Alterations in the Mitochondrial Displacement Loop in Lung Cancers

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ABSTRACT

Purpose: Alterations of the noncoding displacement (D) loop of mitochondrial DNA are present in many cancers. These alterations include numerical changes in a homopolymeric C tract (PCT) at positions 303–309 and single base substitutions (SBS). We determined the frequency of D-loop alterations in lung cancer cell lines and tumors, and related them to clinicopathologic features.

Experimental Design: We sequenced the entire D-loop of 28 lung cancer cell lines [12 small cell lung cancer (SCLC) and 16 non-small cell lung cancer (NSCLC)] and matched B-lymphoblastoid cell lines. In 55 resected NSCLCs and corresponding nonmalignant lungs we determined the length of the PCT.

Results: In nonmalignant cell lines and tissues the most frequent PCT repeat number was seven (36 of 83; 43%) with a range of six to nine. Alterations, often multiple, were present in 17 of 28 (61%) of the cell lines, including 8 of 12 SCLC (67%) and 9 of 16 NSCLC (56%) lines. They consisted of SBS in 8 of 28 lines (29%), all of which were homoplasmic, and PCT changes in 14 of 28 (50%) lines, 8 of which were homoplasmic. Of interest, 95% (40 of 42) of the SBS were present within the two hypervariable regions in the D-loop. Because SBS were more frequent if PCT changes were present, only the PCT number was determined in resected samples. PCT changes were present in 11 of 55 (20%) of the NSCLC tumors. Changes were never noted in tumors when the PCT number in the nonmalignant tissue was seven, and only two tumor cell lines had changes when the PCT number in the matched lymphoblastoid cell line was seven. These changes were higher in squamous cell carcinomas (8 of 25; 32%) than in adenocarcinomas (3 of 30; 10%; P = 0.04) and in large tumors (T3 and T4; 7 of 20; 35%) compared with smaller tumors (T1 and T2; 4 of 35; 11%; P = 0.04). Smoking history, gender, age, and stage were not related to frequency of PCT change.

Conclusions: Our findings indicate that D-loop alterations are frequent in lung cancers and their cell lines, and that these changes are weakly associated with certain clinical parameters. In tumors PCT changes were only present when the corresponding nonmalignant lung demonstrated a variation from the most common repeat number of seven.

INTRODUCTION

mtDNA5 is a 16569 nucleotide pair double-stranded, closed circular molecule, which codes for both small (12S) and large (16S) rRNA, 22 transfer RNAs, and 13 protein-coding genes (1). Most human cells contain hundreds of mitochondria (1), and the vast majority of mtDNA copies are identical (homoplasmic) at birth (2). The mutation rate for mtDNA is ~10 times higher than that of nuclear genomic DNA (1). Somatic mitochondrial mutations and other changes were identified recently in various human cancers (3), with the highest frequency reported in the noncoding D-loop region (4–7).

The D-loop, which is 1124 bp in size (positions 16024–576), is a noncoding region, and acts as a promoter for both the heavy and light strands of the mtDNA, and contains essential transcription and replication elements. The D-loop region is a hot spot for mtDNA alterations, and it contains two hypervariable regions (HV1 at positions 16024–16383 and HV2 at positions 57–372; Ref. 8). There are two previous reports (from one laboratory) that have analyzed alterations of the D-loop in lung cancer (5, 9). These alterations consist of two major categories: (a) numerical changes in PCT at positions 303–309; and (b) SBS.

In this study, we examined alterations of the D-loop region in lung cancer cell lines and resected NSCLC tumors, and related our findings to clinicopathological features.

MATERIALS AND METHODS

Cell Lines. Human lung cancer cell lines (12 SCLC and 16 NSCLC) and corresponding B-lymphoblastoid lines (n = 28) were established by us (Ref. 10; Table 1). Tumor cell lines were selected because of the availability of matched B-lymphoblas-
The number of C repeats in the PCT are stated for TM and BL.

Table 1

<table>
<thead>
<tr>
<th>Change of D-loop sequence in lung cancer cell lines compared with paired lymphoblastoid cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
</tr>
<tr>
<td><strong>NCI-H2195</strong></td>
</tr>
<tr>
<td>16069 C to T</td>
</tr>
<tr>
<td>16126 C to T</td>
</tr>
<tr>
<td>16278 C to T</td>
</tr>
<tr>
<td>16292 T to C</td>
</tr>
<tr>
<td>16294 T to C</td>
</tr>
<tr>
<td>16296 T to C</td>
</tr>
<tr>
<td>16304 T to C</td>
</tr>
<tr>
<td>16362 C to T</td>
</tr>
<tr>
<td>150 T to C</td>
</tr>
<tr>
<td>152 T to C</td>
</tr>
<tr>
<td>295 C to T</td>
</tr>
<tr>
<td>16126 C to T</td>
</tr>
<tr>
<td>16187 T to C</td>
</tr>
<tr>
<td>16209 T to C</td>
</tr>
<tr>
<td>16294 T to C</td>
</tr>
<tr>
<td>16296 T to C</td>
</tr>
<tr>
<td>16304 C to T</td>
</tr>
<tr>
<td>73 G to A</td>
</tr>
<tr>
<td>151 T to C</td>
</tr>
<tr>
<td><strong>NCI-H1339</strong></td>
</tr>
<tr>
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</tr>
<tr>
<td>16127 T to C</td>
</tr>
<tr>
<td>16147 T to C</td>
</tr>
<tr>
<td>16187 T to C</td>
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<tr>
<td>16209 T to C</td>
</tr>
<tr>
<td>16294 T to C</td>
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<tr>
<td>16296 T to C</td>
</tr>
<tr>
<td>16295 C to T</td>
</tr>
<tr>
<td>73 G to A</td>
</tr>
<tr>
<td>151 T to C</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Postsurgical-stage</th>
<th>No. of PCT changes</th>
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</thead>
<tbody>
<tr>
<td>Postsurgical N factor</td>
<td>Gender</td>
</tr>
<tr>
<td>*</td>
<td>Male (n = 46)</td>
</tr>
<tr>
<td>**</td>
<td>Female (n = 9)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Never (n = 14)</td>
</tr>
<tr>
<td>History</td>
<td>Ever (n = 41)</td>
</tr>
<tr>
<td>Logistic</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>ever</td>
<td></td>
</tr>
<tr>
<td>never</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
</tr>
<tr>
<td>Post-surgical T factor</td>
<td></td>
</tr>
<tr>
<td>T1 and T2 (n = 35)</td>
<td></td>
</tr>
<tr>
<td>Post-surgical N factor</td>
<td></td>
</tr>
<tr>
<td>N1 (n = 31)</td>
<td></td>
</tr>
<tr>
<td>N1 and N2 (n = 24)</td>
<td></td>
</tr>
<tr>
<td>Post-surgical-stage</td>
<td></td>
</tr>
<tr>
<td>Stage I and II (n = 27)</td>
<td></td>
</tr>
<tr>
<td>Stage III and IV (n = 28)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1** Changes of D-loop sequence in lung cancer cell lines compared with paired lymphoblastoid cell lines

No alterations were detected in SCLC cell lines (NCI-H1672, NCI-H1184, NCI-H1607, NCI-H2028), or in NSCLC cell lines (NCI-H2122, NCI-H2126, HCC15, HCC44, HCC78, HCC366, HCC515). The number of C repeats in the PCT are stated for TM and BL.

Clinical Samples. Tumor samples from 55 primary previously untreated NSCLC (24 squamous cell carcinomas and 31 adenocarcinomas) and corresponding nonmalignant lung tissues were obtained from surgical resections at Chiba University Hospital in Japan from 1999 to 2000 (Table 2). Appropriate Institutional Review Board permission was obtained from the institute, and informed consent was obtained from all of the subjects. Tissues were stored at −80°C before testing.

Direct Sequencing of the D-Loop Region of mtDNA. To cover the entire D-loop sequence, two overlapping fragments of 613 and 679 bp were PCR amplified and sequenced in the 28 lung cancer cell lines and corresponding B-lymphoblastoid lines. Primers were carefully designed to avoid amplifying the nuclear-encoded pseudogenes reported previously (12, 13). Forward and reverse primers for the first segment were: 5'-ACTC-CACCATTAGCGCACCCAAAAGC-3' and 5'-GTTAATAGGTT-GATAGACCTGTGAT-3', respectively, and for the second segment 5'-CACCATTAGCACCCAAAGC-3' and 5'-GTTAATAGGTT-GATAGACCTGTGAT-3'.

Table 2 Correlation between PCT alterations and clinicopathologic features in resected NSCLCs (n = 55)

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>No. of PCT changes (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 46)</td>
<td>10 (22)</td>
<td>0.67</td>
</tr>
<tr>
<td>Female (n = 9)</td>
<td>1 (11)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64 &gt; (n = 26)</td>
<td>4 (16)</td>
<td>0.51</td>
</tr>
<tr>
<td>64 ≤ (n = 29)</td>
<td>7 (24)</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never (n = 14)</td>
<td>2 (14)</td>
<td>0.7</td>
</tr>
<tr>
<td>Ever (n = 41)</td>
<td>9 (22)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>3 (10)</td>
<td>0.045</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>8 (32)</td>
<td></td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-surgical T factor</td>
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<td></td>
</tr>
<tr>
<td>T1 and T2 (n = 35)</td>
<td>4 (11)</td>
<td>0.042</td>
</tr>
<tr>
<td>T3 and T4 (n = 20)</td>
<td>7 (35)</td>
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</tr>
<tr>
<td>Post-surgical N factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0 (n = 31)</td>
<td>6 (19)</td>
<td>0.99</td>
</tr>
<tr>
<td>N1 and N2 (n = 24)</td>
<td>5 (21)</td>
<td></td>
</tr>
<tr>
<td>Post-surgical-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I and II (n = 27)</td>
<td>4 (15)</td>
<td>0.27</td>
</tr>
<tr>
<td>Stage III and IV (n = 28)</td>
<td>7 (25)</td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test.

a C#; number of poly C repeats, BL; B-lymphoblastoid cell line, TM; tumor cell line, del; deletion, ins; insertion.

b The number of PCT in matched lymphoblastoid cell line was other than 7.

c PCT showed heteroplasmy and the number mentioned indicates the dominant population.
Mitochondrial Alterations in Lung Cancer

For DNA amplification, 100 ng of DNA was subjected to PCR protocol: 95°C for 12 min, 1 cycle; 95°C for 30 s, 62°C for 1 min, and 70°C for 30 s, 30 cycles; and a final extension at 72°C for 7 min. PCR products were excised from a 1% agarose gel and purified with SUPREC-01 (TaKaRa, Tokyo, Japan), and then sequenced by Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA).

Analysis of the PCT and Cloning in NSCLC Patients.

For analysis of the D-loop region in surgically resected samples, a 336-bp fragment containing the PCT at positions 303–309 was PCR amplified from tumor and nonmalignant lung DNA and subsequently cloned. Primers and method of cloning were as described previously (9). The sequences of the primers were: forward, 5′-ACAATTGAATGTCTGCACAGCCACTT-3′ and reverse, 5′-TGTGGGGGGTGTCTTTGGGG-3′. After an initial denaturalization step at 95°C for 12 min, samples were cycled 30 times as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The final extension was at 72°C for 7 min. PCR products were purified and sequenced as described above.

The amplicon obtained from the above-described PCR reaction was also used for clonal analysis of heteroplasmy. The PCR products were cloned into plasmid vectors using the TOPO TA-PCR Cloning kit (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. Plasmid DNAs were purified using the Wizard Plus miniprep kit (Promega, Madison, WI) and then sequenced.

To rule out possible artifacts or mistakes in the PCR reaction, the assay was repeated for all of the samples that showed alterations in the tumor DNA. We also confirmed that lung cancer and corresponding lymphoblastoid cell lines belonged to the same individual by comparing the patterns of polymorphic DNA microsatellite markers. In addition, the cell lines have been deposited with the American Type Culture Collection (Manassas, VA), and they have informed us that their polymorphic marker analyses have confirmed that each pair used by us is indeed derived from the same individual.

Statistical Analysis. Fisher’s exact probability test was used to examine the association between two groups. Two-sided binominal tests were performed to examine whether mutations occurred randomly. Values of $P < 0.05$ were regarded as statistically significant.

RESULTS

Alterations in Lung Cancer Cell Lines. The D-loop region was analyzed in 12 SCLC cell lines, 16 NSCLC cell lines, and matching B-lymphoblastoid lines by direct sequencing (Fig. 1). Results of the analyses are summarized in Table 1. Alterations (either PCT change or SBS) were present in 17 (61%) of the cell lines, including 8 SCLC cell lines (67%) and 9 NSCLC cell lines (56%). Multiple alterations (both PCT change and SBS, or multiple SBS) were present in 6 cell lines (21%). The number of alteration events in these cell lines ranged from 2 to 13 (median 6.5). Interestingly, 95% (40 of 42) of the SBS were present in the sequences of the two hypervariable regions (HV1 and HV2; 676 bp) within the D-loop (1124 bp). Binominal tests confirmed that these SBS do not occur randomly across the entire D-loop length but are concentrated in HV1 and HV2 ($P < 0.0001$). PCT changes were present in 14 cell lines (50%) including 7 SCLC cell lines (58%) and 7 NSCLC cell lines (44%). These numerical changes consisted of either gains or losses from the number present in constitutional DNA. SBS were present in 8 cell lines (29%). All of the SBS and 57% (8 of 14) of PCT changes were homoplasmic. However, SBS without PCT change were present only in 3 cell lines (11%). So for analyses of tumors changes in only the PCT number was determined.

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* Internet address: http://www.mitomap.org/
PCT in NSCLC. We determined whether PCT changes in the D-loop were also present in primary tumors. We analyzed 55 NSCLC cases by comparing the sequences of a region designated PC (Fig. 1), which consisted of 336 bp of HV2 and included the PCT. PCT changes were present in 11 cases (20%) of NSCLC, and that number was not significantly different from the results of NSCLC cell line analysis. Fifty five percent (6 of 11) of PCT changes were homoplastic. There were no SBS alterations in the remaining part of the PC amplicon.

After completion of the detailed sequence analysis in all of the samples, clinicopathological data were correlated with the molecular analyses (Table 2). Higher percentages of PCT change were present in males, in the higher age group, ever-smokers, and in advanced stage tumors, but these were not significant. There were significant differences in the distribution of patients with or without PCT change in terms of histology and postsurgical T factor (a reflection of tumor size and local invasion). The PCT change rate was higher in squamous cell carcinomas (32%) than in adenocarcinomas (10%; \( P = 0.045 \)), and higher in advanced T stages (T3 and T4; 35%) than in localized stages (T1 and T2; 11%; \( P = 0.042 \)).

We also analyzed the correlation between the numbers of PCT in tumors and in matched control tissues (Table 3). The number of cytosines in the 7-bp stretch varied from six to nine in nonmalignant tissues. The most frequent C tract repeat number was seven, as expected, in B-lymphoblastoid cell lines (12 of 28; 43%) and in nonmalignant lung tissues (24 of 55; 43%; Table 4). Except for 2 cell lines, PCT changes in tumor cells (compared with their respective control tissues) were only present if the PCT number in the control tissue was a number other than 7 (\( P = 0.0001 \)). In cell lines, only 2 tumor lines had numerical changes in PCT when the PCT number in the corresponding B-lymphoblastoid cell line was 7 (\( P = 0.003 \)).

Detection of Heteroplasmy at the PCT in Nonmalignant Lung Tissues from Cancer Patients. Sequence analysis on nonmalignant lung tissue occasionally demonstrated variation of the precise PCT number (Fig. 2). In the example displayed, at positions 310 and 311 there appears to be a major T peak and a minor C peak. After analysis of 7 subclones, we determined that there was heteroplasmy, and the C number of the sample was scored as 8 (dominant population) or 9 (minor population).
Heteroplasmy of the PCT number was noted in 2 of 28 B-lymphoblastoid cell lines (7%) and in 9 of 55 nonmalignant lung tissues (16%).

**DISCUSSION**

Our results confirm and extend the findings of two reports published previously (from the same laboratory) regarding mitochondrial alterations in lung cancers (5, 9). These reports describe SBS frequencies within the D-loop of 7–29% and 16% PCT changes in resected NSCLC. We analyzed SCLC and NSCLC cell lines (and corresponding B-lymphoblastoid cell lines), and resected NSCLC tumors (and corresponding lung tissue). Of the cell lines, 61% had alterations, often multiple, and the differences between SCLC and NSCLC were not significant. The SBS rate, often multiple, in lung cancer cell lines was 29%. The distribution of SBS was not random across the entire length of the D-loop but was concentrated in hypervariable regions HV1 and HV2. Whereas this has been described previously as a general tumor finding (14), to our knowledge this is the first report that SBS in the D-loop of lung cancers occur in a nonrandom distribution. In addition PCT changes were frequent (50%) in lung cancer cell lines. Only one tumor cell line (NCI-H1450) showed a previously undescribed alteration (G deletion at position 71), not listed in the mtDNA database.6 The tumor cell line data were compared with corresponding B-lymphoblastoid cell lines. These lines were immortalized with EBV, and allelic changes caused by lymphocyte immortalization have been reported by Weber and Wong (15). However, Richard et al. (16) reported that the mutation rate in the D-loop due to lymphoblastoid transformation was very low (0.2%). Thus, we believe that lymphoblastoid cell lines were an appropriate source of constitutional DNA.

Because PCT numerical changes were more frequent than SBS in cell lines, we limited our analysis of the tumors to a 336-bp stretch of hypervariable region 2, which encompassed the PCT. Of the 55 NSCLC cases analyzed, we found a frequency of PCT changes in 20%, compared with 16%, in a previous study (9) and not statistically different from our finding in NSCLC cell lines. There were no SBS alterations in the remaining part of the PC amplicon. Of interest, changes in PCT number in cell lines and tumors were, with two exceptions, restricted to cases in which the constitutional DNA was other than the modal PCT number of seven cytosines. These findings suggest that constitutional variation in PCT from the modal number reflects an inherent or acquired instability that results in additional numerical changes in corresponding tumors.

When alterations of the D-loop were identified in lung cancer tumors and cell lines, they usually demonstrated homoplasy (100% of SBS and 56% of PCT numerical changes). The percentages of PCT changes in cell lines and tumors were similar, suggesting that the reason for heteroplasy was not due to contamination with nonmalignant tissue. The possible mechanisms by which homoplasy may occur has been discussed by others (9, 17). The frequent finding of homoplasy suggests that it imparts some growth or survival advantage to the tumor cell.

From reports published previously, the frequency of PCT change in human cancers varies from 0% in colorectal cancers to 42% in breast cancers (9, 13, 18–23). Thus, the frequency of PCT in NSCLC (20%), as reported by us, is in the mid-range for human cancers. However, we have identified several previously unreported features, including the relationship of PCT numerical change to variations in the PCT number in corresponding constitutional DNA and the nonrandom distribution of SBS. Whereas the significance of the high intrinsic rate of alterations in mitochondria is not fully understood, the finding of homoplasy suggests that they play a role in cancer pathogenesis.

**REFERENCES**

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