Genomic and Mutational Profiling to Assess Clonal Relationships Between Multiple Non–Small Cell Lung Cancers

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Abstract Purpose: In cases of multiple non–small cell lung cancer, clinicians must decide whether patients have independent tumors or metastases and tailor treatment accordingly. Decisions are currently made using the Martini and Melamed criteria, which are mostly based on tumor location and histologic type. New genomic tools could improve the ability to assess tumor clonality.

Experimental Design: We obtained fresh-frozen tumors specimens from patients who underwent surgery on at least two occasions for presumptively independent NSCLC. We did array comparative genomic hybridization (aCGH), mutational profiling of select genes, and detailed clinicopathologic review.

Results: We analyzed a total of 42 tumors from 20 patients (6 patients with synchronous tumors, 14 patients with metachronous tumors, 24 potential tumor pair comparisons); 22 tumor pairs were evaluable by aCGH. Surprisingly, classification based on genomic profiling contradicted the clinicopathologic diagnosis in four (18%) of the comparisons, identifying independent primaries in one case diagnosed as metastasis and metastases in three cases diagnosed as independent primaries. Matching somatic point mutations were observed in these latter three cases. Another four tumor pairings were assigned an “equivocal” result based on aCGH; however, matching somatic point mutations were also found in these tumor pairs. None of the tumor pairs deemed independent primaries by aCGH harbored matching mutations.

Conclusion: Genomic analysis can help distinguish clonal tumors from independent primaries. The development of rapid, inexpensive, and reliable molecular tools may allow for refinement of clinicopathologic criteria currently used in this setting. (Clin Cancer Res 2009;15(16):5184–90)

Lung cancer is the leading cause of cancer-related death worldwide (1). For patients with localized non–small cell lung cancers (NSCLC), surgery remains the best curative option (2). However, when two lung lesions are found, clinicians must decide whether patients have independent tumors or metastases and tailor treatment accordingly. With the increase in use of computed tomography scans of the chest for screening or surveillance, the identification of multiple primary lung cancers is becoming an increasingly common clinical problem (3).

In the 1970s, Martini and Melamed (4) developed a set of clinical and pathologic guidelines for the diagnosis of multiple primary lung cancer (Supplementary Table S1). The criteria rely upon tumor location and histology. These do not incorporate any molecular biological tools (5). Some studies have compared mutational and/or molecular profiles of NSCLC at candidate markers for this purpose (6–8), but only a few have actually correlated the molecular findings in the context of the Martini and Melamed criteria (9–11). Importantly, these studies have analyzed only a limited number of genetic markers, theoretically not enough to distinguish independent clones from metastatic foci.

In this study, we did a comprehensive molecular and clinicopathologic analysis of 20 patients originally presumed to have...
multiple primary lung cancers. Tumors were analyzed by array comparative genomic hybridization (aCGH), a method that allows identification of copy number changes across the genome (12). By identifying precise regions of allelic gains and losses, aCGH has the potential to establish whether tumors are independent (i.e., lack matching gains/losses) or clonal (i.e., contain matching gains/losses) with very high confidence (13). Tumors were also profiled for the presence of multiple somatic mutations commonly found in lung cancer.

Patients and Methods

Tissue procurement. Tumor specimens from patients with lung cancer who underwent surgical resection at Memorial Sloan-Kettering Cancer Center from January 1999 to December 2007 were obtained with patients' consent under institutional review board–approved protocols (#92-055 and #06-107). During the study period, a total of 3,846 NSCLC tumors were resected; frozen specimens from 1,508 (39%) tumors were collected either in the operating room or in the Department of Pathology and stored at -80°C in institutional tumor banks. We included only those who received an operation for more than one NSCLC and who had available individual tumor specimens with >70% tumor content from at least two tumors banked separately. At the time of

Translational Relevance

The incidence of multiple primary lung cancers is increasing. When patients are found to have multiple primary lung lesions, clinicians must decide whether they have independent tumors or metastases and tailor treatment accordingly. Decisions are currently made using clinical and pathologic criteria. Here, we did a comprehensive molecular and clinicopathologic analysis of 20 patients originally presumed to have multiple primary lung cancers. Tumors were analyzed by array comparative genomic hybridization (aCGH), a method that allows identification of copy number changes across the genome (12). By identifying precise regions of allelic gains and losses, aCGH has the potential to establish whether tumors are independent (i.e., lack matching gains/losses) or clonal (i.e., contain matching gains/losses) with very high confidence (13). Tumors were also profiled for the presence of multiple somatic mutations commonly found in lung cancer.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Case</th>
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<th>Tumor C</th>
<th>Martini Melamed criteria</th>
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<td>Metastases</td>
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NOTE: No patient presented with systemic metastases. Stage was assigned as per reference 15. Abbreviations: RUL, right upper lobe; RLL, right lower lobe; RML, right middle lobe; LUL, left lower lobe; LLL, left lower lobe; ADC, adenocarcinoma; LCNEC, large-cell neuro-endocrine carcinoma; N/a: not applicable.
*Tumor A vs tumor B.
†Tumor B vs tumor C.
‡Tumor A vs C.
diagnosis, these patients were considered to have multiple primaries on a clinicopathologic basis.

Clinical and pathologic data review. To differentiate multiple primary NSCLCs from metastatic NSCLC, we used the clinicopathologic criteria established by Martini and Melamed (Supplementary Table S1; ref. 4). All available pathologic materials were reviewed by an expert pathologist (W.D.T.) for tumor classification (14). Disease status was staged according to the American Joint Committee on Cancer staging system for NSCLC (15).

Verification of the identity of paired samples. DNA was extracted from tissue specimens using a kit (DNeasy; Qiagen) or standard phenol extraction. In all cases, the banked tumors from individual patients were verified to belong to the same patient by genotyping of specimens for 23 highly informative single-nucleotide polymorphisms using mass spectrometry-based genotyping (16).

Genomic profiling. DNA was hybridized to Agilent 244K comparative genomic hybridization arrays (Agilent Technologies; see Supplementary Materials and Methods). The resulting arrays consisted of markers that estimate copy number at each of 244K locations across the genome. The patterns of gains and losses for each pair of tumors were compared using statistical methodology developed specifically for this purpose. The method calculates a measure that represents the relative chances that at least one of the observed concordant mutations is of clonal origin. Closely matching allelic changes contribute to a high value of this measure. These measures are then calibrated for the patients using a reference histogram based on the pairwise comparisons of all pairs of tumors that come from different patients in the study (which are definitively independent). The individual patients’ tumors are then classified as clonal metastases if the measure exceeds the upper limit of this reference histogram, and as independent if the measure decreases below the 95th percentile of the reference distribution, with the intermediate results being considered “equivocal.”

Fig. 1. A, genomic profiles of multiple tumors from case 7. Both tumors displayed matching amplification and losses. B, closely matching allelic changes for 8 chromosome arms. aCGH array segmentation for tumor A (top) and B (bottom) are shown. Paired tumors exhibit similar genomic alterations, suggesting the diagnosis of metastases.
**Mutational profiling.** Mutational profiling was done using mass spectrometry–based genotyping (Sequenom; ref. 17). All tumor samples were analyzed for a total of 101 distinct activating mutations in 9 genes encoding components of the epidermal growth factor receptor signaling pathway: EGFR, KRAS, HRAS, NRAS, BRAF, PIK3CA, AKT1, ERBB2, and MEK1 (Supplementary Table S2). Because the corresponding mutant protein products display gain of function and are often mutually exclusive (18, 19), these genetic alterations are considered to be “driver” mutations required for both tumor initiation and maintenance. In addition, EGFR exon 19 and all exons encoding the tumor suppressor, P53, were analyzed using exon-specific PCR amplification followed by dideoxynucleotide-based sequencing (Supplementary Materials and Methods).

**Results**

**Patient characteristics.** Between 1999 and 2007, 559 (14.5%) of 3,846 patients underwent operations for multiple NSCLC; 175 had synchronous tumors, and 384 had metachronous tumors (Supplementary Fig. S1). In total, 42 tumor samples were available from 20 patients, including 18 patients with 2 different tumors, and 2 individuals with 3 different tumors. In total, there were 24 possible tumor pair comparisons (8 in synchronous tumors and 16 in metachronous tumors).

Clinical characteristics of the 20 patients are listed in Table 1. Patients with synchronous tumors had bilateral disease; all but one had no local or regional lymph node involvement. Metachronous tumors occurred in the ipsilateral lung in nine cases; all but one tumor showed no lymph node involvement. The histologic type was similar in matching tumors for 19 pairs, and different for 5 pairs (Table 1).

**Martini and Melamed characterization.** Using the criteria established by Martini and Melamed (Supplementary Table S1), matching tumors were characterized as multiple primary NSCLCs in 7 of 8 synchronous tumor pairs and in 14 of 16 metachronous tumor pairs (Table 1). The remaining cases were classified as intrapulmonary metastases.

**Genomic profiling.** We did aCGH on all 42 tumor samples, but had to eliminate 2 cases from evaluation because of poor array quality. Thus, 22 tumor pairs were evaluable by aCGH.

Case 7 illustrates the power of this approach. The tumors were considered to be independent multiple primaries by clinical criteria (Table 1), but the genomic profiles were remarkably similar (Fig. 1A). Both tumors displayed a matching amplification of the whole arm on 1q and matching whole arm losses on 5q, 14q, and 21q. More strikingly, both tumors harbored within-arm gains and losses with plausibly identical start and stop locations. For example, closely matching allelic changes were identified on eight separate chromosome arms (Fig. 1B). Conversely, case 4 is an example where the patterns of gains and losses were seemingly random (Fig. 2). These results suggest that the tumors arose independently and were thus multiple primaries.

Overall, among the 22 paired comparisons, genomic profiling led to a diagnosis of metastases and multiple primaries in 4 and 14 cases, respectively. The conclusion was equivocal in the remaining four comparisons. Plots of allelic changes for all cases are displayed in Supplementary Fig. S2, and the reference histograms are displayed in Supplementary Fig. S3.

**Mutational profiling.** Results of mutational profiling are listed in Table 2. Matching point mutations were observed in two synchronous tumor pairs and in six metachronous pairs (including a matching KRAS mutation for all three tumors of case 20). Discordant mutations were observed in nine paired tumors. Interestingly, the matched mutations occurred in the four paired tumors that were classified as metastases by genomic profiling (cases 6, 7, 9, and 13) and also in the four “equivocal” cases (comparison of tumors A and C for case 5 and all 3 pairings of case 20). Conversely, there were no matching mutations in any of the pairings diagnosed by genomic profiling as multiple primaries. Collectively, these data suggest that tumor mutation status is highly concordant with results from aCGH studies. They also suggest that our predetermined methodologic strategy for determining an equivocal region of diagnostic uncertainty may be inappropriately conservative in failing to classify cases as clonally related.

**Comparison of genomic and mutational profiling with clinicopathologic criteria.** The results from all of the classification schemes are summarized in Table 3. The genomic classification contradicted the clinical diagnosis in 4 (18%) of 22 comparisons,
identifying clonal patterns (metastases) in 3 (16%) cases diagnosed as independent primaries (cases 7, 9, and 13), and no evidence of clonality in 1 case diagnosed clinically as a metastasis (case 19). If the equivocal cases are considered to be a tentative diagnosis of metastases, then the genomic approach contradicted the clinical diagnosis in 7 of 22 comparisons (32%). In metachronous multiple primary cases that were misclassified, the size of the first resected tumor was generally larger than the corresponding tumor in cases that were correctly classified (means, 7.0 cm versus 3.2 cm; \( P = 0.04 \), Wilcoxon Mann-Whitney test, two sided). Similarly, the misclassified cases had somewhat shorter intervals from first to second primary diagnosis, although this difference was not significant (means, 25 versus 37 months; \( P = 0.21 \), Wilcoxon Mann-Whitney test).

### Discussion

Emerging data indicate that molecular analysis of patterns of somatic changes within tumor DNA has the potential to improve diagnostic classifications of second tumors as either metastases or independent second primaries. Thus far, studies have focused primarily on cancer sites where second primaries are common, such as those involving the bladder, head and neck, and breast. The typical strategy has been to genotype markers at candidate loci where somatic mutations are known to occur commonly in the tumor under investigation. More recently, the use of aCGH has been applied to this problem, in recognition of the attractiveness of using a comprehensive genome-wide strategy (20, 21), especially in breast tumors (13, 22–26). Studies of lung cancer using this technology have been rare and not directly targeted at the classification of multiple lung tumors (27, 28). Our goal in this study was to apply aCGH and mutational profiling to the problem of multiple primary NSCLCs to determine their usefulness in assessing clonality of tumors.

Our results provide strong evidence that genomic profiling can help distinguish clonal tumors from independent primaries. However, the application of aCGH in normal clinical practice would be challenging. First, the need for fresh frozen tissue would make the technique more immediately applicable for evaluating synchronous tumors. For metachronous tumors, frozen tissues would need to be stored and accessible in an easy manner. Patients who have tumors resected at different institutions would require additional coordination for genomic profiling. Second, standardized software for routine automated statistical comparisons of the array data would need to be widely available, although software for the application of the methods in this article is available from the authors. Third, aCGH is expensive and can be a time-consuming and labor-intensive procedure. Fourth, aCGH requires relatively large amounts of DNA and array quality can be variable, depending upon differences in tumor content, tissue handling, specimen storage, and specimen processing. For example, despite using fresh-frozen tumor samples in an experimental (nonclinical) setting, we found arrays from 2 of the 42 tumors to be of insufficient quality to reach a diagnosis with confidence. The requirement for abundant DNA also may preclude such analysis on DNA derived from needle biopsies before surgery, where such results may actually be more useful in deciding whether patients should undergo surgery in the first place.

A more practical solution may be to develop strategies based on mutational profiling that can be done using DNA extracted from formalin-fixed, paraffin-embedded tissue rather than frozen samples. Although mutational profiling is less comprehensive than aCGH, it may yet have sufficient sensitivity for improved diagnoses, as evidenced by the strong concordance of the genomic and mutational profiling results in this study. The strength of the evidence from individual mutational matches depends on the rarity of the mutation. For example, three of our matching cases involved matching KRAS G12V

### Table 2. Mutational profiling of synchronous and metachronous multiple NSCLC

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<tr>
<th>Case</th>
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<th>EGFR</th>
<th>KRAS</th>
<th>BRAF</th>
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<td>V600E</td>
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**NOTE:** HRAS, NRAS, AKT1, ERBB2, and MEK1 were wild-type (wt) in all tumors.

**Tumors with similar mutations.**

**Truncation.**
mutations. This specific KRAS alteration occurs at a frequency of 4.4% in lung adenocarcinomas (29). Thus, the probability of a match in independent tumors, given that the first tumor has the mutation, is 0.044. Viewed in this context, the simultaneous independent occurrence of this mutation in all three tumors for case 20 is an unlikely event with a probability of 0.002 (0.044^2), providing further evidence that these three tumors are of clonal origin. The corresponding population frequencies of the other matches in our study have been estimated to be 5.2% for KRAS G12D and 1.2% for BRAF V600E (30).

Conversely, mutational profiling is limited in practice by the infrequency of the occurrences of specific mutations. Indeed, for the panel of markers in our study, none of the mutations were observed for 20 of the 42 tumors, and none were observed in either tumor for 7 of the 20 patients. Mutational profiling would be more powerful if assays could detect all known loci that are commonly mutated at the somatic level in NSCLC. A panel of about 20 commonly occurring markers would have good statistical power for detecting clonal tumors, provided that the preponderance of the somatic events observed occur in the originating clonal cell (31). Additionally, mutational profiling would allow the use of partially fragmented DNA, such as can be obtained from paraffin-embedded tissue, or of whole genome-amplified DNA, when starting tumor DNA quantities are limited (i.e., derived from bronchoscopic or needle biopsies). Importantly, one of us (ML) has shown that similar results are obtained from mutation testing of EGFR and KRAS using matching formalin-fixed and frozen specimens.9

Our results suggest surprisingly that diagnostic misclassifications may occur frequently when using the Martini-Melamed guidelines with histologic classification according to traditional WHO criteria. Out of 22 evaluable comparisons of tumor pairs, the genomic classification contradicted the clinical diagnoses in four (18%), identifying clonal patterns of metastasis (as in the case highlighted in Fig. 1) in three cases diagnosed as independent primaries. Based on our statistical algorithm, it is highly unlikely that such identical or near-identical allelic changes would have occurred in the tumors independently by chance. In another three cases deemed multiple primaries by clinical criteria but diagnosed as equivocal by aCGH, the identification of matching somatic point mutations by mutational profiling suggested that the tumors were of clonal origin. Thus, potentially up to 7 of the 22 cases (32%) were misclassified by the traditional clinical/histologic criteria alone.

We recognize that we cannot state with absolute certainty that the diagnoses based on genomic profiling are “correct” and that the clinical diagnoses were “wrong.” However, the

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**Table 3. Integration of genomic and mutational profiling with clinicopathologic characterization (using the Martini-Melamed criteria)**

<table>
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<td>Equivocal</td>
<td>Multiple primary</td>
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</table>

*Indicates discrepant results between genomic profiling and clinical evaluation; N/A: not applicable.

†We could not reach a genomic diagnosis in these two cases due to an excessively high level of noise in one of the paired arrays.

9 M. Ladanyi, unpublished data.
kinds of precise matching of regions of allelic losses and gains that we observed seem comprising as a definitive basis for diagnosis from a logical standpoint. Prospective studies with much larger sample sizes will be necessary to validate the usefulness of genomic analyses in relation to patient survival. We further note that this study was based on a highly selected, small sample size. To be included for study, the metastatic patients all had to survive long enough to develop multiple primaries and undergo resection.

Distinguishing multiple primary tumors from metastases may actually not change the initial management of these patients, as surgery has been shown to increase survival in these instances (4, 32). However, in the cases initially considered as independent primaries, reclassification as metastases using genomic profiling could have led to consideration of adjuvant chemotherapy and/or to a closer surveillance monitoring plan. Conversely, patients found to have multiple primaries rather than metastases could be spared from adjuvant chemotherapy.

In summary, our results suggest that genome-wide examination of copy number changes using aCGH and mutational profiling of multiple loci can help distinguish multiple independent primaries from metastases. Molecular diagnostics such as these should be honed for practical application in the clinic.

Disclosure of Potential Conflicts of Interest

W. Pao receives consulting fees from Molecular MD.

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