Primary Adenocarcinomas of the Lung in Nonsmokers Show a Distinct Pattern of Allelic Imbalance

Maria Pik Wong, Wah Kit Lam, Elaine Wang, Shui Wah Chiu, Chi Leung Lam, and Lap Ping Chung

Departments of Pathology [M. P. W., L. P. C.] and Medicine [W. K. L., C. L. L.], University of Hong Kong, Queen Mary Hospital, Hong Kong, and Department of Pathology [E. W.] and Cardiothoracic Unit [S. W. C.], The Grantham Hospital, Hong Kong

ABSTRACT

Lung cancer development in nonsmokers, particularly in females, has long been observed, but the genetic pathways of oncogenesis are still unclear. The purpose of this study was to identify important targets of chromosomal alteration involved in non-tobacco-related adenocarcinomas of lung. In this study, loci of recurrent allelic imbalance (AI) were identified by microsatellite analysis, focusing on tumors with low frequencies of AI (FAL) relative to the mean level. We reasoned that studying such tumors would facilitate the identification of essential genetic changes needed for the malignant phenotype, which could be masked by genomic instability and widespread nonspecific alterations, especially in tumors showing high FAL. Forty-two adenocarcinomas from nonsmokers (NT-ADs) were analyzed by a broad spectrum of 84 markers covering all nonacrocentric chromosomal arms. Using the mean AI frequency (40%) as the threshold, loci in 7q31, 8p23.2, 10p14-p15, 13q12.3, 16q24, 17p13.1-p13.3, 17q22, 19q13.3, and Xq11.2-q12 showed recurrent AI in the low-FAL tumors, which suggested that essential targets of carcinogenesis may be present. To analyze whether loci, frequently altered in NT-ADs, were uniquely involved in these tumors, 43 loci were also studied in 29 adenocarcinomas from smokers. 2q, 6p, 10p, 13q, 16q, 17q, 19p, 19q, 20p, and 20q showed frequent aberrations in NT-ADs, whereas 1q, 2p, 3p, 3q, 7q, 8p, 9p, 9q, 10q, 11q, 13q, 14q, TP53, 17p, 18q, and 21q were commonly altered in both of the tumor groups. Further comparison of their low-FAL tumors showed that AI involving 16q24, 17q22, and 19q13.3 were significantly associated with NT-ADs; whereas those involving 7q31, 8p23.2, 10p14-p15, 13q12.3, and 17p13.1-p13.3 were observed in both. The findings suggest that oncogenesis in the lung of smokers and nonsmokers involve overlapping yet distinct genetic pathways, whereas the recurrent loci of alteration in NT-ADs may provide a basis for the further mapping of critical molecular targets in these pathways.

INTRODUCTION

Lung cancer is a common malignancy etiologically related to smoking. Genotoxic tobacco metabolites form bulky DNA adducts in the genome resulting in frequent chromatid breaks and mutations (1). In recent years, it is increasingly recognized that factors not related to direct cigarette smoking, such as passive smoking, toxicity from environmental tobacco smoke exposure, and environmental tobacco smoke exposure, and she was designated as a passive-smoker, but subsequent information revealed possible environmental tobacco smoke exposure, and she was designated as a passive-smoker. Chronic smokers had 15–60 pack-years of cigarette-smoking history and included those who had stopped smoking for <6 months. The ex-smokers had stopped smoking for <10 years, and those beyond this duration were excluded from the study. Smokers of tobacco products other than cigarettes were also excluded. The nonsmokers were predominantly women, and the patients were recruited from the Grantham Hospital, Hong Kong, during the period 1992–1999. All of them were ethnic Chinese, and none had received any preoperative radiation or chemotherapy. Demographic data were obtained through patient interviews conducted by the designated clinician-in-charge (S. W. C.) at the first hospital admission according to a standardized protocol and were verified by a review of the hospital charts recorded in subsequent visits. For never-smokers, only patients who were lifetime nonsmokers and not exposed to smoking spouses were recruited. One patient was originally recruited as a never-smoker, but subsequent information revealed possible environmental tobacco smoke exposure, and she was designated as a passive-smoker. Chronic smokers had 15–60 pack-years of cigarette-smoking history and included those who had stopped smoking for <6 months. The ex-smokers had stopped smoking for <10 years, and those beyond this duration were excluded from the study. Smokers of tobacco products other than cigarettes were also excluded. The nonsmokers were predominantly women, and the smokers were predominantly men (P < 0.0001). Significant differences in age, tumor grade, and pathological stages were not present between the two populations (Table 1). Tumor classification was according to the WHO historical classification of lung tumors (1991), and the study cases included all subtypes of adenocarcinoma but not adenosquamous carcinoma. The control samples consisted of macroscopically normal lung taken from a portion of the surgical specimen farthest removed from the tumor and/or peripheral blood mononuclear cell pellets obtained before any blood transfusion. Freshly obtained resection specimens were snap-frozen in liquid nitrogen and kept at −70°C until used. The tumor samples were examined histologically before use to ensure at least 80% of tumor by area, and normal lungs were examined to ensure no tumor presence. Selective cases, including those containing normal mononuclear cell admixture or presenting with interpretative difficulties.

MATERIALS AND METHODS

Study Samples. Seventy-one primary lung adenocarcinomas were collected after informed consent from 42 nonsmokers (41 never-smokers, 1 passive-smoker) and 29 smokers (25 chronic smokers, 4 ex-smokers), including 28 men and 43 women ages 38–81 (mean ± SD, 59.8 ± 10.4). The patients were recruited from the Grantham Hospital, Hong Kong, during the period 1992–1999. All of them were ethnic Chinese, and none had received any preoperative radiation or chemotherapy. Demographic data were obtained through patient interviews conducted by the designated clinician-in-charge (S. W. C.) at the first hospital admission according to a standardized protocol and were verified by a review of the hospital charts recorded in subsequent visits. For never-smokers, only patients who were lifetime nonsmokers and not exposed to smoking spouses were recruited. One patient was originally recruited as a never-smoker, but subsequent information revealed possible environmental tobacco smoke exposure, and she was designated as a passive-smoker. Chronic smokers had 15–60 pack-years of cigarette-smoking history and included those who had stopped smoking for <6 months. The ex-smokers had stopped smoking for <10 years, and those beyond this duration were excluded from the study. Smokers of tobacco products other than cigarettes were also excluded. The nonsmokers were predominantly women, and the smokers were predominantly men (P < 0.0001). Significant differences in age, tumor grade, and pathological stages were not present between the two populations (Table 1). Tumor classification was according to the WHO historical classification of lung tumors (1991), and the study cases included all subtypes of adenocarcinoma but not adenosquamous carcinoma. The control samples consisted of macroscopically normal lung taken from a portion of the surgical specimen farthest removed from the tumor and/or peripheral blood mononuclear cell pellets obtained before any blood transfusion. Freshly obtained resection specimens were snap-frozen in liquid nitrogen and kept at −70°C until used. The tumor samples were examined histologically before use to ensure at least 80% of tumor by area, and normal lungs were examined to ensure no tumor presence. Selective cases, including those containing normal mononuclear cell admixture or presenting with interpretative difficulties.

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‡ To whom requests for reprints should be addressed, at Department of Pathology, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong. Phone: 852-2855-4881; Fax: 852-2872-5197; E-mail: lpmchung@hkucc.hku.hk.
The percentages of agreement of results were generally more than 90%. Cases distinguished in the control DNA and as noninformative when only one allele polyacrylamide gel containing urea and formamide, and exposed to X-ray PCR products were radiolabelled by nucleotide incorporation, resolved in 6% according to individual markers, and extension at 72°C (30 cycles) at 94°C Inc.) and 1–2.5 mM MgCl$_2$, forward and reverse primers (0.2 well-known hot spots of allelic loss in lung cancers, were analyzed (Table 2; as well as loci in chromosomes 3p, 9p21-p23, and 11q22-q24 that contain using commercial primers (Research Genetics, Huntsville, AL). For T-ADs, a common carcinomas were selected after literature review and were analyzed and spanning common regions of genetic gain or loss in lung and other odors. For NT-ADs, 84 markers covering all nonacrocentric chromosomal arms and ethanol precipitation according to standard meth-

**RESULTS**

**AI in NT-ADs.** Microsatellite analysis of 84 markers was performed in NT-ADs from 39 women and 3 men, and the mean AI frequency was 37.0 ± 12.3% (range, 10–64%). The extent of chromosomal alteration in each NT-AD was evaluated by the FAL, which ranged from 3 to 76% (mean, 40.0 ± 20.4%). No significant correlation was found between the FAL and age, tumor grade, tumor stage, nodal stage, metastasis, or pathological stages. The mean FAL was used as the threshold to designate two groups, each of 21 tumors, with high or low FAL. No significant differences in the distribution of sex and other clinicopathological parameters between the low or high-FAL tumors were found. The low-FAL tumor group was examined for loci of high AI frequency, defined as those with AI of >40% using the approximated overall mean (37%) as the cutoff. The loci of frequent AI comprised 10 loci in 7q31, 8p23.2, 10p14-p15, 13q12.3, 16q24, 17p13.1-p13.3, 17q22, 19q13.3, and Xq11.2-q12 (Table 2; see loci with footnote c citation). These loci also showed AI of 40% or above in the high-FAL tumors, and a significant difference with the low-FAL tumors was observed in 13q12.3 (P = 0.002) only. The remaining 74 loci showed relatively infrequent AI (40% or less) in the low-FAL tumors compared with the high-FAL tumors, with the difference reaching statistical significance in 26 loci (P of <0.0001 to 0.05; χ² test, Table 2).

**AI in T-ADs.** To investigate the uniqueness of involvement of the frequently altered loci in non-tobacco-related tumors, a selective panel of markers (Table 2; see loci with footnote c citation), including those with AI >40% in NT-ADs, were then analyzed in 29 T-ADs from 25 male and 4 female patients for comparison. The FAL of the T-ADs ranged from 4 to 84% (mean, 44.5 ± 24.7%). No statistical correlation was found between the FAL and the clinicopathological variables of age, tumor grade, tumor stage, nodal stage, metastasis, or pathological stage. Loci in 2q33, 6p23-p24, 10p14-p15, 13q14.1, 16q24, 17q11-q12, 17q22 (P = 0.06), 19p13.2, 19q13.3, 20p12, and 20q12 showed more frequent alterations in NT-ADs than in T-ADs (Fig. 1, solid bars). Although the differences did not reach statistical significance, these loci were relatively infrequently altered in T-ADs: they showed AI frequency lower than the mean level (49.7 ± 14.5%) for T-ADs. Loci in 1q24-q25, 2p24-p25, 3p14.2, 3p22.3, 3p22.3, 3p22.3, 4q34.5, 7q31, 8p23.2, 9p22, 9q34, 10q26, 11q23.1, 13q12.3, 14q32, 17p13.1-p13.3, 18q23, and 21q22 showed closely similar AI frequencies in both groups (within 10%; Fig. 1, dotted bars), or more frequent alterations in T-ADs (Fig. 1, hatched bars).

**Comparison of Low-FAL NT-ADs and T-ADs.** The low-FAL tumors of NT-ADs and T-ADs, consisting of 21 NT-ADs and 13 T-ADs both designated according to the mean FAL values calculated from the panel of 43 markers analyzed in both tumor groups as cutoff, were compared for recurrent aberrations. There were more women than men in the NT-ADs (P < 0.0001; Table 3). No significant differences in other clinicopathological parameters were detected. In the low-FAL NT-ADs, the same 10 loci as those identified in the analysis of all of the markers showed AI frequency of >40%. Three of 10 loci showed significantly more frequent AI in the low-FAL NT-ADs than in the T-ADs (16q24, P = 0.03; 17q22, P = 0.04; and 19q13.3, P = 0.04), whereas no significant difference was observed for 7q31, 8p23.2, 10p14-p15, 13q12.3, and 17p13.1-p13.3 (Table 3).

### Table 1: Comparison of clinicopathological data and FAL in adenocarcinomas from nonsmokers (NT-AD) and smokers (T-AD)

<table>
<thead>
<tr>
<th></th>
<th>NT-AD</th>
<th>T-AD</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>FAL</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>F</td>
<td>39</td>
<td>38 ± 21</td>
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<tr>
<td>M</td>
<td>3</td>
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<td><strong>Tumor differentiation</strong></td>
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<td>Well</td>
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<td>33 ± 21</td>
</tr>
<tr>
<td>Moderate</td>
<td>26</td>
<td>43 ± 20</td>
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<tr>
<td>Poor</td>
<td>3</td>
<td>23 ± 10</td>
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<td><strong>Tumor stage</strong></td>
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<td>42 ± 25</td>
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<td><strong>Nodal stage</strong></td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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<td><strong>Metastasis</strong></td>
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<tr>
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<td>I</td>
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<td>36 ± 22</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>30 ± 18</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>49 ± 15</td>
</tr>
<tr>
<td>IV</td>
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</tr>
<tr>
<td>Loci</td>
<td>Markers</td>
<td>Overall Het&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>10q24</td>
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<td>14q12</td>
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<td>71.4</td>
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<td>14q22</td>
<td>D14S 267</td>
<td>76.2</td>
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<td>15q11-q12&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>15q22</td>
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<td>61.6</td>
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<td>80.3</td>
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<td>18q11.22-pter</td>
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<td>D20S 119</td>
<td>69</td>
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*<sup>a</sup>*Overall Het: Overall heterozygosity. <br>**<sup>b</sup>*p: p-value. 

Table 2. List of loci analyzed and comparison of frequencies of AI in low- and high-FAL adenocarcinomas from nonsmokers.
DISCUSSION

We have analyzed the pattern of AI in a broad spectrum of microsatellite markers in adenocarcinomas from nonsmokers to identify common genetic targets of tumor development. In view of the considerable amount of data that result from studies using genome-wide approaches, we have used two strategies to help us focus on alterations that are most likely to be important. Firstly, we have used the mean AI percentage of all of the markers in NT-ADs (40%) as our threshold to designate loci of frequent AI throughout this study. Secondly, we propose that essential and critical alterations that drive malignant progression could be found in tumors that show fewer genetic changes; therefore, we searched for these essential aberrations by examining tumors with low FAL. No FAL correlation with the patients’ age, tumor grade, tumor, nodal, metastasis, or pathological stages is found in either NT-ADs or T-ADs, implying that differences between tumors with low or high FAL are unlikely to be caused by variations in these clinicopathological parameters. Accordingly, 10 loci in 7q31, 8p23.2, 10p14-p15, 13q12.3, 16q24, 17p13.1-p13.3, 17q22, 19q13.3, and Xq11.2-q12 showed AI of 40% in both low- and high-FAL tumors, which suggests that these loci are important in all lung cancers arising in nonsmokers. The remaining loci with low AI frequencies in the low-FAL group, especially the 26 loci with significantly more frequent AI in the high-FAL tumors, might represent loci that are not critical for tumor development.

To investigate whether frequently altered genetic loci identified in NT-ADs were uniquely involved in non-tobacco-mediated carcinogenesis, loci with frequent AI were also analyzed in 29 adenocarcinomas from smokers (T-ADs) for comparison. The results indicated a wide overlap in genetic aberrations when all tumors of both groups were analyzed, including 11 regions (2q33, 6p23-p24, 10p14-p15, 11q13.3, 13q12.3, 16q24, 17p13.1-p13.3, 19p13.3, and Xq11.2-q12).

Table 3 Comparison of low-FAL adenocarcinomas of nonsmokers and smokers

| Markers | NT-AD | T-AD | P
| --- | --- | --- | ---
| Sex | | | 0.0001
| F | 20 | 3 | <0.0001
| M | 1 | 10 | ns
| Tumor differentiation | | | 0.47
| Well | 8 | 3 | ns
| Moderate | 10 | 9 | ns
| Poor | 3 | 1 | ns
| Tumor stage | | | 0.23
| 1 | 13 | 6 | ns
| 2 | 5 | 4 | ns
| Pathological stage | | | 0.65
| I | 21 | 13 | ns
| II | 4 | 3 | ns
| III | 3 | 3 | ns
| IV | 0 | 0 | ns

Recurrent loci of AI (%)

| Loci | Markers | NT-AD | T-AD | P
| --- | --- | --- | --- | ---
| 1q424 | D16S 534 | 11/19 (58) | 2/12 (17) | 0.03
| 17q22 | D17S 791 | 8/18 (44) | 1/13 (8) | 0.04
| 19q13.3 | D19S 217 | 8/14 (57) | 1/9 (11) | 0.04
| 7q31 | D7S 522 | 7/11 (64) | 1/3 (33) | 0.54
| 8p23.2 | D8S 307 | 11/21 (52) | 1/10 (10) | 0.11
| 10p14-p15 | D10S2325 | 7/17 (41) | 1/13 (8) | 0.09
| 13q12.3 | D13S 267 | 7/16 (44) | 5/11 (45) | 1
| 17p13.3 | D17S 926 | 6/13 (46) | 2/7 (29) | 0.64
| 17q13.1-p13.3 | D17S 695 | 7/17 (41) | 8/13 (62) | 0.46
| Xq11.2-q12 | AR | 7/15 (47) | 0/2 (0) | nd

*a* ns, statistical analysis is not applicable; nd, analysis was not done because only two female T-AD were involved in this locus.
important tumor suppressor genes for human lung cancer could be developing in this animal model have been shown to share pathoge-
mouse model of lung adenocarcinoma (6). Because lung cancers
involved in the genesis of the urethane-induced
specific regulatory mechanisms of the lung, and so forth. Interest-
ly, 17q22 is located in a syntenically conserved region in the
mouse genome that contains the mouse pulmonary adenoma resist-
ance gene 1 (PAR1) involved in the genesis of the urethane-induced
model of lung adenocarcinoma (6). Because lung cancers
developing in this animal model have been shown to share pathoge-
etic and biological behavior that is similar to that in human lung cancers (7), our finding of frequent AI in this region suggests that
important tumor suppressor genes for human lung cancer could be
located in 17q22. Feng et al. (8) have also reported 42% alterations of
17q in NSCLC, a frequency similar to that in our findings. 16q23-q24,
which spans the common fragile site FRA16D, shows frequent allelic loss or homozygous deletions in cancer cell lines or primary cancers
(9). The H-cadherin gene located in this region shows frequent

down-regulation and methylation in NSCLC, but mutations have not
been identified (10, 11). 10p15 and 19q13.3 are novel regions of

genetic alterations identified in a genome-wide screening study of
lung cancer cell lines using high-density marker sets (12, 13). Fre-
quent alterations in 7q31 have not been previously reported in allelo-
type studies; however, karyotyping and comparative genomic hybrid-
ization studies have consistently reported polymy of chromosome 7
or amplification of 7q31 in NSCLC (14, 15). Because amplified

copies of a DNA fragment may manifest as AI, our results may be a
reflection of the polymy and may indicate the presence of potential
oncogenes in this region.

Because of the small number of male nonsmoking and female
smoking patients who present with excisable lung cancers in our
population, the effect of gender differences on AIs cannot be inde-
pently evaluated in our study. The high proportion of nonsmoking
women with lung cancers may reflect potential influences of female-
specific factors or differences in susceptibility to common carcinoma-
genic agents. In a study of Chinese subjects, significantly higher DNA
adduct levels were found in the nontumor lungs of female nonsmokers
than in those of male nonsmokers (16), which suggests that female
susceptibility to DNA damage derived from environmental carcino-
gen exposure may be a confounding factor in lung cancer develop-
ment. A higher expression level of gastrin-releasing peptide receptor
in women, which is involved in the regulation of cellular proliferation
and is induced in smokers, has also been proposed as a mechanism for
the increased susceptibility (17).

Our findings of frequent genetic changes in NT-ADs, with a mean
AI frequency of 40%, and the involvement of an overlapping spectra of
loci compared with T-ADs, are different from those reported by
Sanchez-Cespedes et al. (18) in a recent study in which AI was
infrequent in 18 adenocarcinomas from nonsmokers. None of 54
markers from 28 chromosomal arms showed alteration of $\geq 25\%$.
Changes at 9p21, 12p, and 19q13.3, each occurring at $22\%$, were the
most common alterations found. Our data support frequent involvement
of 19q13.3 (48%) and 9p21 (45%) in NT-ADs in general, but we have not found 12p alterations to be a frequent event. Furthermore, 19q13.3
involvement is also frequent in our low-FAL tumors, which suggests
that genes that are critically essential for lung cancer oncogenesis in
nonsmokers may be linked to this region. The reason for the discrep-
ant findings between the two studies is not clear, but variations in
genetic, environmental, and lifestyle factors may be involved. It
would be interesting to compare the patient data, particularly sex
distribution, of nonsmoking subjects in the reported study.

In summary, we have shown that AI in 16q24, 17q22, and 19q13.3
may represent essential alterations in lung adenocarcinomas arising in
nonsmoking patients, particularly for females. They represent useful
sites for additional mapping of genetic targets in non-tobacco-associ-
ated lung carcinogenesis by high-density microsatellite marker sets.

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