Comparison of Accumulated Allele Loss between Primary Tumor and Lymph Node Metastasis in Stage II Non-small Cell Lung Carcinoma: Implications for the Timing of Lymph Node Metastasis and Prognostic Value

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ABSTRACT

Although the Tumor-Node-Metastasis staging of non-small cell lung carcinoma (NSCLC) is the most effective predictor of survival, the clinical outcome of patients at each stage is variable on an individual case basis. We tested the value of incorporating information about the tumor heterogeneity of NSCLC into microsatellite allelotyping in a cohort of 48 node-positive stage II patients (T1N1M0 and T2N1M0). Microsatellite allelotyping involved microdissection of the invasive component of primary tumor and lymph node metastasis at multiple target sites followed by loss of heterozygosity (LOH) analysis at specific regions on chromosomes 1p, 3p, 5q, 7q, 8q, 9p, 10q, 17p, and 18q using 16 markers. All microsatellites manifested LOH ranging from 44 to 76% in primary tumor and showed various degree of heterogeneity between primary tumor and lymph node metastasis. LOH on 3p and 5q in the lymph node metastases was associated significantly with shorted survival of the patients ($P = 0.033$ and 0.004, respectively), whereas no single LOH in the primary tumors showed association with prognosis. For the analysis of the accumulated load of allele loss, fractional allele loss (FAL) was calculated for each sample. The maximal FAL of lymph node metastasis was significantly lower than that of primary tumor ($P = 0.0015$), possibly reflecting the early lymphatic spread. High maximal FAL of lymph node metastasis was significantly correlated with an adverse outcome ($P = 0.012$), whereas maximal FAL of primary tumor did not show any prognostic significance ($P = 0.552$). A composite mutational profile for each patient based on the allelotyping of the primary tumor and lymph node deposits may make a significant contribution to a more accurate prognosis of stage II NSCLC.

INTRODUCTION

The prognosis of NSCLC is critically dependent on the extent of the metastatic spread of tumor cells at the time of diagnosis reflected by the clinicopathologic stage of the neoplasm (1). It is apparent that the malignant tumor acquires the metastatic phenotype through the process of clonal evolution occurring during the multistep tumor progression (2, 3). Various molecular genetic events are known to be involved in the process of progression of NSCLC. However, the genetic or epigenetic factors truly critical to a metastatic event still remain unclear. On the other hand, it is unlikely that a single genetic aberration by itself can fully account for the development, progression, and phenotypic expression of cancer. Rather, it is the cumulative effect of acquired gene damage that is likely to be a better determinant of biological tumor aggressiveness, including metastatic spread (4, 5).

Allelic loss, which is one of the common genetic events involved in the process of the tumorigenesis and progression, is known to be accumulated throughout the process of tumor evolution (6–8). The recent development of microdissection-based genotyping using routinely processed archival samples has shown the presence of multiple subpopulations of cells with different type and degree of genetic alteration, including allelic imbalance within individual tumors (9–14). This technique has currently turned out to be the one best suited in drawing the outline of the cross-sectional view of the clonal evolution occurring within individual tumors.

Pathologic staging of malignant tumor, including NSCLC, is based on the concept that primary tumor growth and local invasion precede lymphatic dissemination, which is regarded as a later event in lung cancer progression. From the point of view of molecular genetics, the metastatic event is also considered to be a relatively late event after multiple sequential and selective steps of clonal evolution. However, the timing of the appearance of metastatic variants and its metastatic spread in relation to primary tumor still remain unclear. Recent advances in immunohistochemistry and PCR-based molecular analysis have shown that micrometastatic bone marrow and lymph node involvement occurs even at a fairly early stage of NSCLC (15–18). Furthermore, the correction of conventional staging of NSCLC by incorporating the information of micrometastasis has been shown to predict patients’ survival more accurately (15, 16). Given that not a few populations of NSCLC show metastatic spread at a considerably early stage of the disease, it could be hypothesized that the genetic alteration in surgically resected primary tumor and lymph node metastasis may show different characteristics. These could reflect the evolution process occurring during clonal selection before metastasis or occurring under the different environments after metastatic seeding. It is also hypothesized that molecular characterization of metastatic variant of the tumor cells may provide more accurate information for patients’ prognostication, because the vast majority of recurrence after curative surgery of NSCLC may arise from micrometastasis.

This study was designed to analyze the difference of type and number of allelic loss at multiple sites within individual tumors, including metastatic deposits using a panel of 16 polymorphic microsatellite markers located on nine chromosomal arms. To investigate the timing of lymphatic spread and a prognostic utility of FAL, homogenous cohort of stage II node-positive NSCLC (T1N1M0 and T2N1M0) was selected for study. This group of patients, having earliest tumor with lymph node metastasis, was chosen also because of current interest in prognostic markers for stage II NSCLC and the likelihood that there would be sufficient recurrences in this group to detect significant differences.

MATERIALS AND METHODS

Patients and Tissue Preparation. Forty-eight cases of NSCLC were gathered from the paraffin block archives of the University of Pittsburgh Medical Center. Cases were limited to those occurring between 1994 and 1998 with a minimum of two years follow-up. Histology slides on each case were re-
viewed, and the diagnosis of NSCLC and stage II pathologic status were confirmed using the World Health Organization histological classification (19) and Tumor-Node-Metastasis staging system (1). In all cases but one, a minimum of two topographically distinct sites within the primary tumor was chosen for mutational genotyping to assess intratumoral heterogeneity. Additionally, a minimum of one bronchopulmonary lymph node deposit, if available, was genotyped to afford a genotypic comparison between primary tumor and lymphatic metastasis. The study was performed with the approval and compliance of the University of Pittsburgh Medical Center institutional review board. Four serial sections (4-μm thickness) were obtained from tissue blocks of primary and metastatic lung tumor, as well as normal lung parenchyma. Using histological features as a guide, tumor and normal lung tissue were microdissected by hand from unstained recut sections as described previously (20). Tumor purity was judged to be no <90% using manual microdissection approaches. The range of microdissected tumor tissue size in this study was temporarily defined as 2–6 mm for the convenience of manual microdissection. Normal lung parenchyma was sampled in each case to serve as an internal normal control and for allelic dropout (21). Microdissection of normal lung tissue was carefully performed to be no larger in size than that of any tumor sample for a given case. In view of the fact that normal lung is relatively less cellular than lung tumor, this ensured that all microdissected normal samples were equally or less cellular than tumor samples. Microdissected tissue was digested with protease K as described previously (22).

PCR Amplification and LOH Analysis. Aliquots of the sampled tissue were used in individual PCR amplification reactions targeting microsatellites situated in proximity to specific genes of interest. Mutational genotyping was based on allelic loss using polymorphic microsatellites situated within or adjacent to known tumor suppressor genes or genomic sites potentially involved in human pulmonary carcinogenesis (23–32). Sixteen different microsatellite markers were chosen, being located on 1p, 3p, 5q, 7q, 8q, 9p, 10q, 17p, and 18q, as detailed in Table 1. The use of more than one microsatellite marker ensured a higher yield of information for each genomic locus. Determination of informativeness and allelic imbalance were carried out by \(^{33}P\) autoradiography as described previously (20). Each case of NSCLC yielded a panel of microdissected tumor tissue size in this study was temporarily defined as 2–6 mm for the convenience of manual microdissection. Normal lung parenchyma was sampled in each case to serve as an internal normal control and for allelic dropout (21). Microdissection of normal lung tissue was carefully performed to be no larger in size than that of any tumor sample for a given case. In view of the fact that normal lung is relatively less cellular than lung tumor, this ensured that all microdissected normal samples were equally or less cellular than tumor samples. Microdissected tissue was digested with protease K as described previously (22).

### Table 1 Locations of microsatellite markers and adjacent genomic sites

<table>
<thead>
<tr>
<th>Microsatellite markers</th>
<th>Genetic locations(^a)</th>
<th>Adjacent genes</th>
<th>Genetic locations(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p DIS407 3p DIS1559 5q DIS592</td>
<td>1p36.21 3p26.3 5q23.3</td>
<td>39.47 7.52 115.94</td>
<td>1p36.22 3p25.3 5q23.1</td>
</tr>
<tr>
<td>3p DIS2303</td>
<td>3p25.3</td>
<td>40.42 7.4 182.90</td>
<td>37.35 58.96 111.02</td>
</tr>
<tr>
<td>5q DIS5615</td>
<td>5q23.3</td>
<td>43.95 183.07</td>
<td>36.24 170.09 111.22</td>
</tr>
<tr>
<td>7q D7S1530</td>
<td>7q22.3</td>
<td>155.47 182.90</td>
<td>95.76 166.25 111.22</td>
</tr>
<tr>
<td>8q D8S373 9p D9S254 10q D10S520</td>
<td>8q24.3 9p23</td>
<td>215.95 15.24 10q23.1</td>
<td>188.66 33.35 89.7</td>
</tr>
<tr>
<td>17p D17S1163</td>
<td>17q23.31</td>
<td>49.81 49.1</td>
<td>116.64 146.33 27.71</td>
</tr>
<tr>
<td>18q TP53</td>
<td>18q22.1</td>
<td>125.86</td>
<td>58.93 92.76</td>
</tr>
</tbody>
</table>

\(^{a}\) Data were obtained from http://cedar.genetics.soton.ac.uk/public_html/lbd.html.

\(^{b}\) MCM, male centimorgans; FCM, female centimorgans.

\(^{c}\) Microsatellite marker situated within a target gene locus.

\(^{d}\) NA, not available.

FAL was defined when either the B or T bands were estimated as less than one-half the intensity of the other polymorphic band. LOH 1 was used for the remaining cases showing asymmetry in polymorphic band intensity compared with normal. In this study, allelic loss was finally designated as positive only when a clear-cut band intensity was manifested corresponding to a majority of tumor cases showing asymmetry in polymorphic band intensity compared with normal. The presence of distinct additional alleles in a tumor specimen not detected in the normal control was interpreted as MSI. Samples found to have MSI at a given chromosomal locus were not evaluated for LOH because LOH could not be precisely assessed in MSI-positive samples.

### Determination of LOH in Each Patient

In each case, the presence or absence of LOH in primary tumor and lymph node metastasis was determined separately. For each tumor component, LOH was determined as positive when two observers was 100%. The presence of distinct additional alleles in a tumor specimen not detected in the normal control was interpreted as MSI. Samples found to have MSI at a given chromosomal locus were not evaluated for LOH because LOH could not be precisely assessed in MSI-positive samples.

### Determination of FAL

FAL was defined as the ratio of chromosomes affected by LOH in the informative chromosomes and calculated for every microdissected sample. FALmax of the primary tumor and lymph node metastasis was determined separately in each case by selecting the largest FAL value from those of all microdissected samples in each component. FALmin was also calculated in the same manner.

### Statistical Analysis

Paired Student’s t test was used for the comparison of FAL values between primary tumors and lymph node metastases. Other categorical data were analyzed using Fisher’s exact probability test. A P < 0.05 was taken as the level of significance. Patient survival was analyzed.

**Fig. 1.** Microdissection-based allelotyping of the D10S520 locus (case 7, AD). The normal lung tissue (Lane 1) is informative and shows idoseline allelic bands. All three samples from primary tumor (Lanes 2–4) show LOH of the bottom allele, whereas two samples from lymph node metastasis (Lanes 5 and 6) show LOH of the top allele. Thus, the pattern of LOH at this chromosomal locus is heterogeneous (discordant pattern) between primary tumor and lymph node metastasis.
Informative rate and overall frequency of LOH of each microsatellite

<table>
<thead>
<tr>
<th>Microsatellite markers</th>
<th>Informative rate % (n)</th>
<th>Primary tumor % (n)</th>
<th>Lymph node metastasis % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S407</td>
<td>71% (34/48)</td>
<td>44% (15/34)</td>
<td>45% (13/30)</td>
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<tr>
<td>MYCL1</td>
<td>81% (39/48)</td>
<td>54% (21/39)</td>
<td>43% (15/35)</td>
</tr>
<tr>
<td>D3S1539</td>
<td>77% (37/48)</td>
<td>62% (23/37)</td>
<td>50% (16/32)</td>
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<tr>
<td>D3S3203</td>
<td>79% (38/48)</td>
<td>66% (25/38)</td>
<td>53% (18/34)</td>
</tr>
<tr>
<td>D5S392</td>
<td>65% (31/48)</td>
<td>48% (15/31)</td>
<td>41% (12/29)</td>
</tr>
<tr>
<td>D5S1515</td>
<td>83% (40/48)</td>
<td>60% (24/40)</td>
<td>48% (16/33)</td>
</tr>
<tr>
<td>MCC</td>
<td>50% (24/48)</td>
<td>50% (12/24)</td>
<td>52% (12/23)</td>
</tr>
<tr>
<td>D7S5350</td>
<td>60% (29/48)</td>
<td>45% (13/29)</td>
<td>35% (9/26)</td>
</tr>
<tr>
<td>D8S537</td>
<td>72% (33/46)</td>
<td>36% (12/33)</td>
<td>23% (7/30)</td>
</tr>
<tr>
<td>D15S54</td>
<td>65% (31/48)</td>
<td>71% (22/31)</td>
<td>59% (17/29)</td>
</tr>
<tr>
<td>D9S251</td>
<td>69% (33/48)</td>
<td>48% (16/33)</td>
<td>38% (12/32)</td>
</tr>
<tr>
<td>D10S1173</td>
<td>79% (38/48)</td>
<td>50% (19/38)</td>
<td>40% (14/35)</td>
</tr>
<tr>
<td>D10S520</td>
<td>60% (29/48)</td>
<td>76% (22/29)</td>
<td>64% (16/25)</td>
</tr>
<tr>
<td>D17S1163</td>
<td>90% (43/48)</td>
<td>36% (15/42)</td>
<td>32% (12/38)</td>
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<tr>
<td>TPS3</td>
<td>58% (28/48)</td>
<td>75% (21/28)</td>
<td>67% (16/24)</td>
</tr>
<tr>
<td>D18S814</td>
<td>56% (27/48)</td>
<td>70% (19/27)</td>
<td>42% (10/24)</td>
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</table>

RESULTS

Clinicopathologic Features. The clinicopathologic data are summarized in Table 2. Of the 48 cases studied, 20 were AD, and 23 were SQ. Others include one collision tumor of AD and SQ; one adenocarcinoma, one large cell carcinomas, and two undifferentiated carcinomas. The mean age at the time of surgery of 16 females and 32 males was 66.4 years (sex ratio, 1:2). A follow-up was available in 45 cases (three deaths within 30 days of operation were excluded) with a mean duration of 31.7 months (median 30.3 months with a range of 2.3–81 months).

LOH Analysis and Case Presentation. The informative rate and frequency of allelic loss for each microsatellite marker is shown in Table 3. Informativeness for individual microsatellites ranged from 50 to 90%. All microsatellites selected for study manifested varying
degrees of allelic loss ranging from 36 to 76% in primary tumor samples. More than 70% of the primary tumors showed allelic loss of 9p (D9S254), 10q (D10S520), 17p (TP53), and 18q (D18S814), whereas allelic loss in lymph node metastases was detected most frequently at 10q (D10S520) and 17p (TP53) in >60% of the cases. Representative examples of LOH analysis using genotyping at multiple microdissected sites are shown in Figs. 2, A–C. As shown in these figures, virtually all of the cases showed various degrees of heterogeneity in LOH markers between primary tumor and lymph node deposits, as well as among different areas of the primary tumor,
and this gave each tumor a complex and unique profile. This provides support for a process of continuous clonal and subclonal evolution of mutant tumor cell populations arising in various regions of individual neoplasms becoming predominant over a given area captured by microdissection sampling.

**Intratumoral Heterogeneity of LOH.** All microsatellite markers showed considerable intratumoral heterogeneity of LOH ranging from 13 to 65% (Fig. 3A). In >70% of cases, LOH of 1p (D1S407), 3p (D3S1539 and D3S2303), 5q (MCC), 9p (D9S254 and D9S251), and 17p (TP53) was manifested homogeneously in all different samples of primary tumors. These heterogeneous patterns of allelic losses were consistent with either genetic progression or genetic diversion occurring during the clonal evolution of NSCLC.

**Heterogeneity of LOH between Primary Tumor and Lymph Node Metastasis.** The relationship of LOH between primary tumors and lymph node metastases in each microsatellite marker was shown in Fig. 3B. All of the microsatellite markers showed various degrees of genotypic differences between primary tumor and lymph node metastasis. Among 16 microsatellites, six markers on 1p (D1S407), 3p (D3S1539 and D3S2303), 9p (D9S254), 10q (D10S520), and 17p (TP53) showed relatively consistent changes between the primary tumor and its metastasis in >75% of the cases. Of particular note was that 89% of the cases shared concordant LOH at the TP53 locus. In some microsatellite markers, new allelic loss events were noted in the metastasis that were not present in the primary tumor at the sites sampled. The converse was also observed consisting of lymph node metastasis lacking mutations present in the primary tumor. A few microsatellite markers also showed loss of different alleles on the same locus (discordant LOH) in a few cases.

**Prognostic Value of LOH in Primary Tumor and Lymph Node Metastasis.** Patients whose lymph node metastases demonstrated LOH at D3S1539 or MCC had a shorter survival than those whose lymph node metastases did not (P = 0.037 and 0.011, respectively). When the patient survival was analyzed at the chromosome level, the presence of LOH at 3p or 5q locus in lymph node metastases indicated a worse survival (P = 0.033 and 0.004, respectively). Loss of any single microsatellite or chromosomal locus in the primary tumor did not influence patient survival.

**Comparison of Accumulated Chromosomal Damage between Primary Tumor and Lymph Node Metastasis.** The accumulated mutational damages were analyzed using FAL. FAL values of primary tumor and lymph node metastasis in each case are listed in Table 2. The average and median values of FALmin and FALmax in each tumor component are shown in Table 4. Although the FALmin was not statistically different between primary tumors and lymph node metastases, the FALmax of lymph node metastases was lower by ~0.1 than that of primary tumor, and this difference was statistically significant (P = 0.0015, with paired Student’s t test). Fig. 3C shows the relationship of FALmax between primary tumors and metastatic lymph nodes in a case-by-case manner. FALmax in the lymph node metastasis was equal or lower than that of primary tumor in the majority of cases, and it rarely exceeded that of primary tumor.

**Comparison of Prognostic Value of FAL between Primary Tumor and Lymph Node Metastasis.** On the basis of the finding that the type and degree of chromosomal damage between primary tumor and lymph node metastasis were considerably different, the prognostic value of FALmax in primary tumor and lymph node metastasis was analyzed separately (Fig. 4). When patients were subclassified simply into two categories based on the median value of FALmax, FALmax in primary tumor did not show prognostic significance (P = 0.552). On the other hand, patients whose metastatic deposits showed a high FALmax had a significantly greater risk of an adverse outcome than those with a low FALmax (P = 0.012).

**DISCUSSION**

Recently, molecular pathologic studies of intratumoral and intertumoral heterogeneity, within each individuals using multiple microdissected samples, have increased gradually (9–14, 20, 33, 34). These studies have provided valuable insights into the process of tumor genesis and progression in various human tumors by detecting various patterns of spatial and/or temporal differences of accumulated genetic
For practical clinical analysis, investigation of heterogeneity among histopathologically aggressive tumor components, such as areas of invasion and metastatic deposits, may be important because these components are undoubtedly the most important determinants of a patient’s prognosis. In our study, a substantial amount of mutational heterogeneity was found between primary tumor and lymph node metastasis, as well as among different areas of invasive component in the primary tumor. Among the chromosomal loci tested, allelic loss of 3p (D3S1539 and D3S2303), 9p (D9S254), and 17p (TP53) was relatively frequent and homogeneously manifested throughout the different tumor area and component. In the setting of our study, the high frequency and homogenous manifestation of a certain type of genetic alteration may theoretically indicate: (a) relative early involvement of this alteration in carcinogenesis step; or (b) relative dominant overgrowth of the most aggressive tumor cell clone with this alteration by the second clinicopathologic stage of disease. According to previous studies, allelic loss at the 3p and 17p loci have been shown to be involved at a relatively early stage of NSCLC (12–14, 25, 35). Although allele loss on chromosome 9p could be detected at the early stage of NSCLC or even in the possible precancerous lesions, such as atypical adenomatous hyperplasia or bronchial dysplasia (13, 14), this genetic alteration has also been shown to be associated with advanced stages of disease (12, 36). LOH at 9p locus is probably an important factor in tumorigenesis or the progression of NSCLC; however, the timing of this chromosomal alteration remains unclear in our study.

Recent studies have reported a specific involvement of LOH at certain chromosomal arms, such as 1p, 10q, and 11p loci, in the metastatic process of NSCLC (30, 37–39). It is noteworthy that the frequency of LOH at 10q in our study (71% in primary tumors and 61% in lymph node metastases) was much higher than reported previously in NSCLCs (20–46%; Refs. 28, 40, and 41). Therefore, this finding appears to reflect some unique characteristics of our cohort of NSCLCs. The unusually high frequency of LOH at the 10q locus in our cohort, especially at D10S520, may suggest the possible participation of this locus in lymph node metastasis because all cases of our cohort were node-positive NSCLCs. The association between LOH at the 1p locus and regional lymph node involvement has also been demonstrated in some studies (37, 38). LOH of the D1S407 (not MYCL1) in our study was manifested homogeneously throughout the different area and component, although the frequency of LOH at chromosome 1p was not significantly different from those in other studies. The homogenous manifestation of D1S407 in positive cases may reflect a clonal expansion of aggressive cell subpopulations with this alteration because currently available data do not indicate the early involvement of this locus in NSCLCs. Further investigation using microdissected samples of primary tumors and corresponding lymph node metastases with a larger number of cases is needed to clarify the precise timing and role of these genetic alterations in the metastatic process of NSCLC.

MSI is defined by length mutations in microsatellites in tumor DNA. In the majority of hereditary nonpolyposis colorectal cancer, this phenomenon has been detected in 10–100% of markers tested and is usually linked with defects in a series of DNA mismatch repair genes (42–45). MSI has also been demonstrated in sporadic human

<table>
<thead>
<tr>
<th>Table 4</th>
<th>FAL values in 43 cases of node-positive stage II NSCLCs</th>
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<tbody>
<tr>
<td>Primary tumor (n = 48)</td>
<td>Lymph node metastasis (n = 43)</td>
</tr>
<tr>
<td>Mean ± SD (Median)</td>
<td>Mean ± SD (Median)</td>
</tr>
<tr>
<td>FALmin</td>
<td>0.43 ± 0.26 (0.43)</td>
</tr>
<tr>
<td>FALmax</td>
<td>0.58 ± 0.26 (0.63)</td>
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</table>

Fig. 3. A, intratumoral heterogeneity of allelic loss in stage II NSCLC. a, the pattern of LOH (LOH T or LOH B, see “Materials and Methods”) was concordant across the multiple microdissected samples; b, LOH-positive and −negative samples coexisted, or discordant pattern of LOH was observed. One case of collision tumor (case 44) was excluded from the study. PT, primary tumor. B, heterogeneity of allelic loss between primary tumors and lymph node metastases in stage II NSCLC. One case of collision tumor (case 44) was excluded from the study. a, PT, primary tumor; b, LN, lymph node. C, relationship of FAL values between primary tumor and lymph node metastasis in individual cases in stage II NSCLC.

For practical clinical analysis, investigation of heterogeneity among histopathologically aggressive tumor components, such as areas of invasion and metastatic deposits, may be important because these components are undoubtedly the most important determinants of a patient’s prognosis. In our study, a substantial amount of mutational heterogeneity was found between primary tumor and lymph node metastasis, as well as among different areas of invasive component in the primary tumor. Among the chromosomal loci tested, allelic loss of 3p (D3S1539 and D3S2303), 9p (D9S254), and 17p (TP53) was relatively frequent and homogeneously manifested throughout the different tumor area and component. In the setting of our study, the high frequency and homogenous manifestation of a certain type of genetic alteration may theoretically indicate: (a) relative early involvement of this alteration in carcinogenesis step; or (b) relative dominant overgrowth of the most aggressive tumor cell clone with this alteration by the second clinicopathologic stage of disease. According to previous studies, allelic loss at the 3p and 17p loci have been shown to be involved at a relatively early stage of NSCLC (12–14, 25, 35). Although allele loss on chromosome 9p could be detected at the early stage of NSCLC or even in the possible precancerous lesions, such as atypical adenomatous hyperplasia or bronchial dysplasia (13, 14), this genetic alteration has also been shown to be associated with advanced stages of disease (12, 36). LOH at 9p locus is probably an important factor in tumorigenesis or the progression of NSCLC; however, the timing of this chromosomal alteration remains unclear in our study.

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cancers, including NSCLC (46–49). In our cohort, MSI was found in 9 of 48 cases (19%), and most cases (8 of 9) showed MSI at only one microsatellite marker in a single microdissected sample (data not shown). The presence or absence of MSI was not correlated with the number of accumulated mutational damage or any other clinicopathologic parameters. Additional studies are needed to clarify the role of MSI in NSCLC.

Of particular note in our study was the difference of accumulated amounts of allele loss between primary tumor and lymph node metastasis. Although FALmax was not statistically different between primary tumor and lymph node metastasis, FALmax in the primary tumor was significantly larger than that in lymph node metastasis. At each patient level, FALmax in primary tumor was larger than that in lymph node metastasis in 47% of the cases, whereas FALmax in lymph node metastasis exceeded that in primary tumor only in 16% of the cases (see Fig. 3C). Considering the theory that metastatic subpopulations may result from progressive selection of variant cells within a primary tumor, accumulated genetic damage in lymph node metastasis might be expected to be generally greater than that in primary tumor. However, the authors believe that relatively sparse allelic losses in metastatic deposits of lymph nodes in our study are not a result of artifacts, such as contamination of nonneoplastic cells or underlying statistical error. Even in cases with lower FAL in lymph node metastasis, allelic loss affecting at least one marker was detected in 18 of 20 cases (90%). On the other hand, our result of lower FAL in lymph node metastasis obtained from resection specimens might become different to a varying degree reflecting the period between metastatic event and surgical resection. Even if lower maximal FAL in lymph node metastasis is a reflection of early metastasis, the question must be raised as to why FAL in lymph node metastasis stays at a relatively lower level until the time of surgical operation. It has been shown that the process of cancer metastasis is a highly inefficient one. This concept has recently been comprehensively described and termed “metastatic inefficiency” (3, 50). After infiltration of tumor cells at the primary site and their commencement of metastatic spread, individual cells are required to survive a series of potentially lethal host interactions to successfully form viable and expanding secondary tumor deposits in metastatic target organs (3, 51). Although tumor cells may have metastasized, it is possible that the rate of growth may be lower than that of primary tumor cells given the harsher environment in which they find themselves. This would explain the lower rate of acquisition of mutations we observed in metastatic deposits compared with primary tumor.

Interestingly, it was shown here that mutational profiling based on lymph node deposits was capable of separating stage II NSCLC patients into two groups with a significant difference in survival. A similar analysis performed on the primary tumor, incorporating microdissection sampling of different sites within the tumor manifesting invasive growth, failed to identify these clinically distinct groups. This difference may be related to the observation that having achieved the capacity for metastatic seeding of tumor cells, the primary tumor will continue to accumulate mutational damage. This additional damage may be taking place in cells that otherwise are not representative of the most malignant phenotype within the primary tumor. On the other hand, the metastatic tumor cells within the lymph node may represent a subclone from the original site with a greater tendency for lymphatic spread and may presumably share the genetic profile of undetected microscopic remnants of cancer cells. These cells, more clearly more representative of the malignant phenotype by virtue of their location at a metastatic site, are a much better source of DNA and more likely to provide a truer reflection of the malignant phenotype in an individual case.

Recent studies have suggested that the LOH at specific chromosomal loci 1p, 3p, 5q, 9p, 17q, and 22q is associated with a worse prognosis of NSCLC, although studies of patients from different populations have yielded controversial results (25, 26, 36–38, 52–57). As our results indicate, the prognostic value of LOH at 3p and 5q loci might be attributable to the prognostic significance of FALmax. According to recent genome-wide studies, the chromosome arm 3p may harbor three potential tumor suppressor genes: 3p14/fragile histidine triad, 3p21, and 3p25/VHL (58). In our study, two microsatellite markers in proximity to 3p25/VHL gene were used, and the presence of LOH at this locus in metastatic deposits of regional lymph node was linked to adverse survival. LOH at the APC/MCC gene cluster at chromosome 5q has also been reported to correlate with a worse survival of patients with NSCLC (55). Deletions involving the long arm of chromosome 5 include several other candidate tumor suppres-
sor gene loci, such as the 5q33–35 and 5q11–13 regions (59, 60). Additional studies using high-density markers are necessary to clarify the actual prognostic value of LOH at each of the specific genetic loci because even a single deletion is capable of involving more than one of these loci. It is possible that future analyses of FAL using more precise chromosomal loci will provide more accurate and comprehensive prognostic information for a given patient with NSCLC.

In summary, we created a composite mutational profile for each patient with node-positive stage II NSCLC based on the aggregate allelic loss using primary site microdissected samples and, in a similar fashion, using microdissected lymph node deposits (Fig. 5). In stage II NSCLC, virtually all cases revealed a varying degree of genetic heterogeneity between primary tumor and lymph node metastasis, and the maximally accumulated load of mutational change in lymph node metastasis was significantly smaller than that of primary tumor, presumably reflecting the early occurrence of metastatic spread. In addition, FAL based on microdissected lymph node deposits of tumors accurately predicted patient survival within this homogenous cohort. Our results indicate that future investigations focusing on the prognostic value of genetic alterations in NSCLC should be performed not only in primary tumors but also in their corresponding metastases. The tumor heterogeneity is a ubiquitous phenomenon of human neoplasms, and this essential characteristic of human tumor should be taken into account for the establishment of the optimal system for integration of molecular data for diagnostic and prognostic purposes.

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REFERENCES


Comparison of Accumulated Allele Loss between Primary Tumor and Lymph Node Metastasis in Stage II Non-small Cell Lung Carcinoma: Implications for the Timing of Lymph Node Metastasis and Prognostic Value
