalibration (20).

MuTect was employed to detect potential single nucleotide variations. In addition to the build-in filters, the following filtering criteria were applied: (I) total read count in tumor DNA ≥30; (II) total read count in germline DNA ≥15; (III) presence of variant on both strands; (IV) variant allele frequency (VAF) in tumor DNA ≥5%; (V) VAF in germline DNA ≤1% (21). Single nucleotide variants called by MuTect were used for further analysis.

Statistical analysis

Pearson's correlation test was used to assess the association between mutational burden and various demographic

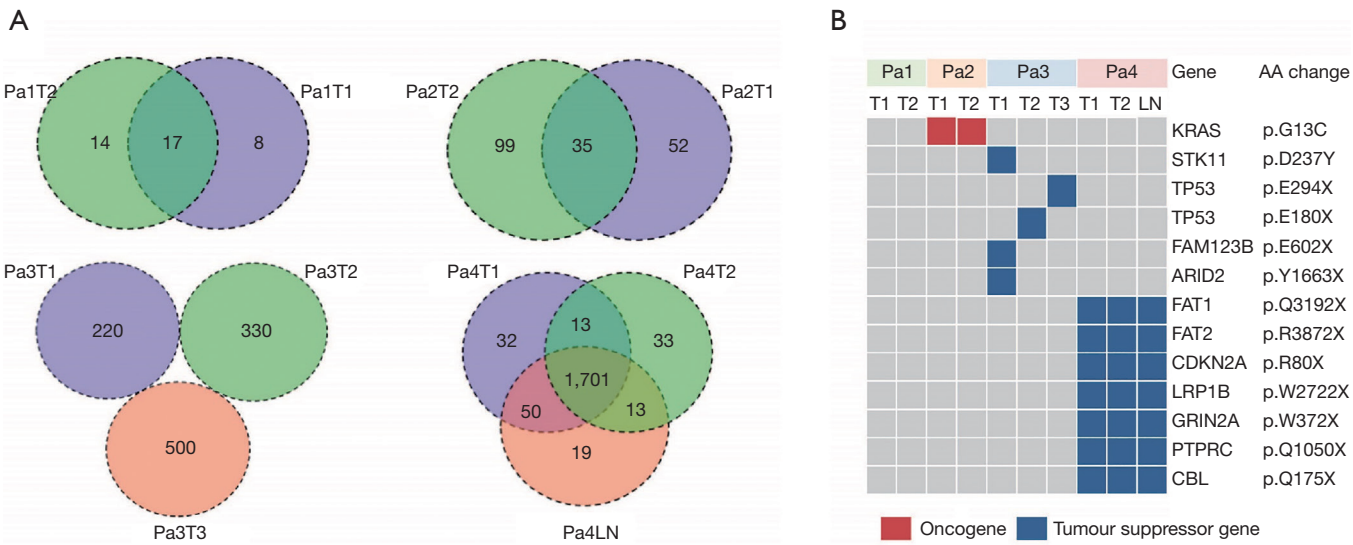

Figure 1 Genomic profiling of different lesions rising from four patients (Pa) with MSLT. (A) Venn diagram illustrating the distributions of functional mutations across 10 lesions. The numbers of mutations identified in only one tumor (T) or shared by two or more lesions are as indicated. Shared mutations were defined as identical nucleotide substitutions at the same genomic coordinates. (B) Heatmap of known cancer gene mutations shared across 10 lesions.

and clinicopathologic factors, as well as to determine the correlation between the mutation spectra of different tumors. Fisher's exact test was used to assess the significance of differences in mutation spectra between different tumors.

Results

Somatic point mutations

A total of 9,839 coding and splice site mutations were detected from 10 tumor samples. Of those mutations, 1,708 were detected in seven adenocarcinomas (median of 163 mutations per sample, ranging from 31–648) and 8,131 in three squamous cell carcinomas (median of 2,725 per sample, ranging from 2,678–2,728) (*Table S2*). All seven adenocarcinoma samples from patients 1, 2, and 3 were of an acinar pattern; the three squamous samples from patient 4 were poorly-differentiated. To determine the genomic heterogeneity of MSLT and the clonal relationships between different tumors within the same patient, we first defined functional somatic mutations as non-synonymous or stop-gain/stop-loss, frame-shift, alternative splicing mutations (*Figure 1A*) in exons leading to potential changes in final proteins. A total of 52 shared functional mutations were detected in the individual lung adenocarcinomas (LUAD) [17 of 39 (44%) mutations from Patient 1 and 35 of 186 (19%) from Patient 2]. No shared mutations were detected between different tumors from Patient 3. In Patient 4, two tumors (T1, T2) and a lymph node metastasis shared 1,701 (91%) of 1,861 mutations.

Known cancer gene mutations

We then characterized canonical cancer gene mutations, defined as nonsynonymous mutations identical to those previously reported in known cancer genes or truncating mutations in known tumor suppressor genes (22-27). In total, 13 known cancer gene mutations were identified, of which eight cancer gene point mutations were shared between any two tumors from the same patient, including *KRAS* p.G13C mutation in Patient 2 (*Figure 1B*). Five other known cancer gene mutations (*STK11* p.D237Y, *TP53* p.E294X, *TP53* p.E180X, *FAM123B* p.E602X, *ARID2* p.Y1663X) were found in different tumors of Patient 3. In Patient 4, seven known tumor suppressor gene mutations (*FAT1* p.Q3192X, *FAT2* p.R3872X, *CDKN2A* p.R80X, *LRP1B* p.W2722X, *GRIN2A* p.W372X, *PTPRC* p.Q1050X, and *CBL* p.Q175X) were shared by three different tumors.

To confirm our findings, we compared the mutations in our cohort and those in 230 unrelated LUAD and 178 unrelated lung squamous cell carcinomas (LUSC) in The Cancer Genome Atlas (TCGA) study (8,9,11). Pairs of any

two unrelated tumors in TCGA were less likely to have shared mutations (*Figure S2*). Among 230 LUAD samples (26,335 pairs): 1 pair of samples had 63 shared functional mutation, 40 pairs of samples had 2 shared functional mutations, and 1,846 pairs of samples had 1 shared functional mutation. In 178 LUSC samples (15,753 pairs): 5 pairs of samples shared 2 functional mutations and 212 pairs of samples had 1 shared functional mutation.

Mutation spectra and mutation signature

Next, we explored mutational processes in the context of independent tumors arising on a fixed genetic background and with shared exposure. The mutation spectra were different between ever smokers and never smokers (22,23,27,28). All tumors (including a metastatic lymph node) from the two never smokers (Patients 1 and 4) exhibited an enrichment for C>T mutations, while the tumors from the two smokers (Patients 2 and 3) had a higher prevalence of C>A substitutions (*Figure S3*). Discordant mutation spectra were observed between adenocarcinomatous tumors derived from the same patient (Patients 1, 2, and 3), while similar mutation spectra were observed between squamous cell tumors from Patient 4, indicating that different mutational processes may be involved during the development of tumors from Patients 1, 2, and 3, but not from Patient 4.

Followed by extraction of the mutational signature, we found that signature profiles differed substantially between individual tumors, except those from Patient 4. In this patient, the two tumors and associated lymph node metastasis had almost identical mutational signatures corresponding to presumptive APBOEC signature (*Figure S4*).

Discussion

MSLT are increasingly diagnosed largely as a result of improved implementation of early detection tools such as multislice spiral computed tomography, fluorescence endoscopy, and positron emission tomography (29,30). MSLT represent a clinical conundrum, as they may indicate multiple primary cancers which are potentially curable, or, on the other hand, could signify intrapulmonary metastases which would symbolize unresectable disease. As a result, many attempts have been made to distinguish these clinical entities. Although they largely represent empirical guidelines with little supporting molecular evidence, the Martini-Melamed criteria and ACCP guidelines are widely adapted clinical tools used to assess MSLT (7,31).

In this report, we performed genomic profiling of nine MSLT and one lymph node metastasis from four patients using WES alone. Initially, upon examination of TCGA data, it is surmised that tumors from unrelated patients are unlikely to share a high proportion of mutational similarity (12). Using this knowledge, we were able to distinguish independent primary lung cancers from intrapulmonary metastases in Patient 3, despite a shared genetic background and exposure history. These three tumors shared no mutations, similar to LUAD of different patients examined in TCGA, a majority of whom shared only a single mutation. These data from Patient 3 provide evidence that multiple molecular processes, resulting in distinct mutational profiles, may be in play during the development of independent lung cancers within the same individual, despite a shared exposure history.

Conversely, in comparison to TCGA data in which tumors from separate patients shared rare genetic markers, samples from Patients 1, 2, and 4 in our cohort shared at least 20% of the same mutations. Taken together, these results suggest that tumors from these three individuals more likely represent a single primary cancer with corresponding metastatic disease.

In addition, closer examination of the *TP53* gene demonstrated disparate mutation events between the MSLT of Patient 3. Analogous intratumoral heterogeneity is observed in clear cell renal cell carcinoma, in which a single known gene implicated in carcinogenesis may have different subclones of the primary tumor. We have furthermore reported the occurrence of different mutations within a single cancer gene found in separate subclones of LUAD, which may be suggestive of convergent selection (12). This suggests a propensity for a single pathway of tumorigenesis, which is, however, driven by different biological mechanisms within a uniform biological environment (32). The data from Patient 3 suggests that, even in the context of a single genetic makeup and exposure history, the development of multiple primary lung cancers can be driven by distinct molecular events, with a possible predilection for certain pathways critical for carcinogenesis in a single patient.

Intrapulmonary metastases, either by hematogenous or local spread, are thought to derive from the same progenitor cells as the matched primary tumors (5). In this study, we profiled 4 patients, all of whom were initially clinically diagnosed with metastatic disease (either intrapulmonary metastases or satellite nodules) according to

ACCP guidelines. While the MSLT from s from Patients 1, 2, and 4 shared similar molecular traits, the three tumors from Patient 3 demonstrated distinct genomic profiles. Our results suggest that the MSLT identified in Patient 3 represent independent primary lung cancers with separate progenitor cells. Conversely, the disease burden found in Patients 1, 2, and 4 most likely represents a single primary cancer with secondary metastatic disease.

During the follow-up period, only Patient 1 experienced disease recurrence after surgical resection after a follow-up duration of 20 months. The remaining patients all had a minimum postsurgical follow-up of at least 59 months (range, 59–73 months), and were without evidence of disease. Previous studies have demonstrated a slightly shorter survival in patients with satellite nodules compared with patients without satellite nodules matched for primary tumor size, lymph node and metastatic stage (33). Although our sample size was small, our data were consistent with previous studies. Our report is further novel in that we have contributed data on an exclusively Caucasian cohort, whereas our prior investigation involved sequencing of MSLT among Asian patients only (12); though we are unfortunately unable to draw direct conclusions from our assembled findings among these Asian and Caucasian patients, we feel our current report contributes to the growing body of literature on the topic of MSLT.

Of importance, all patients examined herein possessed MSLT within a single lobe. By current American Joint Committee on Cancer (AJCC) guidelines, this constellation represents T3 disease. A limitation of these criteria is demonstrated within our cohort, in which the etiology of MSLT is unclear, representing either metastatic disease or distinct primary cancers, though all patients received the benefit of surgical therapy (34). Though our investigation sheds light on this common clinical scenario of a patient with T3 disease identified imaging, our investigation is limited in that we are not able to infer the genomic profiles and molecular events which may lead to MSLT on separate lobes. Future investigations should be directed towards this additional management challenge. Additionally, though we are limited by our sample size, this work represents an exploratory investigation which we feel will still be of great importance to the literature.

To improve the diagnostic accuracy of MSLT, pioneering studies led by Travis et al have presented comprehensive histologic assessments and have shown promising results (35,36). Using a similar approach, we were able to accurately classify the molecular etiology of the 10 tumors observed, further confirming that comprehensive histologic assessment is highly valuable to distinguish multiple primary cancers from intrapulmonary metastases.

Conclusions

While tumor morphology may be controlled by complex molecular mechanisms and is furthermore subject to some degree of variable interpretation, we present a concrete molecular evidence which can help to better characterize MSLT. We demonstrate that multiple primary tumors have distinct genomic profiles, while metastatic lesions usually retain a significant fraction of genomic aberrations from the founding primary tumors (13,37). Therefore, comprehensive genomic profiling at the exome level can provide pivotal information to clinical and histologic assessment to accurately distinguish multiple primary lung cancers from intrapulmonary metastases. Application of genomic profiling in the clinical setting of staging patients with MSLT should be explored in a larger cohort to confirm the utility suggested here. If corroborated, genomic profiling may prove to be an important component of a more precise approach in managing patients presenting with MSLT.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jtd-20-1). CG has a patent named "Markers associated with ribavirin-induced anemia" that was issued in 2012. CB and IIW report grants and personal fees from Genentech/Roche, grants and personal fees from Bayer,

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The collection and analysis of patient samples was approved by the MDACC Institutional Review Board [Cancer Prevention & Research Institute of Texas Multi-Investigator Research Awards (CPRIT-MIRA), RP160668]. Informed consents were obtained from all patients.

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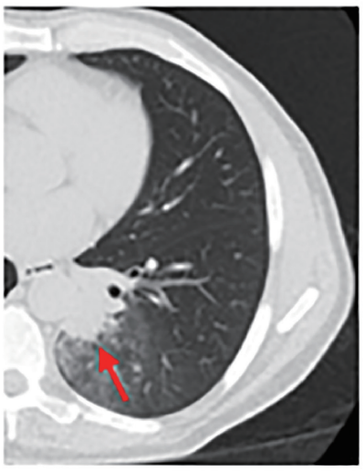
Supplementary

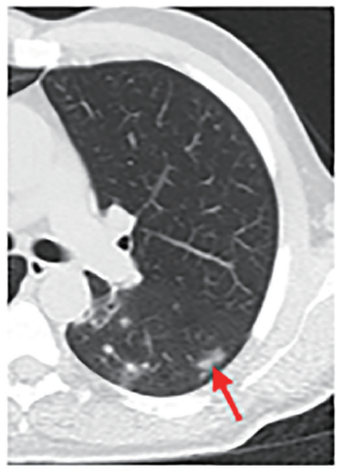
Table S1 Histomorphological subtypes[a] and their percentages in the 10 intrathoracic lesions

Patient ID	Sample ID	Histology	Location	Tumor (HE assessment)	Malignant cell (HE assessment)
Pa1	Pa1T1	ADC	LLL	80	80
	Pa1T2	ADC	LLL	80	80
Pa2	Pa2T1	ADC	LUL	80	80
	Pa2T2	ADC	LUL	>10	>10
Pa3	Pa3T1	ADC	RUL	10	10
	Pa3T2	ADC	RUL	90	90
	Pa3T3	ADC	RUL	30	30
Pa4	Pa4T1	SCC	RUL	90	60
	Pa4T2	SCC	RUL	90	80
	Pa4LN	SCC	Right hilar	10	10

[a], according to the multidiscipline classification criteria for adenocarcinoma in 2011. HE, hematoxylin-eosin; T, tumor; LN, lymph node; ADC, adenocarcinoma; SCC, squamous cell carcinoma; LLL, left lower lobe; LUL, left upper lobe; RUL, right upper lobe.



Pa1T1



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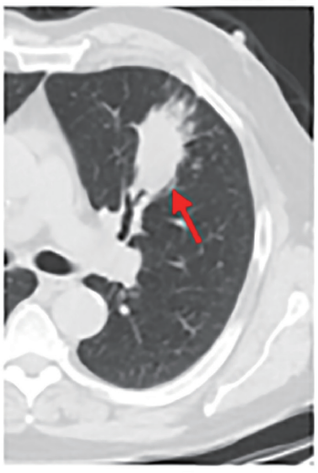

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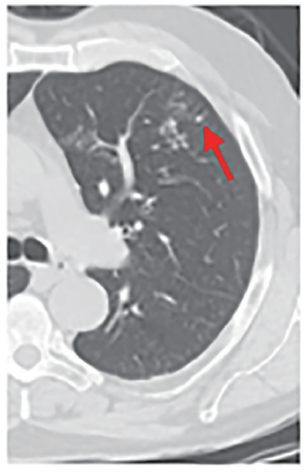
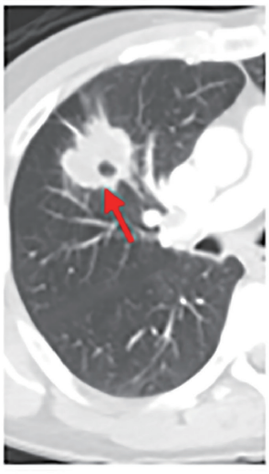
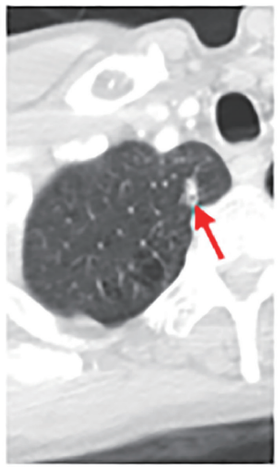


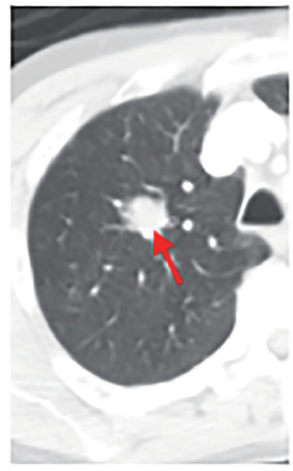
Pa2T2



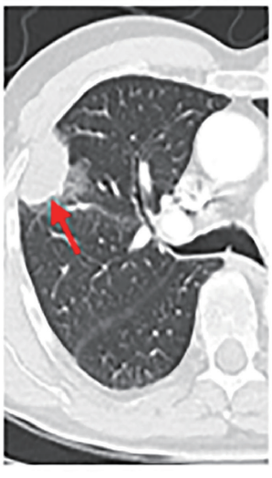
Pa3T1



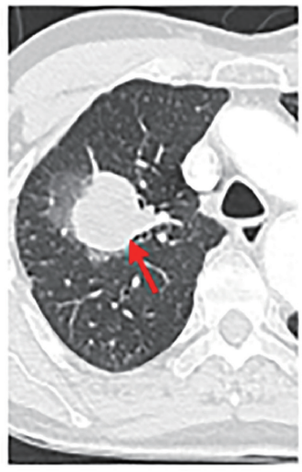
Pa3T2



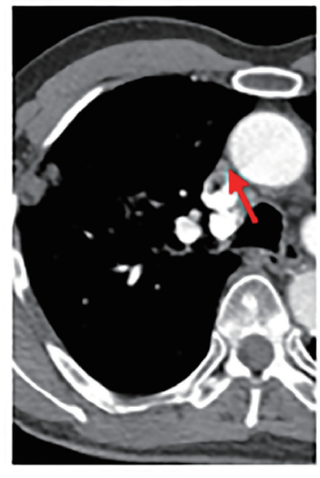
Pa3T3



Pa4T1



Pa4T2



Pa4LN

Figure S1 Representative computed tomography images of 10 intra-thoracic MSLC lesions (red arrow). Pa, patient; T, tumor; LN, lymph node metastasis; MSLC, multiple synchronous lung cancer.

Table S2 Summary of somatic mutations detected in tumors obtained from whole exome sequencing

Single nucleotide variants[a]	Pa1T1	Pa1T2	Pa2T1	Pa2T2	Pa3T1	Pa3T2	Pa3T3	Pa4T1	Pa4T2	Pa4LN
Number	107	132	189	430	684	698	1,339	6,250	6,013	6,385
Coding	31	41	110	159	296	403	633	2,691	2,643	2,689
Non-synonymous	21	26	76	119	201	295	436	1,634	1,603	1,622
Synonymous	6	10	28	30	82	80	149	948	934	958
Stop-loss/stop-gain	4	5	6	10	13	28	47	106	103	106
Unknown	0	0	0	0	0	0	1	3	3	3
Non-coding	15	18	16	48	66	59	118	633	601	641
UTR	9	8	12	30	48	40	78	452	435	471
ncRNA	6	10	4	18	18	19	40	181	166	170
Intronic	41	45	61	171	268	216	496	2,573	2,458	2,723
Splice site	0	0	4	4	5	7	15	37	35	36
Other	41	45	57	167	263	209	481	2,536	2,423	2,687
Intergenic	20	28	2	52	54	20	92	353	311	332

[a], single nucleotide variants called by MuTect. Pa, patient; T, tumor; LN, lymph node; UTR, untranslated region; ncRNA, non-coding RNA.

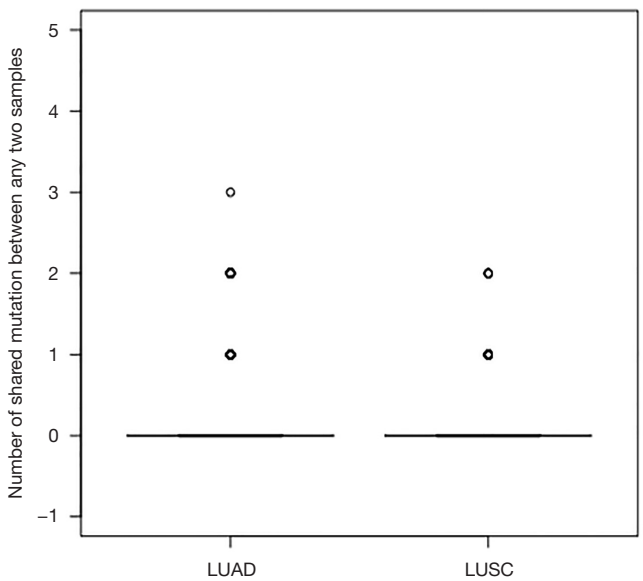


Figure S2 Numbers of shared mutations between any two samples in LUAD and LUSC from TCGA database. This figure shows that pairs of any two unrelated tumors in TCGA were less likely to have shared mutations. In 230 LUAD samples (i.e., 26,335 pairs), one pair of samples shared three mutations, 50 pairs of samples have two shared mutations, and 24,269 pairs of samples have one shared mutation. In 178 LUSC samples (i.e., 15,753 pairs), five pairs of samples have two shared mutations, and 301 pairs of samples shared one mutation. LUAD, lung adenocarcinomas; LUSC, lung squamous cell carcinomas; TCGA, The Cancer Genome Atlas.

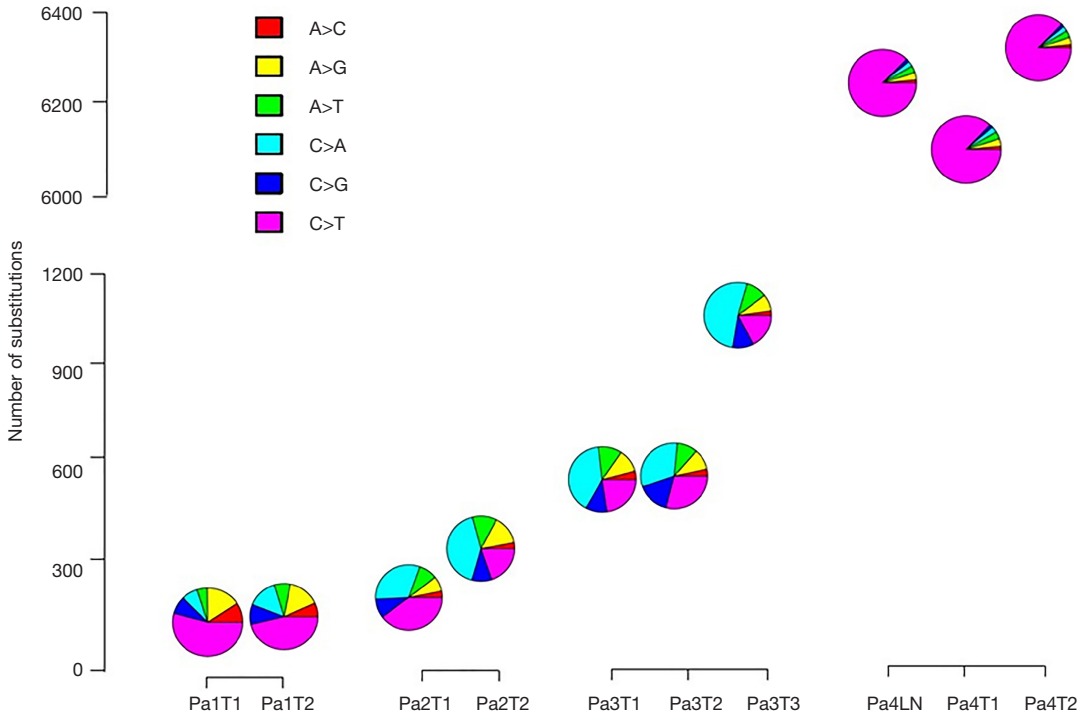


Figure S3 Mutation spectra of all mutations across 10 MSLC lesions. An enrichment of C>T mutations were noted in all tumours of Patients 1 and 4 who were both non-smokers. The other five tumours from Patients 2 and 3 have predominantly C>A substitutions. Similar mutational spectra were observed between tumors in Patient 4. Discordant mutational spectra were observed between same-patient tumors in all adenocarcinoma lung cancer patients. MSLC, multiple synchronous lung cancer.

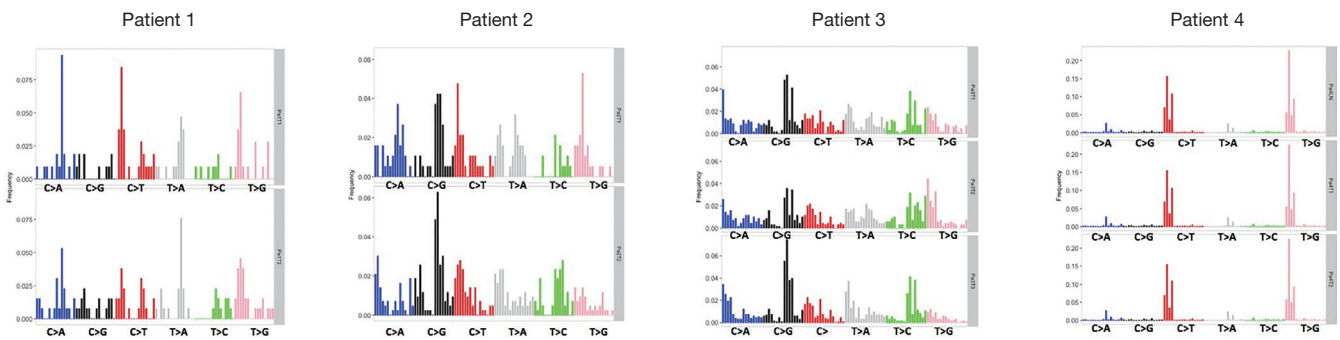


Figure S4 Mutational signature analyses of nine MSLC tumours (T) and a lymph node metastasis (LN) across all four patients. All mutations were included in the analysis. Nucleotide substitutions in all mutations were grouped into six classes of mutations on the x-axis. In each class, mutations were grouped into 16 subclasses according to the bases immediately 5' and 3' to each mutated base. The data were the relative frequencies of the six mutation classes in each 16 tri-nucleotide contexts. MSLC, multiple synchronous lung cancer.