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

Shigeki Shimizu, Yasushi Yatabe, Takashi Koshikawa, Nobuhiro Haruki, Shunzo Hatooka, Masayuki Shinoda, Motokazu Suyama, Makoto Ogawa, Nobuyuki Hamajima, Ryuzo Ueda, Takashi Takahashi, and Tetsuya Mitsudomi

Departments of Thoracic Surgery [S. S., S. H., M. Sh., M. Su., T. M.], Pathology and Clinical Laboratories [Y. Y., T. K.], and Internal Medicine [M. O.], Aichi Cancer Center Hospital, Nagoya 464-8681; Department of Epidemiology [N. H.] and Division of Molecular Oncology [N. H., T. T.], Aichi Cancer Center Research Institute, Aichi Cancer Center, Nagoya 464-8681; Department of Internal Medicine II, Nagoya City University Medical School, Nagoya 467-8601 [S. S., R. U.]; Japan

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 225 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
metastatic spread (15). In our previous clonal analysis of metastatic 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 occurring on chromosome 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 8 of 16 patients, but synchronous multiple lung tumors, genetic diagnosis could be made in the remaining 7 (16).

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 consisted 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-32P]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 autodigestion. 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 [D3S103 (3p14.2), D3S966 (3p21), D3S478 (3p2), D3S1537 (3p25), D3S1351 (3p25), D3S1351 (3p25), INFA (9p21), and D18S546 (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-32P]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-

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at diagnosis</th>
<th>Sex</th>
<th>No. of lesions</th>
<th>Histologya</th>
<th>Tumor 1</th>
<th>Tumor 2</th>
<th>Tumor 3</th>
<th>Distribution</th>
<th>Lymph node metastasis</th>
<th>Martini and Melamed criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>M</td>
<td>2</td>
<td>BAC</td>
<td>BAC</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>M</td>
<td>2</td>
<td>SM</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>+</td>
<td>MC</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>M</td>
<td>2</td>
<td>LA</td>
<td>LA</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>+</td>
<td>PM</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>F</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>+</td>
<td>PM</td>
</tr>
<tr>
<td>5</td>
<td>77</td>
<td>M</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>MC</td>
</tr>
<tr>
<td>6</td>
<td>69</td>
<td>M</td>
<td>2</td>
<td>AD</td>
<td>SQ</td>
<td></td>
<td></td>
<td>different lobe</td>
<td>–</td>
<td>MC</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>F</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>+</td>
<td>PM</td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>F</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>different lobe</td>
<td>–</td>
<td>MC</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>M</td>
<td>3</td>
<td>SQ</td>
<td>SQ</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>+</td>
<td>PM</td>
</tr>
<tr>
<td>10</td>
<td>76</td>
<td>M</td>
<td>2</td>
<td>SQ</td>
<td>SQ</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
<tr>
<td>11</td>
<td>66</td>
<td>M</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
<tr>
<td>12</td>
<td>35</td>
<td>F</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
<tr>
<td>13</td>
<td>53</td>
<td>F</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
<tr>
<td>14</td>
<td>56</td>
<td>M</td>
<td>2</td>
<td>AD</td>
<td>AD</td>
<td></td>
<td></td>
<td>same lobe</td>
<td>–</td>
<td>PM</td>
</tr>
</tbody>
</table>

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 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,
The probability of the given pattern in case 1 was 0.36, 0.16, 0.61, and 0.45, respectively, in the order of P53, E7, E5A, and E5B. Patients in whom LOH patterns did not conflict with p53 analysis were grouped in a, whereas those in whom there was discordance were grouped in b.

Table 2  p53 gene mutations in synchronous multiple lung cancers

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Tumor</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Tumor 1</td>
<td>Intron 4 to exon 5</td>
<td></td>
<td>24-bp deletion</td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>No mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tumor 1</td>
<td>Exon 7</td>
<td>249</td>
<td>AGG→TGG(Arg→Trp)</td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>Exon 7</td>
<td>249</td>
<td>AGG→TGG(Arg→Trp)</td>
</tr>
<tr>
<td>5</td>
<td>Tumor 1</td>
<td>Exon 5</td>
<td>155</td>
<td>ACC→ATC(Thr→Ile)</td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>Exon 5</td>
<td>155</td>
<td>ACC→ATC(Thr→Ile)</td>
</tr>
<tr>
<td>9</td>
<td>Tumor 1</td>
<td>Exon 5</td>
<td>169</td>
<td>ATG→AATG</td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>Exon 5</td>
<td>169</td>
<td>ATG→AATG</td>
</tr>
<tr>
<td></td>
<td>Tumor 3</td>
<td>Exon 5</td>
<td>169</td>
<td>ATG→AATG</td>
</tr>
<tr>
<td>10</td>
<td>Tumor 1</td>
<td>No mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>Exon 5</td>
<td>172</td>
<td>GTT→TTT(Val→Phe)</td>
</tr>
<tr>
<td>14</td>
<td>Tumor 1</td>
<td>Exon 7</td>
<td>246</td>
<td>ATG→GTG(Met→Val)</td>
</tr>
<tr>
<td></td>
<td>Tumor 2</td>
<td>Exon 7</td>
<td>246</td>
<td>ATG→GTG(Met→Val)</td>
</tr>
</tbody>
</table>
Molecular Diagnosis of Synchronous Multiple Lung Cancers

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 metastatic cancers. 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 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.

REFERENCES

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

Shigeki Shimizu, Yasushi Yatabe, Takashi Koshikawa, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/10/3994

Cited articles
This article cites 28 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/10/3994.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/6/10/3994.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.