Genetic Alterations in Bronchial Lavage as a Potential Marker for Individuals with a High Risk of Developing Lung Cancer

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ABSTRACT

Using 12 microsatellite markers, we have studied DNAs from the bronchial lavage of 90 individuals who were referred to an early-lung-cancer clinic in the Northwest of England with suspected lung cancer. Genetic alterations were detected in 15 (35%) of 43 patients with lung cancer but also in 11 (23%) of 47 patients with no cytological or radiological evidence of bronchial neoplasia. No significant differences were found between the referring symptoms in any of the second group of individuals with and without genetic alterations. No correlation was found between smoking exposure and loss of heterozygosity (LOH)/microsatellite alterations (MAs) in the microsatellite markers. On comparing LOH with MAs based on cytology review, we found that the prevalent type of alteration in specimens with cytological evidence of malignancy was LOH; in contrast, the individuals with no cytological evidence of malignancy showed a preponderance of MAs (P = 0.01). Our results indicate that a substantial proportion of cells in the bronchial lavage from suspected lung cancer patients carry identifiable genetic alterations. However, the presence of genetic alterations in the bronchial lavage of individuals with no clinical evidence of lung cancer raises the question whether instability is a phenomenon solely associated with cancer or represents a feature of nonneoplastic diseases. Our results suggest that microsatellite PCR-based assays can be developed as tools for the earlier identification of genetic changes in cells exfoliating in the bronchus.

INTRODUCTION

Lung cancer is responsible for more deaths than any other cancer, accounting for 785,000 deaths per year worldwide. It is the most common malignancy in males in the United Kingdom, and the highest incidence rates of lung cancer for both men and women are found in the Merseyside region of the Northwest of England. Current clinical techniques are not capable, in the majority of lung cancer cases, to detect tumors before they have already progressed beyond effective treatment; consequently, there is a high mortality rate with a five-year overall survival rate of 5% (1). Thus, increased attention on earlier detection and intervention management is imperative.

Genomic instability is considered to be a hallmark of cancer. It has been proposed as a novel mechanism in the multistep process of carcinogenesis (2–4). Genomic instability in cells is found in many forms of genetic alterations. Allelic imbalance or LOH (5) mainly represents chromosomal instability (4) whereas MI, also found in the counterpart tissue has become the most widely used method to determine such genetic alterations. Genomic instability is a common phenomenon in lung cancer (5–9) and, in some reports, has been associated with prognosis (10, 11). In a previous study from our group (8), 42 (93%) of 45 of NSCLC specimens were found to carry LOH or MAs in at least one of the 92 markers examined. Furthermore, genetic alterations have been detected in the plasma (12), bronchial mucosa, and sputum specimens of lung cancer patients (6, 13, 14). It is of note that genetic alterations were detected in specimens presenting with minimal atypia (6) and, moreover, that LOH and MAs have also been demonstrated in bronchial specimens from chronic smokers who do not have lung cancer (15, 16). These findings suggest that these genetic alterations precede morphological transformation of the cells. In this study, we have assayed DNA from 90 BL specimens for LOH and MAs, and we pose the question as to whether genetic alterations can be identified in BL from individuals with no cytological or radiological evidence of lung cancer. To date this question has not been answered.

MATERIALS AND METHODS

Patient Selection and Clinical Samples

We have collected 90 BL and control blood samples from individuals with suspected lung cancer who have been referred to the Cardiothoracic Center in Liverpool over a twelve-month period. The criteria for referral to the Cardiothoracic Center included: (a) unresolved chest infection; (b) abnormal chest X-ray; (c) cough (>4 weeks); (d) nonspecific weight loss; (e) stridor; (f) persistent (>3 weeks) hoarse voice; and (g) other suspicious features that would prompt referral to the lung cancer clinic. Each patient underwent a full clinical workup for lung cancer including a chest X-ray, spirometry, and bronchoscopy. BL specimens were obtained from all of these patients; the choice of site was based on bronchoscopic findings within the large airways, into which ~50 ml of saline was introduced via the bronchoscope and then aspirated.

The selection of patients was undertaken on the basis of an adequate cytology preparation and blood sample availability. The age of the patients selected ranged between 38 and 89 (average, 65). Thirty-three of the individuals were females, and 57 were males. Smoking data were available for 85 individuals (74 smokers, 11 nonsmokers). The total smoking exposure was calculated as follows:

\[ \text{Pack-years} = (\text{age at presentation} - \text{age started} - \text{years stopped}) \times \frac{\text{pack}}{40}\text{day} \]

A differential cell count was undertaken for all of the BL samples that were reported as NMCS and for the lung cancer patients with genomic instability. The epithelial cells present varied between samples (20–90%).

DNA Extraction

BL. BL (1 ml) was transferred into 1.5-ml tubes and centrifuged for 5 min, 10,000 rpm, at room temperature. The resulting pellet was resuspended in 400
mm Tris-HCl (pH 7.0), 150 mM NaCl, 60 mM EDTA, 1% SDS, and 100 μg/ml proteinase K and incubated at 42°C for 12–15 h in an orbital shaker. Deproteinization included the addition of 150 μl of 5 M sodium perchorotate and 500 μl of chloroform. After mixing and microcentrifugation for 2 min, the aqueous phase was transferred to a fresh tube, and DNA was precipitated by the addition of an equal volume of isopropanol. After incubation at ~20°C for 12–14 h, DNA was recovered with microcentrifugation for 15 min at 4°C, washed with 70% ice-cold ethanol, and resuspended in 50 μl of sterile distilled H₂O.

Blood. Blood (3 ml) was washed three times with 10 mM Tris-HCl (pH 8.0), 320 mM sucrose, 1% Triton X-100, and 5 mM MgCl₂ to remove red cells. Lysis of the resulting WBCs was similar to that of BL. Deproteinization was carried out by the addition of 500 μl of phenol/chloroform. After mixing and microcentrifugation for 2 min, the aqueous phase was transferred to a fresh tube, and DNA was precipitated by the addition of an equal volume of isopropanol. DNA was recovered with a sterile loop, washed with ice-cold 70% ethanol, and resuspended in 200 μl of sterile distilled H₂O.

**PCR Amplification of Microsatellite Loci**

The selection of microsatellite markers was based on the previous work of our group (7, 8, 17, 18) and other workers’ published results (5, 6, 9). We chose 12 markers; ACTBP, D14S50, D3S1215, D3S1339, D3S1351, D4S194, D4S392, FGA, D6S271, D9S286, TCRD, and TP53. These markers have been previously shown to exhibit a high frequency of instability in lung cancer.

Oligonucleotide primers purchased from Research Genetics (Huntsville, AL). The reaction mixture contained 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 0.1% Tween 20, 200 mM dNTPs, 0.1 mM of each primer, 2 mM MgCl₂ and 0.5 units BIOPRO polymerase (BIOLINE, United Kingdom). The amplification parameters were: initial denaturation for 2 min, followed by 28–30 cycles at 94°C for 30 s, 55–58°C for 30 s, and 72°C for 30 s, and final extension step at 72°C for 5 min. PCR products were analyzed by electrophoresis on 10% non-denaturing polyacrylamide gels and stained with silver. MA was judged from the presence of an allele (band) with different electrophoretic mobility. LOH was judged visually on the basis of a >40% relative reduction of one allele intensity. MA was judged from the mobility shift of one of the alleles or the presence of an additional allele on the gel. Positive samples were scored only when the LOH/MA results were confirmed at least twice in separate PCR reactions.

**Sensitivity and MA Detection**

To assess the sensitivity of LOH and MA of our method, we prepared DNA samples of various tumor:normal (T:N) ratios from tumor specimens with known LOH and MAs. The analysis showed that LOH may be detected in 1:10 dilution (T:N), and MA was detected in 1:16 dilution (Fig. 1).

**Statistical Analysis**

Fisher’s exact test was used to analyze the data.

**RESULTS**

**Genetic Alterations in Lung Cancer Patients.** The clinical findings (cytology, radiology, biopsy) revealed 43 patients with lung cancer, of which 18 individuals were reported with malignant cells present in the cytological specimen; 25 individuals were reported as NMCS but did have a radiological and/or histological diagnosis of lung cancer. The remaining 47 individuals had no clinical evidence of lung cancer (Fig. 2).

Genetic alterations were identified in 8 (44%) of 18 of the positive cytology cases (Fig. 2). All of the eight individuals demonstrated LOH, and three specimens had LOH and/or MAs in more than one marker (Table 1). The remaining 10 of 18 carcinomas of the positive cytology group with no evidence of genetic alterations included 9 squamous cell carcinomas and 1 adenocarcinoma.

Seven (28%) of the 25 individuals who were diagnosed with bronchial carcinoma but had no malignant cells in their BL exhibited genetic alterations (Table 1). The remaining 18 carcinomas in this group with no genetic alterations were composed of 9 squamous cell carcinomas, 1 adenocarcinoma, and 8 individuals without histological diagnosis (diagnosed radiographically). No association was found between the radiological findings and genetic alterations.

In nine of the cases with lung cancer, tumor tissue was also available. Three of nine tumors demonstrated genetic alterations. Of these, two (BL114 and BL176) had the same aberration in the BL (Fig. 3b and c), whereas in patient BL154, the aberration was not detectable in the BL specimen, although it was present in the tumor (Fig. 3a). We have examined the relationship between genetic alterations and confirmed cytology/histopathology and found that LOH/MAs tend to be more frequent among the BL of patients with adenocarcinomas (6 of 8) than squamous carcinomas (5 of 25; P = 0.069).

On comparing LOH with MAs based on the cytology review, we found that the prevalent type of alteration in specimens with cytological evidence of malignancy is LOH (Table 1). In contrast, the individuals with negative cytology show a preponderance of MAs (Fisher’s exact test, P = 0.01). To examine this, we compared the number of LOH/MA findings against the differential cell count in the cytology specimens from patients with no clinical evidence of cancer by any diagnostic method. In the BL specimens with >50% epithelial cells, 3 had with LOH and five with MAs, whereas those samples with <50% epithelial cells in the BL had two with LOH and four with MAs. No significant difference was found between LOH/MA frequency and the epithelial cell content of the BL specimen.

**Genetic Alterations in the BL of Individuals with No Clinical Evidence of Lung Cancer.** Eleven (23%) of 47 of the individuals did not have cytological or radiological evidence of lung cancer but did demonstrate genomic alterations. Nine individuals had evidence of MA/LOH, and two individuals were found to have LOH alone (Table 1). The statistical analysis in the group of individuals with no clinical evidence of lung cancer revealed an association (6 of 9) between genomic alterations and individuals with rheumatoid arthritis, clinical goiter, cardiac problems, and fibrosing alveolitis compared with those (4 of 31) with common chest disease (e.g., asthma, COPD, or chest infections; P = 0.003). No significant differences were found between the referring symptoms in the individuals with and without genetic alterations. On examination of the prescribed drugs to these 47 patients over the past 5 years, no association was found between genetic alterations and any specific drugs. Cytology review of these 47 individuals showed 9 with squamous metaplasia of regular type, of whom 4 had genetic alterations, but this trend was not found to be significant (Fisher’s exact test, P = 0.09).

The statistical analysis on either the total number of individuals or the separate groups of lung-cancer and non-lung-cancer individuals did not produce any significant correlation between genetic alterations of the markers examined with age or gender. Similar was the case with the smoking history or tobacco exposure in patients with lung cancer because, of 42 with complete smoking data, we had only two non-smokers (one patient with squamous cell carcinoma of the lung...
metastatic melanoma; Table 2). In the group of 47 patients with no clinical evidence of lung cancer, smoking data were available for 43 individuals (34 smokers, 9 nonsmokers). Seven of the smokers (two with LOH only, three with MAs only, and two with LOH/MAs) and three of the nonsmokers (one with LOH/MAs and two with MAs only) demonstrated genetic alterations. The clinical diagnosis for these three patients was rheumatoid arthritis, fibrosing alveolitis, and one individual with no evidence of any disease.

DISCUSSION

We have analyzed 90 BL specimens and blood samples from individuals with suspected lung cancer with twelve microsatellite markers that were found to exhibit the most frequent MAs and LOH in previous publications in NSCLC and SCCHN (5–9, 17, 18). Genetic alterations (LOH and MAs) were detected in the BL of 35% (15 of 43) lung cancer patients. It is of particular interest that genetic alterations were detected in BL specimens from patients who were reported as NMCS on cytological examination. This finding indicates that a proportion of the cells in the BL carried genetic aberrations but presented with no morphological evidence of malignancy. However, an alternative source of LOH/MAs may be naked tumor DNA in the BL sample. This may also explain the fact that LOH/MAs were detected in samples with a cytology report indicating possible target epithelial cells in very low abundance, lower than the sensitivity we have demonstrated for this assay (1:10 for LOH and 1:16 for MAs; Fig. 1). Naked DNA released in body fluids is also suggested to be a source of genetic alterations detected in the plasma of lung cancer patients (12). It should be noted that the sensitivity of MA detection in our experiments is in agreement with that of Foucault et al. (19), who reported detection of the less abundant allele when it represents 6% of the total DNA. Genetic alterations have also been detected in body fluids such as urine (20), plasma (12), and sputum (6) and may, therefore, be considered as an attractive candidate for the development of strategies for the early diagnosis of cancer, alongside other molecular methods that require no invasive techniques.
Table 1  Clinical details of patients with genetic alterations detected in their BL specimen

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<td></td>
<td>RA</td>
<td>0</td>
<td>H</td>
<td>MA</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
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</tr>
</tbody>
</table>

pk-yr, pack-year; NED, no evidence of disease; SCLC, small cell lung carcinoma; AdenoCa, adenocarcinoma; RA, rheumatoid arthritis; FA, fibrosing alveolitis; RUL, right upper lobe; LLL, left lower lobe; LMB, left middle bronchus; LHC, left hilum consolidation; MD, mediastinal; H, heterozygous; ND, not determined; R, Hilum, right hilum.
In an allelotype study of NSCLC undertaken with 92 markers, we have showed that 93% of the samples exhibited LOH/MA in at least one marker (7). Thus, it may be argued that the majority of NSCLC cases contain such genetic damage because it may be considered to be a fundamental feature of carcinogenesis. The detection of LOH/MA in 35% of lung cancer patients in this study can, therefore, be considered as an underestimate, and a larger number of markers are, therefore, required to increase the detection efficiency. In addition, a possible underestimation of the LOH/MA frequency may be due to the visual identification. MAs are easier to detect because they are represented by an additional band, whereas LOH demands quantitation of the relative density of the two alleles. The detection method that we used (i.e., silver staining of the PCR products analyzed on polyacrylamide gel), has been widely used for the detection of genomic instability. However, it is clear that there are certain limitations to its applicability on clinical specimens, such as BL, with a possible low number of target cells. In the future, biomagnetic separation for epithelial cell enrichment may be a solution to this problem. In addition, the use of fluorescent chemistry will increase the sensitivity of the assays because the quantitation of band intensities would become far more accurate.

It is of note that 23% (11 of 47) of the patients referred for investigation with no cytological or radiological evidence of lung cancer do have genetic alterations in the same microsatellite markers as found in patients with proven neoplasia. These patients, however, cannot be considered as a “control group,” based on their referral criteria and are still under long-term follow-up. Our results are in agreement with recent reports demonstrating genetic alterations in biopsy specimens from current and former smokers who do not have lung cancer (15, 16) and MI in cytological material from patients with COPD (21). In this group of 47 patients, genetic alterations were more common in rheumatoid arthritis, goiter, fibrosing alveolitis, and cardiopulmonary problems (MCI, LVF, and angina) than in common chest disease. Thus, this may imply that genetic alterations occur in disease processes such as cardiac and autoimmune conditions and, thereby, suggests a possible genetic link between certain neoplastic and non-neoplastic diseases. Moreover, LOH/MA in this group may just represent molecular damage due to tobacco exposure in smokers or other carcinogens in nonsmokers. Such genetic changes have been previously reported in smokers in a range of histological types including normal epithelium (15, 16). The individuals included in this study are currently being followed up in the context of the Liverpool Lung Project (22), and it remains to be shown whether those with genetic alterations will develop lung cancer at a future date.

In this study, no statistical correlation was found between genomic instability and age or gender. Because there were only 11 nonsmokers (2 in the lung cancer group and 9 in the noncancer group) in this study, we are unable to produce significant comparisons between genetic alterations and smoking history. LOH tends to be the predominant genetic alteration found among smokers, and MA is more frequent in nonsmokers, although not significantly (Fisher’s exact test, P = 0.19; Table 2). This might reflect the exposure to different carcinogens between smokers and nonsmokers. However, among patients with no malignancy, nonsmokers did not demonstrate a significant difference in the frequency of total genetic alterations (LOH + MA; Table 2). This is in contrast to the findings of Mao et al. (15) and Wistuba et al. (16). One hypothesis for this observation may lie in the argument that this population in the Northwest of England may be exposed to additional carcinogens. This hypothesis is consistent with our previous observations on NSCLC and SCCCHN patients in this region, who demonstrated a p53 mutational profile with prevalence of GC→AT transitions, which is not typical of a smoking population (23, 24).

We have observed a trend for LOH/MA to be found more frequently in BL patients with adenocarcinomas (6 of 8) than with SqCCL (5 of 25). The reason for this is unclear, mainly because adenocarcinomas are usually associated with peripheral airways whereas SqCCL is usually associated with large-airway disease and is more efficiently sampled by BL. Furthermore, the prevalent type of genetic alteration found in specimens with cytological evidence of malignancy was LOH (Table 1), whereas individuals with NMCS at cytology mainly demonstrated MA. This finding implies that LOH (i.e., chromosomal imbalance) may be associated with neoplastic cell transformation and, thus, altered cytological appearance of the cell, whereas MA may not be associated with identifiable morphological changes. MA may, therefore, represent genetic changes of subclones arising from a cell population with high genetic diversity. It may be argued that this difference may be due to the fact that MA is more

Table 2 Genomic instability and smoking history of the lung cancer patients investigated in this study

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>LOH</th>
<th>LOH + MA</th>
<th>MA</th>
<th>No evidence of genetic alterations</th>
</tr>
</thead>
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<tr>
<td>Smokers</td>
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</tr>
<tr>
<td>Lung cancer</td>
<td>9</td>
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<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Noncancer*</td>
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<td>2</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Nonsmokers</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
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<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noncancer</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>No smoking data</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noncancer</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*No clinical lung cancer has been observed in these patients on follow-up to date.
References of MA in samples with low numbers of target cells, such as the cytological specimens that have been reported as NMCS. However, our results indicate that there was no bias in the detection of MA in samples with <50% epithelial cells. We also investigated whether this bias was due to the different histological composition of the NMCS group (prevalence of adenocarcinomas) compared with the cytology-positive group (prevalence of SqCCL: Table 1). However, the absence of a significant difference in the type of genetic alteration seen in adenocarcinomas (four with LOH, two with MA) and SqCCL (four with LOH, one with MA) does not support this argument.

In this study, we have demonstrated that genetic alterations can be detected in the BL. In time, additional markers and advances in technology may increase the present success rate. Additional studies and long-term follow-up are required to clarify the value of such genetic alterations as a tool for the identification of individuals with a high risk of developing the disease.

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References

Genetic Alterations in Bronchial Lavage as a Potential Marker for Individuals with a High Risk of Developing Lung Cancer

John K. Field, Triantafillos Liloglou, George Xinarianos, et al.


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