Comparison of Molecular Changes in Lung Cancers in HIV-Positive and HIV-Indeterminate Subjects

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Context.—Human immunodeficiency virus (HIV) infection has been associated with an increased incidence of malignancy, and HIV-infected persons have an increased incidence of primary lung carcinoma compared with the general population.

Objective.—To investigate the molecular changes present in HIV-associated lung tumors and compare them with those present in lung carcinomas arising in HIV-indeterminate subjects (“sporadic tumors”).

Design.—Convenience sample.

Subjects.—Archival tissues from 11 HIV-positive persons and from 35 persons of indeterminate HIV status.

Setting.—University-based medical centers and affiliated hospitals.

Main Outcome Measures.—Analysis of frequency of loss of heterozygosity (LOH) and microsatellite alteration (MA) using polymerase chain reaction and 16 polymorphic microsatellite markers at 8 chromosomal regions frequently deleted in lung cancer. Presence of HIV and human papillomavirus (HPV) sequences.

Results.—The overall frequency of LOH at all chromosomal regions tested and the frequencies at most of the individual regions were similar in the 2 groups. Frequency of MA present in the HIV-associated tumors (0.18) was 6-fold higher than in sporadic tumors (0.03) (P<.001). At least 1 MA was present in 10 (91%) of 11 HIV-associated tumors vs 17 (48%) of 35 sporadic tumors (P=.02). Molecular changes were independent of tumor stage and gender. HIV and HPV sequences were not detected in the HIV-associated lung carcinomas.

Conclusions.—Microsatellite alterations, which reflect widespread genomic instability, occur at greatly increased frequency in HIV-associated lung carcinomas. Although the mechanism underlying the development of increased MAs is unknown, it may play a crucial role in the development of many HIV-associated tumors.

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HUMAN immunodeficiency virus (HIV) infection has an established predisposition to certain malignancies (acquired immunodeficiency syndrome [AIDS]-defining neoplasms), including Kaposi sarcoma, primary central nervous system lymphoma, non-Hodgkin lymphoma, and cervical carcinoma. As the AIDS epidemic advances, the number of HIV-infected subjects developing AIDS-related neoplasms has increased, and the spectrum of malignancies is expanding. Several non-AIDS-defining cancers, including lung cancer,2 are being reported at increasing incidences in HIV-infected persons.

Although substantial progress has been made in understanding the mechanism involved in the development of AIDS-related neoplasms, the pathogenesis of these malignancies is still not fully understood. Studies on the mechanism of tumorigenesis in AIDS have focused on loss of immune surveillance and possible involvement of certain infectious agents. Epstein-Barr virus has been implicated as an agent in the development of non-Hodgkin lymphoma, and recently a novel human herpesvirus has been linked to a predisposition for Kaposi sarcoma. However, it is unknown whether HIV plays a direct role in the genesis of AIDS-related neoplasms.

Lung cancer is the most frequent cause of cancer deaths in men and women in the United States, and tobacco smoking is accepted as the major cause. Although the association of lung cancer and HIV infection is rare, more than 180 cases of lung carcinoma in HIV-positive patients have been documented in as many as 22 reports since the first case was described in 1984. Because the subject has been reviewed recently, only key references describing multiple cases are mentioned herein. Parker et al, in a recent population-based epidemiological study in Texas, identified primary lung cancers in 36 subjects with HIV infection or AIDS. Although no adjustment was made for age at onset or smoking history, the observed/expected ratio for primary lung cancer compared with that of the US population was 6.5. As with the AIDS-defining neoplasms, lung carcinomas arising in HIV-positive persons are characterized by aggressive clinical behavior with more advanced stage and shortened survival vs similar neoplasms (“sporadic cancers”) in HIV-seronegative or HIV-indeterminate persons.

Lung cancer pathogenesis is characterized by multiple molecular changes, including activation of oncogenes and loss of known and putative tumor suppressor genes (TSGs). Many mutations and allelic losses, especially those involving TSGs, have been described in clinically evident lung cancers. Microsatellites are highly polymorphic short tandem repeat DNA sequences. Because they are abundantly and evenly distributed throughout the genome and are easily analyzed by polymerase chain reaction (PCR)-based methods, they are fre-
**Table 1.**—Clinicopathological and Molecular Data of HIV-Associated Male Lung Cancer Cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Lung Cancer Histology</th>
<th>Age. y</th>
<th>Clinical Stage</th>
<th>Smoking History, Pack-years</th>
<th>CD4 Count</th>
<th>Prior Chemotherapy</th>
<th>FRL Index</th>
<th>MA Index</th>
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<tbody>
<tr>
<td>1</td>
<td>Adenocarcinoma (BAC)</td>
<td>49</td>
<td>I</td>
<td>30</td>
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<td>1.0</td>
<td>0.06</td>
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<td>2</td>
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<td>45</td>
<td>I</td>
<td>10</td>
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<td>Zidovudine</td>
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<td>4</td>
<td>Adenocarcinoma</td>
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<td>24</td>
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<td>0.00</td>
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<tr>
<td>5</td>
<td>Squamous cell carcinoma</td>
<td>49</td>
<td>I</td>
<td>15</td>
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<td>6</td>
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<td>38</td>
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<td>7</td>
<td>Squamous cell carcinoma</td>
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<td>Small cell carcinoma</td>
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<td>Extensive</td>
<td>Yes</td>
<td>...</td>
<td>...</td>
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<td>0.36</td>
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<tr>
<td>11</td>
<td>Small cell carcinoma</td>
<td>60</td>
<td>Extensive</td>
<td>Yes</td>
<td>...</td>
<td>...</td>
<td>0.50</td>
<td>0.36</td>
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*HIV indicates human immunodeficiency virus; BAC, bronchoalveolar carcinoma; FRL, fractional regional loss; MA, microsatellite alteration; and ellipses, information not available.
†Metastasis of lung squamous cell carcinoma to a cerebral lymph node.

Materials and Methods

**Study Populations and Tumor Specimens**

Paraffin-embedded archival materials from 11 HIV-associated lung carcinomas were obtained from 4 institutions in the United States (10 cases) and from 1 institution in France (1 case). We selected all cases for which archival paraffin-embedded tumor, along with nontumor tissue (as a source of constitutional DNA), were available to us. The tumors represented 10 primary lung carcinomas and 1 metastasis of a lung cancer in a cervical lymph node. Further clinicopathological information is presented in Table 1. Additional details of the French case are available from a published report. Six of the tumor specimens were received from the AIDS Malignancy Bank, a National Institutes of Health (NIH)-sponsored resource. We compared the findings from the HIV-associated carcinomas with those from 35 sporadic primary lung carcinomas representing all major histological types from patients undergoing curative intent surgical resections. The latter consisted of 10 adenocarcinomas, 11 squamous cell carcinomas, and 14 small cell lung carcinomas.

**Microdissection and DNA Extraction**

Microdissection and DNA extraction were performed using the nonoverlapped slides. Precisely identified areas of invasive carcinoma were microdissected under microscopic visualization without contamination with normal cells. Nondisorgan lung tissue or lymphocytes from the same sections were used as a source of constitutional DNA. After DNA extraction, 5 µL of the proteinase K–digested samples, containing DNA from at least 100 cells, was used for each multiplex PCR reaction.

**Polymorphic DNA Markers and PCR for LOH and MA Analyses**

To evaluate LOH and MA, we used primers flanking dinucleotide (n=11) and polynucleotide (n=5) microsatellite repeat polymorphisms located at the following genes or chromosomal regions: D3S1274, D3S1403 at the FHT gene, D3S1029, D3S1478, D3S1447, ITH (K.C.A.), D3S22-24, D3S2452, D3S15351, D3S15371, L5.S71 in the APC-MCC region, D3S1274, D3S1478, D3S1447, microsatellite-repeat at the RB gene [RB CA], and Tp53 (TPS3 dinucleotide [TPS3 CA] and pentanucleotide-repeat [TPS3 penta]). Primer se-
quences can be obtained from the Genome Database [http://gdbwww.gdb.org], with 5 exceptions: *ITH1*-22, pentamethcycloolactone,22 and dinucleotide repeats in the *TP53* gene, dinucleotide repeat in the *RB* gene,23 and the KLA dinucleotide marker identified in our laboratory (forward primer 5'-CATCCTCTCAACATGTAACG-3; reverse primer 5'-CAGGGGACAGA GACTTTG-3').

Nest PCR20 or 2-round PCR (using the same primers in 2 consecutive amplifications) methods were used. Multiplex PCR was done during the first amplification, followed by uniplex PCR for individual markers. In the multiplex PCR, 6 markers were amplified during the same reaction. Volumes of 50 µL were used for each multiplex reaction, containing 20-mmol/L Tris buffer (pH 8.3), 50-mmol/L potassium chloride, 2.5-mmol/L magnesium chloride, 400 µmol of each deoxy nucleotide triphosphate (dATP, dCTP, dGTP, dTTP) per liter, 0.5 µmol of each forward and reverse primer per liter, and 3.5 U of AmpliTaq Gold (Perkin Elmer, Foster and reverse primer per liter, and 3.5 U of AmpliTaq Gold (Perkin Elmer, Foster Heights, Calif), 0.25 µL of dCTP tagged with 3'-fluorescein (Perkin-Elmer, Branchburg, NJ), utilizing the SK38 and SK39 primers and targeting a highly conserved 115-base pair segment of the HIV-1 Gag gene. The positive control was supplied by the manufacturer. This method is sensitive enough to detect 10 input copies of HIV-1–positive control DNA per reaction in paraffin-embedded tissue.

The presence of HPV sequences was assessed for using general and type-specific primers designed for paraffin-extracted DNA as previously described.45 Specific primers were used to identify high (HPV 16 and 18) and intermediate (HPV 31 and 33) oncogenic risk–HPV strains.46 DNA extracted from human cell lines CaSkii (HPV 16) and HeLa (HPV 18) (obtained from the American Type Culture Collection, Rockville, Md) was used as a positive control for HPV analyses.

**Data Analysis**

To compare the total frequencies of LOH and MA in lung carcinomas, we devised 2 indices,46 calculated as follows: (1) Fractional Regional Loss Index = Total No. of Chromosomal Regions With LOH/Total No. Informative Regions (2) Microsatellite Alteration Index = Total No. of Loci Demonstrating MA/Total No. Loci Analyzed

The fractional regional loss index indicates LOH for all informative chromosomal regions per case (maximum of 8 regions per case). In some instances, we were able to increase the number of regions that were informative by using multiple markers to analyze individual regions (when available and suitable for analysis of archival paraffin-embedded materials). If a marker for a region was informative (ie, heterozygous), the region was regarded as informative, and if 1 or more of the markers showed LOH, we regarded the region as demonstrating loss. The MA index indicates the total frequency of MA expressed as a fraction per case (n = 16 markers tested). Because MAs at individual markers occur independently of chromosomal region and informativeness, data from all markers were used.

Statistical analyses were performed using the nonparametric Wilcoxon and Fisher exact tests. Probability values of P < .05 were regarded as statistically significant.

**RESULTS**

**Clinical-pathological Data for HIV-Associated and Sporadic Lung Carcinomas**

As presented in Table 1, the 1I HIV-associated lung carcinomas consisted of 4 (36%) adenocarcinomas, 4 (36%) squamous cell carcinomas, 2 (18%) small cell carcinomas, and 1 (9%) large cell carcinoma. Seven of these tumors were surgically resected, and the rest were obtained by bronchoscopy (n = 2) or autopsy (n = 2). We compared the findings from the HIV-associated carcinomas with those from 35 sporadic primary lung carcinomas from patients undergoing curative intent surgical resections. All of the HIV-associated lung carcinoma patients were male and relatively young, with a median age of 45 years (range, 26-60 years). All 10 patients from whom data were available were current or former smokers (median exposure, 20 pack-years). Tumor stage varied from early (6 patients, stages I and II) to late (4 patients, stages III and IV), and, for the small cell lung carcinoma patients, extensive. In

<table>
<thead>
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<th>Microsatellite Sequence</th>
<th>N</th>
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<th>N</th>
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<th>N</th>
<th>T</th>
<th>N</th>
<th>T</th>
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<tr>
<td>TP53 penta</td>
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<td></td>
<td></td>
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</table>

Figure 1.— Representative examples of loss of heterozygosity (LOH) and microsatellite alteration (MA) in human immunodeficiency virus–associated lung carcinomas. Each panel demonstrates microsatellite marker analysis of microdissected DNA of paired nonmalignant (N) and tumor (T) tissue samples from individual patients. A, Tetranucleotide repeat marker D3S1537 (3p22-24.2) shows LOH of the upper allele in the tumor sample. B, Tetranucleotide repeat marker D3S1351 (3p22-24.2) shows loss of 1 tumor allele and a mobility shift of the remaining allele. C, The TP53 penta nucleotide repeat marker shows a mobility shift of the lower tumor allele. D, Tetranucleotide repeat marker D3S2432 (3p22-24.2) shows a mobility shift of the upper tumor allele. E, Tetranucleotide repeat marker D3S1537 (3p22-24.2) shows a single allele in the nonmalignant tissue and an additional tumor allele (gain of heterozygosity). F, Dinucleotide repeat marker D3S1478 (3p21) shows a single allele in the nonmalignant tissue that has undergone a mobility shift in the tumor sample.
6 of 7 patients from whom information was available, HIV seropositivity was noted at the time of tumor diagnosis, while in the remaining patient, HIV seropositivity was detected 4 years prior to tumor diagnosis. Of the 8 patients from whom information was available, 5 had AIDS as defined by CD4 counts below 0.20x10^9/L, and 1 of these had received therapy with zidovudine for 4 years prior to tumor diagnosis.

The sporadic lung cancer patient group showed a nearly equal gender distribution. All but 1 were heavy smokers with a median exposure of 37 pack-years (range, 0-120). The 1 nonsmoker was a woman with a long history of heavy passive exposure to cigarette smoke. Their median age was 61 years (range, 40-75 years), which is closer to that of persons with lung cancer in the general US population, for whom the median age at lung cancer diagnosis is 68 years.

**LOH Frequency in Lung Carcinomas**

Analyses were performed on tumor samples that had been carefully microdissected and LOH was detected by complete absence of 1 of the 2 alleles present in constitutional DNA of informative cases (Figure 1). Frequencies of LOH and the pattern of allelic losses were similar in HIV-associated and sporadic lung carcinomas (Figure 2, A, Table 2). Almost identical relatively high frequencies of total LOH at the 8 chromosomal regions analyzed were present in the 2 groups, as represented by the fractional regional loss indices (0.67 and 0.68, respectively) (Figure 2, A). In addition, the frequencies at individual regions were similar in the 2 groups with high frequencies of allelic loss at chromosomal regions 3p12, 3p21, 3p22-24.2, 9p21, 13q (RB gene), and 17p (TP53 gene) (Table 2). Differences were noted at 2 sites: allelic loss frequencies were higher at 5q22 (APC/MCC gene region) in HIV-associated than in sporadic carcinomas (86% vs 39%; P = .007) but were lower at the FHIT gene (25% vs 77%; P = .03, by the Fisher exact test).

**MA Frequency in Lung Carcinomas**

Although there was considerable variability, the mean MA index of the HIV-associated tumors (0.18) was 6-fold greater than the mean index of the sporadic cases (0.03) (Figure 2, B) and the difference was highly significant (P = .001, Wilcoxon test). Examples of various MA patterns are presented in Figure 1. Because artifacts resulting from PCR amplification may be mistaken for MAs, especially when minute amounts of input DNA are utilized, all examples of MAs were confirmed using DNA microdissected from a replicate microsection. Using 16 polymorphic markers, at least 1 MA was identified in 10 (91%) of 11 HIV-associated tumors vs 17 (48%) of 35 sporadic tumors (P = .02). The patient who received zidovudine for 4 years prior to the diagnosis of lung cancer had a relatively low MA index (0.06).

Of the MAs identified, insertions (61% and 50%, respectively) and deletions (39% and 50%, respectively) occurred at similar frequencies in HIV-associated and sporadic tumors. We determined the frequency at which both LOH and MA occurred at individual informative loci. In both HIV-associated and sporadic tumors, the frequencies were similar (20% and 24%, respectively) (P >.05). For 12 (75%) of the 16 markers, the rates were higher in the HIV-associated group (up to 9-fold higher), while for the other 4 markers the rates were either similar (n = 2) or slightly higher in the sporadic group (n = 2) (Figure 3). Although no obvious differences were detected in the MA frequencies between dinucleotide or polynucleotide microsatellite repeat markers (Figure 3), the frequencies varied considerably for individual markers.

Because all the HIV-associated lung carcinoma patients were male, we also compared their MA index (0.18) with that of cases of sporadic lung tumors (0.04) arising only in males (n = 20), and the differences were still significant (P = .004). To exclude the possibility that tumor stage influenced the frequency of MA, we determined that the MA index of 10 non–small cell lung carcinoma cell lines from early stage cases (stages I and II, mean MA index = 0.03) was similar to that from 8 cases of late-stage tumors (stage IV, mean MA index = 0.02, P >.05).

**MA in Nonmalignant Tissues**

In 2 HIV-associated cases, multiple samples of nonmalignant tissue were available for analysis (Table 3). Of these cases, 1 tumor had a relatively high MA index (0.38), while the other had a low index (0.06). In 15 samples of nonmalignant tissue from these 2 cases, no MAs were detected.

**Viral Sequences in HIV-Associated Lung Carcinomas**

Human immunodeficiency virus or HPV sequences were not detected in any of the microdissected lung carcinomas from the HIV-positive persons.
COMMENT

Because no information is currently available about the molecular changes involved in lung carcinoma arising in HIV-positive patients, we investigated the frequencies of LOH and MA present in 11 lung carcinomas from 11 HIV-positive persons and compared them with those from sporadic lung carcinomas of 35 persons. After careful microdissection of the tumors, extracted DNA was analyzed by PCR for LOH and MA at multiple chromosomal regions frequently deleted in sporadic lung cancer.

Reports of HIV-associated lung carcinomas suggest that the natural history of the disease is different in persons with lung cancer in the general population.2,7,13 Lung carcinomas arising in HIV-positive patients are characterized by young age at diagnosis (median, 38 years), occurring almost exclusively in males, slightly increased frequency of adenocarcinoma histology, late stage at presentation, and shortened survival.7 Although cigarette smoking appears to be as prevalent among HIV-positive lung cancer patients as in other lung cancer patients, the former usually have a lesser history of smoking exposure (usually <20 pack-years).2 Our cohort of 11 subjects with HIV-associated lung cancer fits this profile. In contrast, the profile of our 35 cases with sporadic lung cancer more closely resembled that of lung cancer arising in the general population.

Many mutations, especially those in hereditary nonpolyposis colon cancer, size changes affecting minisatellites present in noncoding regions of the genome and are probably represent evidence of some form of genomic instability.5 Most microsatellite alterations have also been described in other HIV-associated tumors and may be involved in their pathogenesis.4,11

Although there are several theories, the mechanism by which increased rates of MA occur in HIV-associated tumors is not known. While increased rates occur (probably in all somatic tissues) after radiation exposure in humans50 and experimental animals,52 none of our HIV-positive subjects had received prior radiotherapy. The possibility also exists that zidovudine, a thymidine analog, could interfere with the DNA repair mechanism. However, only 1 of our patients with HIV infection had received zidovudine prior to the diagnosis of lung cancer, and the MA index of his tumor was relatively low. A relatively high frequency of MA has been described in lung cancers arising in young nonsmoking subjects,22 suggesting a genetic predisposition. Microsatellite alterations are not due to direct effects of HIV viral integration into the genome of affected cells, as HIV sequences were absent in all of the HIV-associated lung carcinomas, a finding also reported for most other HIV-associated tumors (reviewed by Bedi et al).55 Our failure to identify MAs in nonsmugnant tissues diminishes the possibility that HIV induces a general somatic effect on the mutation repair system throughout the body. Human papillomavirus sequences, which may be present in
HIV-associated cervical and anogenital carcinomas (and in a subset of sporadic lung carcinomas), were absent in the HIV-associated lung carcinoma. We cannot exclude a direct role of other viruses described in HIV-associated tumors including human herpesvirus 8 frequency. Immunosuppressive states may result in minisatellite instability. Thus, immunosuppression, which develops in most HIV-infected subjects, may play a role in tumor development via multiple mechanisms.

Our findings suggest that widespread genomic instability, as manifested by the increased frequency of MA, occurs frequently in HIV-infected lung carcinomas. We did not detect MA in nonmalignant tissue from 2 HIV-positive cases and it remains to be determined at what stage MAs develop during the multistage pathogenesis of lung cancer. If they develop relatively early, during the preneoplastic process, determination of MA frequency may provide a useful method for cancer risk assessment in HIV-positive persons. Although the mechanism underlying the development of increased numbers of MAs is unknown, it may play a crucial role in the development of many HIV-associated tumors.

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Six of the HIV-associated tumor specimens were obtained from the AIDS Research and Referral Bank, a resource supported by the National Institutes of Health, Bethesda, Md.

References