Prognostic Implication of Microsatellite Alteration Profiles in Early-Stage Non-small Cell Lung Cancer

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INTRODUCTION

The lung is the number one cancer mortality site overall and is one of the top four incidence sites for each racial and ethnic group (1). In the United States in 1998, there were an estimated 160,000 deaths from lung cancer, and an estimated 172,000 new cases were detected. Despite improvements in the detection and treatment of lung cancer in the past two decades, the overall 5-year survival rate remains less than 15% (2). Lung cancer is classified into NSCLC (1) and small cell lung cancer. About 80% of primary lung cancers are NSCLCs, including two major histological subtypes: (a) SQCC; and (b) ADCA. For stage I NSCLC, the current standard treatment is surgical resection of primary tumors. Although the treatment is effective and may cure about 60% of the patients, the other 40% of the patients will die of the disease. Unfortunately, current clinical means alone cannot precisely predict the outcomes of these patients, thus hampering further improvement of survival times. Importantly, with advances in the early detection of lung cancer, for example low-radiation-dose computed tomography diagnosis (3), more patients may be diagnosed at earlier stages of the disease. Novel and clinically applicable strategies must therefore be formulated to complement the current methods for further classification of early-stage NSCLC. Patients who might not be cured by surgery alone and may therefore require alternative treatment plans or additional therapies may then be identified before the disease progresses.

The development of NSCLC is a multistep process in which genetic alterations including activation of proto-oncogenes, inactivation of tumor suppressor genes, and inactivation of mutator genes accumulate (4–6). Inactivation of tumor suppressor genes is the most frequently identified alteration in human solid tumors, including NSCLC. In most tumor suppressor genes, both alleles are inactivated in tumor cells, usually by a mutation in one allele and a deletion of genetic material containing the other allele. The latter alteration may be detected by microsatellite analysis.

Microsatellite analysis has been widely used to detect heterozygosity at 10q24 and retention of at least one of the other two loci, none died of lung cancer within 5 years after surgery, whereas 28% (19 of 67 patients) of the patients outside these profiles did so (P = 0.01). Our results support the hypothesis that microsatellite alterations can be used as biomarkers for the genetic classification of pathological stage I NSCLC, which may in turn influence treatment decisions dependent on an accurate forecast of patient survival time.

ABSTRACT

Development of non-small cell lung cancer (NSCLC) is a result of multiple accumulated genetic abnormalities. Profiles of genetic abnormalities may determine tumor behavior and impact on patient outcome. We used microsatellite markers at 3p14, 9p21, and 10q24 to analyze tumor samples from 91 patients with pathologically confirmed stage I NSCLC for microsatellite alterations. Loss of heterozygosity at any single locus was not significantly associated with length of survival. However, patients whose tumors had microsatellite instability (MI) at 10q24 had shortened disease-specific survival. Among 31 such patients, 32% (10 of 31 patients) had died of the disease within 5 years after surgery compared with 16% (9 of 58 patients) without MI at 10q24 (P = 0.07). Interestingly, in the adenocarcinoma subtype, 71% (5 of 7 patients) of the patients with MI at 10q24 succumbed to the disease compared with only 12% (3 of 26) of the adenocarcinoma patients without such MI (P < 0.001), suggesting the presence of distinct mechanisms in tumorigenesis among different subtypes of lung cancer. It has been noticed that certain microsatellite alteration profiles provide additional values for risk assessment. Of 23 patients who had MI at 10q24 and an alteration at 3p14, 39% (9 of 23 patients) died of the disease within 5 years as compared with only 15% (10 of 66 patients) of the patients without such a profile (P = 0.02). Strikingly, among the 22 patients with no alteration at any loci tested or with loss of
genetic alterations in NSCLC (7–10). Two types of microsatellite alterations have been described: (a) LOH; and (b) MI (11, 12). They are probably the most frequent genetic changes identified in NSCLC and are present in virtually all such tumors, if sufficient chromosomal regions are examined. Recent studies have shown that microsatellite alterations may provide important prognostic information in human cancers including NSCLC (13–16). However, most of these studies contained mixed populations of patients with various stages of disease who had received different treatments. Analysis of genetic alterations in the context of outcome is based mainly on examining alterations at individual chromosomal loci. Because genetic alterations are the basis of NSCLC development, we hypothesize that profiles of microsatellite alterations in early-stage NSCLC can be correlated with the biological behaviors of tumors and can be used as biomarkers to better predict patient outcome. To test this hypothesis, we studied primary NSCLCs (pathological stage I) from 91 patients who underwent complete surgical resection of their primary tumors and received follow-up care for at least 5 years or until death. We analyzed microsatellite alterations at chromosomes 3p14, 9p21, and 10q24 and found such an analysis to be a potentially valuable tool for genetic classification of early-stage NSCLC.

MATERIALS AND METHODS

Study Population. Ninety-six patients who were diagnosed with pathological stage I NSCLC and had undergone lobectomy or pneumonectomy for complete resection of primary tumors at The University of Texas M. D. Anderson Cancer Center between 1975 and 1990 were enrolled into the study. Patient selection was based on sample availability and completeness of clinical and follow-up information. The study was reviewed and approved by the institution’s surveillance committee. All of the patients received regular follow-up care, including a chest X-ray every 3 months for the first 3 years and every 6 months for up to 5 years. None of the patients had received adjuvant therapy before and after surgery. Tissue sections (4-μm thick) were obtained from each tissue block, stained with H&E, and reviewed by our staff pathologist (B. L. K.) to confirm the diagnosis and the presence or absence of tumor cells in these samples.

Microdissection and DNA Extraction. Tissue sections (8-μm thick) were obtained from each patient, fixed in formalin, and embedded in paraffin. The tumor cells and normal tissues were microdissected under a stereomicroscope, as described previously (17, 18). Dissected tissues were digested in 200 μl of digestion buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS, and 0.5 mg/ml proteinase K at 42°C for 36 h. The digested products were purified by treating them twice with phenol-chloroform. DNAs were then precipitated by using the ethanol precipitation method in the presence of glycogen (Boehringer Mannheim, Indianapolis, IN) and recovered in distilled water.

Microsatellite Analysis. Ten ng of DNA were used for each PCR amplification. The microsatellite markers used were D3S1234 and D3S1481 at 3p14, D9S171 and D9S1747 at 9p21, and D10S198 and D10S192 at 10q24 (Research Genetics, Huntsville, AL). For PCR amplification, one of the primers for each marker was end-labeled with [γ-32P]ATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). PCR reactions were carried out in an 8-μl volume containing 3% DMSO, 200 μM deoxynucleotide triphosphate, 1.5 mM MgCl2, 0.4 μM PCR primers including 0.01 μM γ-32P-labeled primer, and 0.5 unit of Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD). DNA was amplified for 35 cycles at 95°C for 30 s, 56°C to 60°C for 60 s, and 70°C for 60 s followed by a 5-min extension at 70°C in a temperature cycler (Hybaid; Omnimgene, Woodbridge, NJ) in 500-μl plastic tubes. PCR products were separated on a 7% polyacrylamide-urea-formamide gel and then exposed to X-ray film. The results were interpreted visually by two independent observers (X. Z. and L. M.); discrepancies were resolved by discussion.

Statistical Analysis. LOH and MI at all microsatellite markers were determined and recorded in a spreadsheet format together with clinical parameters. The survival probability as a function of time was computed using the Kaplan-Meier estimator. The log-rank test was used to compare patients’ survival time differences. The two-sided Fisher’s exact test and the χ² test were used to determine statistical differences. The Cox model was used to adjust risks of microsatellite alterations and other factors including age, gender, and histology.

RESULTS

Patient Characteristics. A total of 96 patients were entered into this study. Five patients were not eligible for the final data analysis because DNA from these patients could not be amplified by PCR at any of the markers tested, therefore excluding these samples from evaluation. The demographic characteristics of the remaining 91 patients are shown in Table 1.

Frequency of Microsatellite Alterations. LOH was defined as a >50% reduction of intensity by visual inspection in one of the two alleles as compared with that seen in the corre-
early-stage NSCLCs, we observed different frequencies of MI among the chromosomal regions. MI frequency was higher at the 10q24 locus than at 3p14 locus ($P = 0.003$ by $\chi^2$ test).

We further analyzed frequencies of microsatellite alterations based on major histological subgroups. It appears that more microsatellite alterations were present in SQCCs than in ADCAs (Table 2), although no statistical significance was observed except for a marginally significant difference found in MI at 10q24 between SQCC and ADCA ($P = 0.06$ by $\chi^2$ test). We also analyzed the associations between microsatellite alterations and smoking status as well as gender, but we did not see any statistically significant difference.

### Prognostic Value of Microsatellite Alterations at a Single Locus.

We studied the potential value of using LOH or MI at each chromosomal locus to predict patient outcomes. Because all of the patients were followed-up for at least 5 years after surgical treatment, and survival information was well documented, we used 5 years as a cutoff point in the patients’ overall and disease-specific survival analyses. Among the 91 patients, the overall death rate was 41% (37 of 91) within 5 years after surgery, whereas the disease-specific death rate (death caused by disease recurrence or metastasis) was 22% (20 of 91). The remaining 17 (19%) patients died of other causes.

Among 81 patients whose tumors were informative at 3p14 markers, 33 (41%) died within the 5-year follow-up period. Although more than 46% (24 of 52) of the patients with LOH died within 5 years and 31% (9 of 29) of the patients without LOH at 3p14 died within 5 years, the difference was not statistically significant by the log-rank test ($P = 0.19$). A similar trend was observed when the disease-specific survival time was used as the end point ($P = 0.22$). There was no difference in outcome between patients with MI and those without MI at 3p14 (data not shown). We also examined whether the genetic alterations could predict survival times in two major histological subtypes, i.e., SQCC and ADCA, and we did not discover any statistically significant result (data not shown).

Among 40 patients whose tumors exhibited LOH at 9p21, 43% (17 of 40) died within 5 years of any cause, whereas 37% (10 of 27) of the patients without LOH at the locus died within 5 years of any cause. When the disease-specific survival rate was analyzed, 28% (11 of 40) of the patients with LOH at 9p21 died of disease recurrence or metastasis within 5 years after surgery compared with only 11% (3 of 27) of the patients without LOH at the locus. However, statistical significance between the two groups was found in neither overall survival rate nor disease-specific survival rate ($P = 0.66$ and 0.14, respectively, by the log-rank test). As with the results from studies of the 3p14 locus, no statistical significance was found when MI or histological subtypes were taken into account (data not shown).

Due to the high frequency of MI at 10q24 markers, only 58 tumors (64%) were informative and were able to be evaluated for LOH status at the locus. Among 34 (59%) patients whose tumors contained LOH at 10q24, the 5-year overall survival rate was 71% (24 of 34), whereas the 5-year overall survival rate for the patients without LOH at 10q24 was 63% (15 of 24). A similar trend was also observed in disease-specific survival rates studied for this locus. Although the differences observed had no statistical significance, the data suggest that a single loss at the
10q24 locus may be a favorable prognostic factor in patients with pathological stage I NSCLC. MI frequency was highest at the 10q24 markers among the three chromosomal loci analyzed. Of 31 patients with MI at this locus, 45% (14 of 31) died within 5 years, as compared with 38% (22 of 58) of the patients without MI at the locus. The difference was not statistically significant. Interestingly, a striking association was found between MI at 10q24 and the overall survival rate in cases of ADCA but not SQCC when histological subtypes were analyzed. In ADCA, among 7 patients with MI, 71% (5 of 7) died within 5 years; only 23% (6 of 26) of the ADCA patients without MI died within 5 years (P = 0.01 by log-rank test). More striking results were obtained when the disease-specific survival time was used as the end point. Overall, among the 31 patients with MI, 32% (10 of 31) died of disease compared with 16% (9 of 58) of the patients without MI (P = 0.07). For ADCA, 71% (5 of 7) of the patients with MI died of disease compared with only 12% (3 of 26) of the patients without MI (P < 0.001).

To determine whether MI at 10q24 was an independent risk factor, we performed a multivariable analysis using the logistic regression model to consider gender, age, and histological types as cofactors. Results showed that MI at 10q24 was the only independent poor prognostic factor (P = 0.02) for disease-specific survival rate among these variables in these patients.

Prognostic Value of Profiles of Microsatellite Alterations. We analyzed several combinations of microsatellite alterations and their associations with clinical outcome. As described above, we found that MI at 10q24 is an independent marker predicting poor outcome. It was therefore used as a core marker in some of the combination analyses. Patients with LOH at 9p21 or 3p14 in their primary tumors also showed a trend toward poorer prognosis. However, we did not find statistically significant differences when examining LOH at 3p14 and 9p21 (data not shown). In contrast, when MI at 10q24 was used as a cofactor, several combinations of microsatellite abnormalities improved the statistical power of disease-specific survival rate predictions. Patients whose tumors contained MI at 10q24 and also had LOH or MI at 3p14 (dashed line) versus patients without this alteration profile (solid line; P = 0.02). b, disease-specific survival times for patients with ADCA showing MI at 10q24 and LOH or MI at 3p14 (dashed line) versus patients without this alteration profile (solid line; P = 0.0004). c, disease-specific survival time for patients showing MI at 10q24 and LOH at 9p21 (dashed line) versus patients without this alteration profile (solid line; P = 0.01). d, disease-specific survival times in patients with ADCA showing MI at 10q24 and LOH 9p21 (dashed line) versus patients without this alteration profile (solid line; P = 0.0015).
profile died of the disease within 5 years, but only 15% (8 of 53) of the patients lacking it did so ($P = 0.01$; Fig. 2c). In cases of ADCA, 2 of 3 patients with the profile succumbed to the disease within 5 years, only 10% (2 of 21) of the patients without this profile did so ($P = 0.002$; Fig. 2d). We also analyzed a group of patients whose tissue samples showed no alterations at all three loci or showed LOH at 10q24 but retained two alleles in at least one other locus. We studied these genetic profiles because tumors with fewer genetic alterations may be less aggressive, and because we saw that patients whose tumor displayed LOH at 10q24 did well clinically. Among 22 patients with these profiles, none developed recurrent lung cancer or metastasis and died of the disease within 5 years after surgery, a most notable finding. In contrast, more than 28% (19 of 67) of the patients lacking these profiles died of the disease within 5 years ($P = 0.01$; Fig. 3a). In a further subtype analysis, 35 of 67 patients lacking these profiles had SQCC, and 15% (8 of 35) of these patients died of the disease ($P = 0.16$; Fig. 3b). However, more than 36% (8 of 22) of ADCA patients lacking these profiles died of the disease ($P = 0.03$; Fig. 3c), indicating that the alterations have great value in forecasting clinical outcome differences, particularly in ADCA.

**DISCUSSION**

Frequent LOH has been recognized at multiple chromosomal loci in NSCLC tumor samples and is believed to inactivate critical tumor suppressor genes and play an important role in tumor initiation and progression. Recent studies have provided promising data suggesting a potential prognostic value of microsatellite alterations at certain chromosomal loci in NSCLC (15, 16, 21). Fong et al. (15) analyzed LOH status at chromosome 11p in 101 patients with NSCLC and found that it correlated significantly with advanced tumor stage and nodal involvement. In SQCC, patients with LOH at 11p13 had significantly lower survival rates than those without LOH, suggesting that a tumor suppressor gene or genes on 11p affect the progression of NSCLC (10). MI at chromosome 2p and 3p has been associated with poor survival in stage I NSCLC (16). We have previously demonstrated that a high frequency of MI could be detected in somatic solid tumors, if selected microsatellite markers at certain chromosomal regions were used (25, 26).

In this preliminary study, we selected three critical chromosomal loci (3p14, 9p21, and 10q24) to test our hypothesis that the profiles of microsatellite alterations in early-stage NSCLC can be correlated with the biological behavior of tumors and can therefore be used as biomarkers to predict patient outcome. FHIT and p16 tumor suppressor genes are located at 3p14 and 9p21, respectively, and have been considered important in lung tumorigenesis (16–20, 27, 28). The LOH frequencies of 63% and 60% at 3p14 and 9p21 are comparable to most reports in the literature. Because the markers used are located either within a gene (FHIT) or close to a gene (p16), frequent LOH found in these regions supports the notion that FHIT and p16 play important roles early in lung carcinogenesis. In fact, previous studies by our group and others have shown that loss of 3p14 and 9p21 can be found frequently even in the normal-appearing bronchial epithelium of smokers (29, 30). In another study, we found LOH at 10q24 in more than 70% of primary small cell lung cancers (21). LOH at 10q in NSCLC was reported in a previous study and associated with advanced stages of the disease. However, the study was not extensive and included only 50 NSCLCs in different stages of the disease (31). In this study, we analyzed a larger number of cohorts with a single stage of the disease. The 60% LOH frequency at 10q24 found in this study suggests that this alteration is also important in early-stage NSCLC. The difference in LOH frequencies between previous reports and this study may be due to the selection of different chromosomal subregions.

In a recent study, Burke et al. (32) analyzed LOH status at the 3p14 (FHIT) region in tumors from patients with NSCLC and found that LOH at the region was associated with non-ADCA histology and short survival time. In this study, we observed a trend toward poor survival rates, but we observed no difference between ADCA and other histological subtypes. This difference may be due to the different patient population, because we analyzed pathological stage I patients. In addition, the
LOH frequency reported in this study was much greater than that reported by Burke et al. (32). Furthermore, although Burke et al. (32) studied about 100 patients, only 38 patients were included in the survival analysis. However, whether LOH at a single 3p14 or FHIT site can independently predict the survival time of patients with NSCLC may require further investigation. Alternatively, Fhit protein expression status may be used to better determine FHIT alteration and as a marker to predict patient outcome. We also did not observe a clear predictive value when LOH at 9p21 was used as a single factor, but that is not surprising because loss of the p16 tumor suppressor gene occurs early in lung tumorigenesis and may not significantly impact tumor progression and invasion.

The only single alteration that was associated with a poorer disease-specific survival rate was MI at 10q24. Although we previously found that MI in somatic tumors is tumor type specific and chromosomal region specific, the high incidence of MI at the locus was striking. However, the identical MI patterns in repeated analyses and the presence of matched microsatellite patterns at other markers for each normal tissue/tumor pair indicate that the phenomenon observed was unlikely to be an artifact or a sampling error. Interestingly, although MI at 10q24 was less frequent in ADCA, it provided a better predictive value for survival rate in this histological type (Fig. 2, a and b). Although the mechanism causing such MI is unclear, it appears to be different from the mismatch repair defect that results in a replication error phenotype (25, 26). Replication error phenotype (RER+), a type of MI found frequently in hereditary nonpolyposis colorectal carcinomas, is a result of the defect of mismatch repair genes (33, 34). Induction of MI by oxidative DNA damage was reported in a previous study (35). However, there is no evidence to support that the defect in the known mismatch repair genes also contributes to MI in somatic tumors. In a previous report, MI at chromosomes 2 and 3 was associated with reduced survival time in patients with stage I NSCLC (16). Because MI was identified in genomic sequences without known function, the manner in which it affects tumor behavior and patient outcome must be explored.

Because cancer development requires an accumulation of genetic alterations, it is hypothesized that different alteration profiles may provide clues for distinct biological behaviors that in turn influence tumor progression, invasion, and metastasis. Therefore, if these profiles can be determined in each tumor, they should serve as valuable parameters in tumor classification. In this study, we found that microsatellite alteration profiles at only three loci already provide promising results, indicating that the study of combinations of microsatellite alterations at critical chromosomal regions may allow a more accurate prediction of tumor behavior and patient outcome in early-stage NSCLC. We have demonstrated that tumors with no LOH at any locus or with favorable alterations and a low frequency of LOH were less invasive and less likely to recur (Fig. 3). However, many patients without these genetic alteration profiles also survived for more than 5 years after initial treatment. By continued study of critical chromosomal regions, additional genetic profiles that may better predict tumor behavior might be identified. When a large number of parameters (chromosomal loci) have been analyzed, it should be possible to develop an appropriate statistical model system to identify the genetic profiles that can best predict patient outcome. The value of these profiles must be further validated in large-scale prospective studies, whereupon the improved ability to classify early-stage NSCLC will lead to more effective treatment and eventually to lengthened survival time for patients with lung cancer.

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