# High Frequency of Clonally Related Tumors in Cases of Multiple Synchronous Lung Cancers as Revealed by Molecular Diagnosis[1]

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## ABSTRACT

In patients with multiple synchronous lung tumors, discrimination of multicentric lung cancers from intrapulmonary metastasis is important for treatment decision, but this is sometimes difficult. The aim of this study was to retrospectively distinguish multicentric lung cancers from intrapulmonary metastases in 14 such cases by loss of heterozygosity (LOH) and p53 mutational status. DNA was extracted from microdissected tumor cells in paraffin-embedded archival tissue, and 3p14.2, 3p21, 3p25, 9p21, and 18q21.1 were investigated for LOH. Exons 5–8 of the *p53* gene were examined for mutations by the PCR, followed by single-strand conformation polymorphism analysis and DNA sequencing. For cases with the same LOH pattern, we calculated a clonality index, the probability of the given LOH pattern when these tumors were hypothesized to be independent in origin. Eleven of 14 cases (79%) were thus diagnosed as having pulmonary metastasis and only one case as having genuinely multicentric lung cancers. Two cases presented difficulty in diagnosis. In several cases, the LOH patterns conflicted with p53 mutation patterns, suggesting that clonal evolution is directly affected by certain genetic

changes. The combination of p53 with LOH helped increase both the sensitivity and specificity of the assay.

## INTRODUCTION

In a clinical practice, it is not rare to see patients with multiple foci of pulmonary malignancies. Auerbach *et al.* (1) examined 255 patients who died of lung cancer and found, by extensive histological evaluation, 37 primary invasive carcinomas in tracheobronchial trees separate from the main tumor mass. McElvaney *et al.* (2) reported that in a consecutive series of 62 lung resections for bronchogenic adenocarcinoma, 12 patients (19%) had two or more adenocarcinomas on careful pathological examination. These multiple tumors could be either multicentric and clonally different, or intrapulmonary metastases, because patients with a primary cancer of the lung have an increased risk of developing other lung tumors, in line with the "field cancerization" concept (3), and the lung is also a common site of hematogenous lung cancer metastases.

Synchronous multiple cancers can be regarded as potentially curable. On the other hand, with intrapulmonary metastases the prognosis is usually poor. Therefore, distinction between the two might be of therapeutic and prognostic importance. In conventional clinicopathological analyses, the incidence of multicentric tumors of the lung is reported to be ~1% (4–7), but lack of definite criteria prevents estimation of an accurate value.

Martini and Melamed (8) in 1975 proposed the criteria that are currently most widely used. Essentially, a diagnosis of multiple primary cancers is made when the histological characteristics are different. Even when the histological features are the same, the second tumor is diagnosed as multicentric tumor, if it is in a different segment, lobe, or lung, or if the other tumor is a carcinoma *in situ*, or if there is no obviously related carcinoma in the lymphatics common and extrapulmonary metastasis is lacking at time of diagnosis. However, these criteria are rather empirical, and no theoretically sound background has been established.

Recent advances in molecular biology have provided several markers that can be used for clonal analysis. These include X chromosome inactivation analysis in female patients, immunoglobulin or T-cell receptor gene analysis for lymphoid tumors, and the occurrence of somatic mutations of oncogenes or tumor suppressor genes (9). Analysis of mutations occurring in the *p53* tumor suppressor gene is particularly useful for lung cancer. Because they are the most frequently observed genetic alteration (10). It is not likely that two independent tumors would have the same p53 mutation by chance alone, because they are widely distributed, involving various codons in exons 5–8 (11, 12). Another advantage is that mutations of the *p53* gene occur relatively early in the development of lung cancer, especially in those of squamous cell type (13). Because they have a role in maintenance of the malignant phenotype (14), once acquired they are well preserved during progression or

*Table 1* Summary of clinicopathological features of patients with synchronous multiple lung cancers

| Patient no. | Age at diagnosis | Sex | No. of lesions | Histology[a] | | | Distribution | Lymph node metastasis | Martini and Melamed criteria |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Tumor 1 | Tumor 2 | Tumor 3 | | | |
| 1 | 62 | M | 2 | BAC | BAC | | same lobe | − | PM |
| 2 | 59 | M | 2 | SM | AD | | same lobe | + | MC |
| 3 | 67 | M | 2 | LA | LA | | same lobe | + | PM |
| 4 | 50 | F | 2 | AD | AD | | same lobe | + | PM |
| 5 | 77 | M | 2 | AD | AD | | same lobe | + | PM |
| 6 | 69 | M | 2 | AD[b] | SQ | | different lobe | − | MC |
| 7 | 32 | F | 2 | AD | AD | | same lobe | + | PM |
| 8 | 68 | F | 2 | AD | AD | | different lobe | − | MC |
| 9 | 69 | M | 3 | SQ | SQ | SQ | same lobe | + | PM |
| 10 | 76 | M | 2 | SQ | SQ | | same lobe | − | PM |
| 11 | 66 | M | 2 | AD | AD | | same lobe | − | PM |
| 12 | 35 | F | 2 | AD | AD | | same lobe | + | PM |
| 13 | 53 | F | 2 | AD | AD | | same lobe | + | PM |
| 14 | 56 | M | 2 | AD | AD | | same lobe | − | PM |

[a] AD, adenocarcinoma; SM, small cell carcinoma; LA, large cell carcinoma; SQ, squamous cell carcinoma; BAC, bronchioloalveolar carcinoma; PM, pulmonary metastasis; MC, multicentric lung cancer.
[b] Tumor 1 of this case had a squamous component.

metastatic spread (15). In our previous clonal analysis of metachronous multiple lung tumors, genetic diagnosis could be made for 9 of 16 patients, but *p53* gene analysis was not informative in the remaining 7 (16).

In this study, we examined 14 patients with synchronous multiple lung tumors for their clonal origin by examining *p53* gene mutations and in addition, evaluated LOH[3] occurring on chromosomal arms 3p, 9p, and 18q. Leong *et al.* (17) showed recently that examination of loss of loci on particular chromosome arms is useful for distinction of second primary tumors from lung metastases in patients with head and neck cancer.

## MATERIALS AND METHODS

**Patients.** During a 3-year-period from 1996 to 1998, 250 patients with primary lung cancers underwent pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital. Of those, 20 patients (8%) had multiple lung tumors that were physically distinct and separate. We analyzed 38 tumors occurring in 18 these patients for whom archival materials were available. Subsequently, we excluded four patients because of inappropriate materials (see below). We could thus examine 29 tumors occurring in 14 patients, 9 men and 5 women. Median age at the time of the operation was 60 years (range, 32–77). Histological types of tumors were the same in 12 patients and different in 2. Tumors were in the same lobe in 12 patients and in two cases in different lobes. Clinicopathological feature are summarized in Table 1. The resected tumors were routinely fixed in 10% formaldehyde and embedded in paraffin. Sections cut at 3 μm were stained with H&E for histological examination.

**Extraction of DNA from Formalin-fixed Paraffin-embedded Materials.** Single 6-μm-thick paraffin sections were used for genomic DNA extraction. After deparaffinization

with xylene, they were stained with hematoxylin and areas were carefully microdissected containing >70% of neoplastic cells. Adjacent normal appearing nonneoplastic tissues were also obtained. The dissected tissue was digested in 50 μl of buffer consisting of Tris-HCl (pH 8.0), 20 mM/L, EDTA (pH 8.0), 1 mM/L, 0.5% Tween 20, and 200 μg/ml proteinase K for 24 h at 37°C. After incubation for 15 min at 95°C to inactivate the proteinase K, essentially as described by Sugio *et al.* (18). Aliquots of 1 μl were used for each experiment.

**PCR-SSCP Analysis of the *p53* Gene.** Mutations of the *p53* gene occurring in exons 5–8 were screened by PCR-SSCP analysis essentially as described earlier (16) with a modification to eliminate nesting-PCR strategy, because we found that this introduces significant artificial mutations. Briefly, 1-μl aliquots of DNA solution were amplified with a pair of primers in a volume of 10 μl including 0.5 μl of [α-$^{32}$P]dCTP (3000 Ci/mmol, 10 mCi/ml; Amersham, Arlington Heights, IL) with AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ). The PCR products were diluted with loading buffer 1:10, heat degenerated, loaded onto nondenaturing 6% polyacrylamide gels, and subjected to electrophoresis at 25 W for 4 h in a cold room (4°C) or at 30 W for 5 h at room temperature. After electrophoresis, the gels were subjected to autoradiography. We repeated at least three experiments to confirm the mutations, because mutation artifacts are relatively common in formalin-fixed materials (19). One case was thereby excluded from the present analysis because of inconsistent results.

**Microsatellite Analysis.** To determine the presence of loss of heterozygosity occurring at 3p, 9p, and 18q, microsatellite analysis was performed using seven microsatellite markers [*D3S4103* (3p14.2), *D3S966* (3p21), *D3S1478* (3p2), *D3S1537* (3p25), *D3S1351* (3p25), *INFA* (9p21), and *D18S46* (18q21)]. One-μl aliquots of solution were amplified with a pair of primers in a volume of 10 μl including 0.5 μl of [α-$^{32}$P]dCTP (3000 Ci/mmol, 10 mCi/ml) with AmpliTaq Gold. The PCR products were diluted with loading buffer, heat degenerated, loaded onto denaturing 8% polyacrylamide gels, and subjected to electrophoresis at 50 W for 3 h at room temperature. After electro-

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[3] The abbreviations used are: LOH, loss of heterozygosity; SSCP, single strand conformation polymorphism; ClnI, clonality index.

phoresis, the gels were dried and used to expose X-ray films at room temperature for 24 h. At least triplicate experiments were performed to exclude PCR artifacts in LOH analysis. Three cases were excluded because results were not reproducible.

**DNA Sequencing.** Samples showing variant p53 patterns upon PCR-SSCP analysis were selected for DNA sequencing. The amplified bands were then cut out of the gel of PCR-SSCP analysis. The gels were suspended in 50 μl of distilled water and heated at 75°C for 15 min. One-μl aliquots of solution were amplified with a pair of primers in a volume of 50 μl with AmpliTaq. PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). They were sequenced by AmpliCycle Sequencing kit (Perkin-Elmer, Alameda, CA).

## RESULTS

**Clinical Diagnoses Using Martini and Melamed's Criteria and Morphological Features.** Following the criteria proposed by Martini and Melamed (8), 11 of the 14 patients were diagnosed to have pulmonary metastasis, and 3 were diagnosed to have multicentric primary tumors (Table 1). The histological combinations in 14 patients were adenocarcinoma-adenocarcinoma (9 patients), squamous cell carcinoma-squamous cell carcinoma (2 patients), large cell carcinoma-large cell carcinoma (1 patient), adenocarcinoma-squamous cell carcinoma (1 patient), and small cell carcinoma-adenocarcinoma (1 patient). Hence, in 12 of 14 (86%) cases, the histological type was the same, whereas in 2 patients, the lesions differed.

**Detection of *p53* Gene Mutations.** Eleven *p53* gene mutations were detected in 11 tumors (35%) occurring in 6 patients (Figs. 1 and 2; Table 2). Four cases (cases 3, 5, 9, and 14) had identical SSCP gel patterns in their pairs of tumors, whereas in 2 cases (cases 2 and 11), only one of the two tumors showed an abnormal bandshift in the SSCP gel. Four cases (cases 3, 5, 9, and 14) had identical p53 abnormalities upon DNA sequencing. With 8 patients, neither of the tumors harbored a p53 mutation.

**Detection of LOH at Chromosomes 3p, 9p, and 18q.** LOH at 3p was detected in 19 of 29 tumors (66%) in total, 11 of 20 (55%) adenocarcinomas, 5 of 6 (83%) squamous cell carcinomas, 2 of 2 (100%) large cell carcinomas, and 1 of 1 (100%) small cell carcinoma (Figs. 1 and 2). The incidence of LOH at 9p21 was 5 of 17 informative tumors (29%) in total, 5 of 15 (33%) adenocarcinomas, and 0 of 4 (0%) squamous cell carcinomas with 12 tumors not informative. The incidence of LOH at 18q21.1 was 2 of 23 informative tumors (9%), and 6 tumors were not informative. In 10 patients (cases 1, 3, 4, 6, 7, 8, 10, 11, and 14), LOH patterns of their tumors were identical, whereas in five cases (cases 2, 5, 9, and 12), they were discordant.

**Interpretation of the Results and Molecular Diagnosis of Clonality (Figs. 2 and 3).** In patients with tumors harboring the same p53 mutation (cases 3, 5, 9, and 14), the diagnosis made was intrapulmonary metastases, because the possibility of those occurring in two independent tumors by chance alone is extremely unlikely.

In contrast, we had to take into account the fact that LOH pattern could be identical by chance alone, even when two
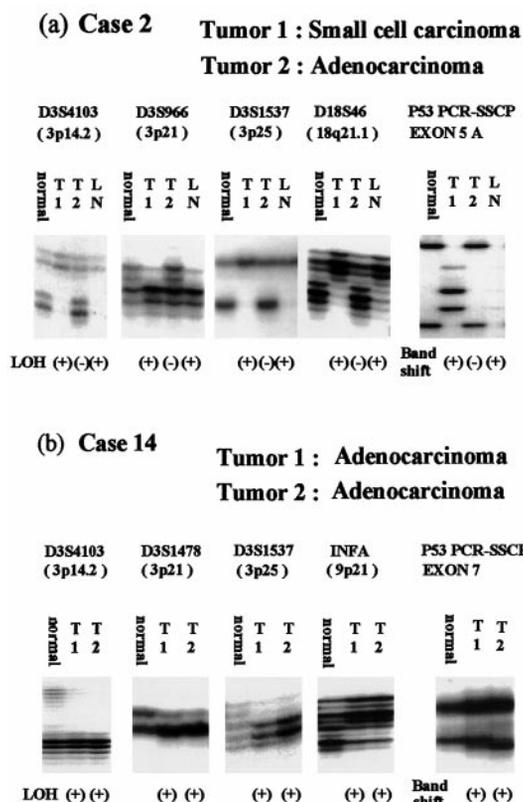


*Fig. 1* Examples of analyses of *p53* gene mutations and microsatellites in patients with synchronous multiple cancers. *a*, a 59-year-old man (*Case 2*) had two tumors in the left lower lobe with hilar lymph node metastasis. Histologically, one tumor and lymph node metastasis was a small cell carcinoma (*Tumor 1*), and the other was an adenocarcinoma (Tumor 2). Tumor 1 (*T1*) exhibited LOH at 3p14 3p21 3p25, 18q21, and a p53 mutation in exon 5, whereas tumor 2 (*T2*) showed no abnormalities at those loci. Furthermore, the metastatic lymph node (*LN*) showed identical patterns, with T1 suggesting this to be its parent. *N,* adjacent normal-appearing pulmonary tissue. *b*, a 56-year-old man (*Case 14*) had two adenocarcinomas in his left upper lobe. Both tumors demonstrated loss of one allele at 3p14, 3p21, 3p25, and 9p21. There were also the same bandshifts in exon 8 of the *p53* gene.

tumors are clonally independent in origin. Therefore, we calculated the probability of given p53 and LOH patterns under the hypothesis that these tumors were independent or multicentric tumors for cases with identical LOH patterns and no p53 mutation (cases 1, 4, 6, 7, 8, 11, and 13). When this probability, which we called ClnI, was <0.05, then the hypothesis was rejected and the patients was diagnosed as having intrapulmonary metastasis. Frequencies of LOH at respective chromosomal loci on which the clonality index was based were estimated as 0.28 at 3p14, 0.64 at 3p21, 0.33 at 3p25, 0.39 at 9p21, and 0.55 at 18q21, in line with previous reports (20–25). Similarly, the frequency of p53 mutations was estimated to be 0.5. For example, the ClnI for patient 1 was calculated as follows: probability of no LOH at 3p21, 1 − 0.64 = 0.36; probability of same paternal (or maternal) loss at 3p25, 0.33 × 0.5 = 0.165; probability of no LOH at 9p21, 1 − 0.39 = 0.61; probability of no LOH at 18q21, 1 − 0.55 = 0.45; probability of no p53 mutation,

**Fig. 2** Summary of the results of molecular analyses for patients with synchronous multiple lung cancers. □, allelic retention; ■, allelic loss; the position of the *black boxes* indicates the relative position of the lost alleles; *NI,* not informative. *E5A,* bandshift of anterior exon 5 in p53 PCR-SSCP; *E5B,* bandshift of posterior exon 5; *E7,* bandshift of exon 7; *E8,* bandshift of exon 8. Patients in whom LOH patterns did not conflict with p53 analysis are grouped in *a,* whereas those in whom there was discordance are grouped in *b.*

*Table 2* *p53* gene mutations in synchronous multiple lung cancers

| Patient no. | Tumor | Exon | Codon | Mutation |
|---|---|---|---|---|
| 2 | Tumor 1 | Intron 4 to exon 5 | | 24-bp deletion |
| | Tumor 2 | No mutation | | |
| 3 | Tumor 1 | Exon 7 | 249 | AGG→TGG(Arg→Trp) |
| | Tumor 2 | Exon 7 | 249 | AGG→TGG(Arg→Trp) |
| 5 | Tumor 1 | Exon 5 | 155 | ACC→ATC(Thr→Ile) |
| | Tumor 2 | Exon 5 | 155 | ACC→ATC(Thr→Ile) |
| 9 | Tumor 1 | Exon 5 | 169 | ATG→AATG |
| | Tumor 2 | Exon 5 | 169 | ATG→AATG |
| | Tumor 3 | Exon 5 | 169 | ATG→AATG |
| 10 | Tumor 1 | No mutation | | |
| | Tumor 2 | Exon 5 | 172 | GTT→TTT(Val→Phe) |
| 14 | Tumor 1 | Exon 7 | 246 | ATG→GTG(Met→Val) |
| | Tumor 2 | Exon 7 | 246 | ATG→GTG(Met→Val) |

$1 - 0.50 = 0.50$. Therefore, the probability of the given pattern in case 1 was $0.36 \times 0.165 \times 0.61 \times 0.45 \times 0.5 = 0.008$. All ClnIs listed in Fig. 2 were <0.05 except for case 3, suggesting that these are intrapulmonary metastases. In case 6, diagnosis of pulmonary metastasis appeared at odds with independent tumors based on histological subtyping (*e.g.,* squamous *versus* adeno-carcinoma) at first sight. However, the fact that tumor 1 of this case had a squamous component did not conflict with molecular diagnosis.

In three cases (cases 5, 9, and 10), the LOH patterns apparently conflicted with the results of p53 analysis (Fig. 2*b*). Cases 5 and 9 showed different LOH patterns but had the same p53 mutation. On the basis that LOH patterns were almost identical with the exception of 1 or 2 loci, they were diagnosed as intrapulmonary metastases. In the other case (case 10), the same LOH pattern (ClnI, 0.02) was found, but only one tumor had a p53 mutation. It is conceivable that this case is intrapulmonary metastasis because the ClnI was

low, and it is possible to acquire p53 mutations late in neoplastic development; but this case presented difficulty in diagnosis.

Case 2 showed different LOH and p53 PCR-SSCP patterns and was therefore diagnosed as suffering from multi-centric lung cancers. Case 12 showed different LOH patterns but was not informative for p53 analysis. Only one tumor showed LOH of 3p25, but both tumors showed the same pattern in other loci. Because the ClnI except for 3p25 was low at 0.01, we assumed that metastatic cells originating from a parental clone had later acquired 3p25, but this case presented difficulty in diagnosis.

Fig. 3 summarizes results of our molecular diagnoses. We were able to evaluate clonal origin of multiple lung tumors in 12 of 14 cases (86%) examined. Two of 14 cases (14%) presented difficulty in diagnosis. Eleven of 12 cases were diagnosed as having pulmonary metastasis, whereas only one case was considered to have genuinely multiple cancers.
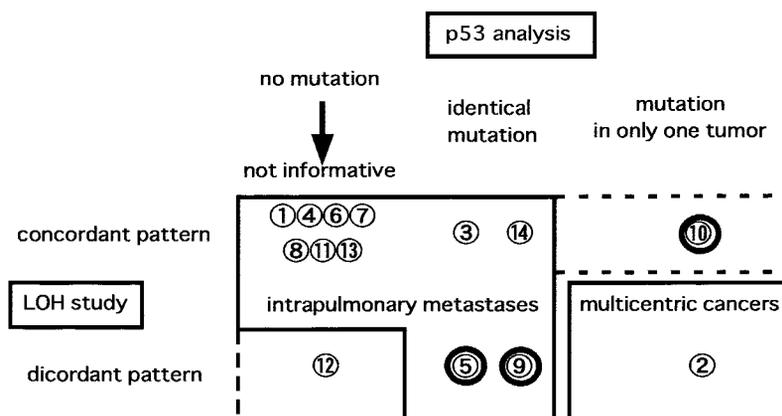
*Fig. 3* Paired lung tumors from all 14 patients were grouped according to patterns of LOH and p53 mutations. In three cases (cases 5, 9, and 10; *double circles*), the LOH results conflicted with those of p53 mutation analysis. However, it was more likely that case 5 and 9 were intrapulmonary metastases because conflict was noted in only one or two loci in each case, whereas there were concordant patterns in 5–7 loci. Case 12 showed different LOH patterns but was not informative for p53 analysis. Because the ClnI except for 3p25 was low at 0.01, we assumed that metastatic cells originating from a parental clone had later acquired 3p25, but this case presented difficulty in diagnosis. In case 10, the same LOH pattern (ClnI, 0.02) was found, but only one tumor had a p53 mutation. It is conceivable that this case is intrapulmonary metastasis, because the ClnI was low, and it is possible to acquire p53 mutations late in neoplastic development; however, this case presented difficulty in diagnosis.

## DISCUSSION

In the present study, we could make a reasonable estimate of the clonal origin of multiple lung tumors in 12 of 14 unselected patients (86%). This was made possible by our strategy of combining p53 mutation analysis with LOH studies. Only 5 of 14 cases (36%) were informative by *p53* gene analysis, whereas 10 of 14 cases (71%) were informative by LOH study alone. p53 status is highly informative when p53 mutation was present in at least one tumor, but the incidence of mutation is not high enough to allow diagnosis for all cases, whereas incidences of LOH were high when examining multiple chromosomal arms, but interpretation of results needed statistical consideration. In this respect, it can be said that the p53 study formed a contrast to those of LOH. To reasonably estimate clonal origin of multiple tumors where LOH patterns were identical, we introduced the ClnI, a probability of the occurrence of a given LOH pattern when these tumors were hypothesized to be independent in origin.

The finding that nearly all patients had tumors diagnosed as intrapulmonary metastatic cancers was somewhat unexpected and contrary to the "field cancerization" concept (3) that multiple cells independently undergo neoplastic transformation with a similar genetic and environmental background, resulting in clonally distinct tumors. However, Leong *et al.* (17) reported that, on examining head and neck squamous cell carcinomas and solitary lung nodules for 3p and 9p LOH, most (12 of 16 cases) were metastases and that independent transforming events were uncommon, in line with our observations.

Although there have been several reports of molecular approaches to clonal distinction of metachronous multiple lung tumors, those on synchronous lesions are relatively few. Matsuzoe *et al.* (26) reported that multicentric lung cancers were more frequent than intrapulmonary metastases after analyzing synchronous multiple lung cancers for p53 mutations. Of seven informative cases in 20, four had mutations in only one of the two tumors, two patients had different p53 mutations, and one had identical p53 mutations. They thus concluded that the first and second cases could be classified as multicentric lung cancers, and that the multicentric cancers were more frequent than the intrapulmonary metastatic cancers in synchronous multiple cancers. This is in contrast with our results and the results of Leong *et al.* (17). However, Matsuzoe *et al.* (26) performed only p53 analysis, and the low rate of informative cases might be misleading for estimation of the real incidence. Four patients with mutations in only one of their two tumors might have had intrapulmonary metastases like our patient 10. Again, we would like to emphasize the importance of combining p53 and LOH analysis for higher specificity and sensitivity as well as repeated confirmation of reproducibility of the results.

Mutations of the *p53* gene or loss of chromosomal arms are in general believed to occur early in the pathogenesis of lung cancers. For example, Reichel *et al.* (15) reported that the p53 mutational status was concordant in the primary tumor and its corresponding multiple metastases in 94% of cases in 9 patients on examining a total of 26 primary lung tumors and 60 metastases. In several cases, the p53 pattern conflicted with the LOH pattern in the present series, and we were forced to interpret some genetic lesions (LOH at 3p25, 3p21, 18q21, or p53 mutation) as occurring later separately after establishment of pulmonary metastases. Case 10 showed p53 mutation in only one tumor but the same LOH pattern (see Fig. 2). This case presented difficulty in diagnosis. Because the ClnI was 0.02, the second tumor was more likely to be a subclone originating from the same parental clone that acquired metastatic potential with p53 mutations than an independent multicentric tumor. In cases 5 and 9, the situation was opposite, *i.e.*, the same p53 pattern but different LOH. However, we also have diagnosed intrapulmonary metastases based on the fact that it is extremely rare to have the same p53 mutations by chance alone. Therefore, if we had not combined p53 analysis with LOH analysis, we might have

reached the wrong conclusion. It is important to bear in mind that there is a danger of misdiagnosis by making distinctions by a single method.

In conclusion, we have shown that molecular biological methods are useful to distinguish between multicentric lung cancers and intrapulmonary metastasis for patients with synchronous multiple tumors. In most cases, tumors were diagnosed as being clonally related, indicating one tumor to be a pulmonary metastasis from the other. Molecular diagnosis was concordant with Martini and Melamed (8) criteria in 10 of 12 (83%) cases. This high concordance does not detract from the utility of our molecular approach. Because materials obtained by transbronchial lung biopsy or transthoracic needle biopsy have been shown to allow molecular analysis (27, 28), information on clonal origin is available at the time of treatment planning. Precise determination of the clonal origin of multiple lung tumors might help rationalize treatment strategy and hopefully might improve prognosis of the affected patients.

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# High Frequency of Clonally Related Tumors in Cases of Multiple Synchronous Lung Cancers as Revealed by Molecular Diagnosis

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