Nucleotide Sequence Variation Is Frequent in the Mitochondrial DNA Displacement Loop Region of Individual Human Tumor Cells

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Abstract
The mitochondrial DNA (mtDNA) displacement loop (D-loop) regions of 76 various tumor cell lines were examined to investigate the existence of a specific relationship between a somatic mtDNA sequence and initiation and/or progression of a tumor. Based on molecular cloning-sequencing analysis, a nucleotide sequence in the D-loop region in each cell line was found to be homoplasmic. Several site-specific nucleotide variations were found in stomach and liver tumor cell lines more frequently than those in other tumor cell lines. Subsequently, 20 pairs of noncancerous and cancerous parts from stomach and liver tumor tissues were examined. In the liver tumor tissue, 80% of the noncancerous parts exhibited slightly higher heterogeneity than the corresponding cancerous parts. Several site-specific nucleotide variations found in 76 tumor cell lines were also detected in noncancerous or cancerous parts of stomach and liver tumor tissues. However, it remains unclear why the mtDNA D-loop sequence is homoplasmic in each tumor cell line. The data indicate that mtDNA exhibits heterogeneity even in the noncancerous part and a slight decrease in heterogeneity during tumorigenesis and/or tumor progression. Homoplasy of the mtDNA population in the tumor cell line would be acquired in the cloning process of establishing a cell line. Site-specific nucleotide substitutions might not be directly involved in the tumorigenesis process. (Mol Cancer Res 2005; 3(1):14–20)

Introduction
Human mitochondrial DNA (mtDNA) consists of 16,569 bp, comprising a circular dsDNA molecule. The mitochondrial genome encodes 2 rRNAs and 22 tRNAs that are required for intramitochondrial translation. It also encodes 13 polypeptides that are components of the respiratory chain enzyme complexes in the inner membrane of the mitochondria (1). There is a displacement loop (D-loop) region in mtDNA (2), which contains the heavy-strand promoter and light-strand promoter regions (3, 4) as well as the initiation site of heavy-strand replication (2).

There are ~10^3 to 10^4 mtDNA copies per human cell (5), and the majority of these copies are identical immediately after birth. Because the mitochondria lack protective histones or highly efficient mechanisms for repairing DNA, mammalian mtDNA accumulates mutations at a 10-fold higher rate than does nuclear DNA (6, 7). Because several somatic mutations have been recently identified in human tumors (8-12), examination of mtDNA mutations in various tumor cells is important for understanding the relationship between somatic mtDNA mutation and the initiation and/or progression of a tumor.

In this study, we first analyzed the frequency of nucleotide variation in the D-loop region of mtDNA in each sample derived from various tumor cell lines. The frequencies of nucleotide variation among 76 cell lines were also analyzed to determine whether tumor-specific or tumor type–specific nucleotide changes could be detected. Tumor-specific change in a particular position could not be detected. The sequence homogeneity of the D-loop region in a tumor cell line could therefore be acquired in the process of establishing the cell line from tissue.

Next, we isolated DNA clones from each cancerous and noncancerous part of stomach and liver tumor tissue samples. Sequence analysis of the D-loop region revealed many sequence variations in tumor tissue samples showing nonclonal features of tumor tissue in both cancerous and noncancerous parts. This suggests that the mtDNA population remained heterogeneous in the cancerous part and that site-specific nucleotide variation might not be directly involved in the mechanism of tumorigenesis.

Results
Nucleotide Sequence of the mtDNA D-Loop Region from a Tumor Cell Line Was Identical
Direct sequencing of the PCR product may lose the potential to detect minor mtDNA mutations in the background of large phenotypic expression. In this study, mtDNA was isolated from 76 culture cell lines derived from human stomach (n = 21), liver (n = 13), breast (n = 12), lung (n = 8), brain (n = 6), colon (n = 5), ovary (n = 5), renal (n = 2), prostate (n = 2), melanoma
(n = 1), and osteosarcoma (n = 1) tumors. The variation of D-loop DNA in each cell line sample was analyzed initially cell by cell. Pooled PCR products of 1.2-kb fragments were molecularly cloned and subjected to DNA sequencing. The 15 independent DNA clones from each of 76 tumor cell lines were isolated and 1,140 DNA clones (76 × 15) in total were sequenced.

Nucleotide sequences detected in 15 individual D-loop DNA clones were homoplasmic in each cell line, with the exception of the poly-C tracks in D16189 [16,184-16,193 nucleotide position (np)] and D310 (303-315 np) regions. Earlier work showed that the D16189 (16,184-16,193 np) and D310 (303-315 np) regions exhibited polymorphic length variation among individuals (1, 13) as well as nucleotide variation within an individual (14, 15). Substitution of T by C in the 16,189 or 310 np site will generate homopolymer tracts, which are error hotspots originated from polymerase fidelity and DNA slippage during DNA synthesis in vitro (16).

Most of the nucleotide variations were found in two hypervariable regions, which are so-called hotspots (17), and were not uniformly distributed in the D-loop region (Fig. 1). When 21 stomach tumor and 13 liver tumor cell lines were compared with each of the remaining 55 and 63 tumor cell lines, respectively, several site-specific nucleotide variations were found in the stomach (Fig. 2) and liver (Fig. 3) tumor cell lines. The frequency of nucleotide variation at sites, such as T146C, C150T, T152C, A16183C, T16189C, C16223T, T16362C, and T16519C, were higher than at other sites. Particularly, at site 16,189 np, the frequency was clearly higher in both cell types. In liver tumor cell lines, 10 of 13 cell lines showed heterogeneity corresponding to 77% and 9 of 21 stomach tumor cell lines were mutated at this site corresponding to 43%. The frequency of some nucleotide variations was higher in the stomach and lower in other tumor cell lines, such as C150T (33%) and T16362C (52%). In contrast, the frequency at site T146C was higher (31%) in the liver and lower (11%) in other tumor cell lines (Fig. 3). Even if some of the nucleotide variations were higher in both stomach and liver tumor cell lines, such as T152C, C16223T, and T16519C, the frequency of nucleotide variation in the two tumor types was different. For instance, at site T16519C, 8 of 21 stomach cell lines were changed corresponding to 38% and 7 of 13 liver cell lines were changed corresponding to 54%. These are considered to be site-specific nucleotide variations of the D-loop regions in stomach and liver tumors. To eliminate the possibility that these nucleotide variations originated from nuclear pseudogenes, we searched the human genome sequence (BLAST; http://www.ncbi.nlm.nih.gov./BLAST/) similar to the D-loop

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![Sequence Variation in mtDNA D-Loop Region](http://www.ncbi.nlm.nih.gov./BLAST/)

**FIGURE 1.** Frequency of nucleotide variation in the D-loop DNAs isolated from 76 human tumor cell lines. D-loop DNA clones from each of 76 tumor cell lines were sequenced and their nucleotide variation at each site was analyzed. X axis, nucleotide position of mtDNA D-loop region. Numbering of the np is based on the Cambridge sequence deposited in Genbank accession no. J01415. Solid rods, number of nucleotide variation at each position of the D-loop DNA from np16,000 to 16,569 (A) and from np 1 to 600 (B). Open arrows, hypervariable regions HV1 (np 16,024-16,383) and HV2 (np 57-372).
Pseudogene-like sequences are present in human nuclear DNA; however, these are all ∼100 bp long and none of the sequences span the whole D-loop region.

Nucleotide Sequence of the mtDNA D-Loop Region from a Tumor Tissue Was Heterogeneous

To examine whether nucleotide variation found in tumor cell lines can be found in the patients’ tumor tissues, we prepared D-loop DNAs from 10 pairs of cancerous and noncancerous parts of 10 stomach and 10 liver tumor tissues. The D-loop DNAs of a total of 40 parts were recovered from microdissected, formalin-fixed, and paraffin-embedded specimens. The mtDNA D-loop region was amplified thrice independently with nested PCR. Three PCR products were mixed as described above. Ten D-loop DNA clones were obtained from each sample, and total 400 DNA clones (40 × 10) were sequenced.

Unlike the tumor cell lines, most of the tumor tissue samples carried intracellular variations in the 10 D-loop DNA clones derived from each tumor sample. More specifically, excluding the common nucleotide changes that are unique to each individual tumor tissue sample, one or more additional nucleotide variations were detected in some clones. As shown in Fig. 4, sample 10 revealed that 50% or 90% of DNA clones derived from the cancerous or noncancerous parts, respectively, of one patient showed nucleotide changes that were different from cell specific variation. In contrast, in sample 2, neither cancerous nor noncancerous D-loop DNA clones carry additional changes from cell-specific nucleotide variation. Only 1 of 20 pair samples from stomach and liver tumor tissues was homoplasmic, whereas the other tumor samples were heteroplasmic as can be seen in Figs. 4 and 5. It was regrettable that we could not conduct a chased-back study to determine whether these cell-specific nucleotide variations detected in individual tumor tissue samples were generated from a germ line polymorphism or from clonal development of a somatic mutation from single mtDNA molecules. Furthermore, when 10 clones from noncancerous and cancerous parts of the same tumor tissue sample were compared, the D-loop regions in some noncancerous parts (open rods) were more heterogeneous than those in the cancerous parts (solid rods) as shown in most liver tumor samples (Fig. 5).

Several site-specific nucleotide diversities detected in stomach and liver tumor cell lines were also found in many D-loop DNA clones derived from the respective tissue samples (Table I). As can be seen in Table 1, the nucleotide change of...
C16223T was detected in all clones derived from stomach tumor samples but was less common in the liver tumor group. The T16519C nucleotide change was detected in all DNA clones from liver tumor samples but was less common in the stomach tumor tissue. Although these nucleotide changes are clustered in Asian groups, the so-called Asian haplogroups (18), at least in this study, not all the tumor tissue samples carried these nucleotide changes, although all 20 patients were Japanese. The nucleotide change, T16362C, was detected in 61% of stomach tumor tissue D-loop clones (cancerous part 57

![Figure 3](image)

**FIGURE 3.** Comparison of the frequency of nucleotide variation in the mtDNA D-loop region between liver and other tumor cell lines. D-loop DNA sequences derived from 13 liver tumor and 63 other tumor cell lines were analyzed to detect sequence variation at each site. X axis, nucleotide position of mtDNA as described in Fig. 1 legend. Solid rods, frequency of nucleotide variation found in liver (red) and other tumor (green) cell lines. Several nucleotide positions of >30% frequency in stomach cell line are presented its nucleotide number at each position of the D-loop regions from np 16,000 to 16,569 (A) and from np 1 to 600 (B).

![Figure 4](image)

**FIGURE 4.** Number of identical D-loop DNA clones among 10 clones derived from stomach tumor tissues. Ten each D-loop DNA clones isolated from every stomach tumor tissue samples were sequenced to analyze the nucleotide variation. Vertical rods, number of the identical clones isolated from cancerous (solid rods) and noncancerous (open rods) parts. X axis, 10 stomach tumor tissue samples.
of 100 clones, noncancerous part 65 of 100 clones). The nucleotide change, T16189C, was detected in 50% of liver tumor tissue D-loop clones (cancerous part 50 of 100 clones, noncancerous part 50 of 100 clones) and T146C was detected in 69% of liver tumor tissue clones (cancerous part 66 of 100 clones, noncancerous part 72 of 100 clones). These data are similar to the results found in tumor cell lines. The nucleotide change from T to C at position 16,362 np was detected in 11 cases among 21 stomach tumor cell lines corresponding to 52% (165 of 315 clones) and also was detected in 5 cases among 55 other carcinoma cell lines corresponding to 9% (75 of 825 clones). Likewise, at position 16,189 np, a nucleotide change from T to C was detected in 10 cases among 13 liver tumor cell lines, whereas 13 of 63 other tumor cell lines carried this nucleotide change corresponding to 77% (150 of 195 clones) and 21% (195 of 945 clones), respectively.

Discussion

Somatic mtDNA mutations have been studied intensively because of their proposed involvement in the initiation and/or progression of tumors (9-12). It was implied that somatic mtDNA mutations derive from a single nucleotide substitution in individual mtDNA molecules, thereby increasing their population (19). Little is known about how these nucleotide variations were generated in the individual mtDNA D-loop region of various human tumor cells. Indeed, the proportion of the mtDNA sequence necessary for their “phenotypic expression” is in the order of 90% as determined in some reports (20, 21). Similarly, detection of a point mutation is difficult by PCR direct sequencing, when the fraction of mtDNA mutation is <10% in the PCR products.

In the present study using molecular cloning-sequencing analysis, the frequency of nucleotide variation in the mtDNA D-loop regions of each tumor cell line or tumor tissue sample was investigated first; then, the variation among different cell lines or tissue samples was analyzed to determine whether the variation in particular positions of the mtDNA exhibited any tumor features or tumor type specificity. Fifteen independent D-loop DNA clones obtained from each of 76 tumor cell lines were isolated and examined. For each tumor cell line, the nucleotide sequence found in 15 individual D-loop DNA clones was homoplasmic in every cell line.

Most nucleotide variations detected in the D-loop regions were in two hypervariable regions, the so-called hotspots (Fig. 1). Several site-specific-like nucleotide variations were detected in the stomach and liver tumor cell lines, because the frequencies of these variations were clearly higher than those in other tumor cell lines (Figs. 2 and 3) as can be seen at the 16,189 np site. Particularly in liver tumor cell lines, 10 of 13 cell lines had substitutions from T to C at this site.

Table 1. Frequency of Nucleotide Variations in the D-Loop DNA Clones Derived from Stomach and Liver Tumor Tissues

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Stomach tumor tissues</th>
<th>Liver tumor tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cancerous parts (%)</td>
<td>Noncancerous parts (%)</td>
</tr>
<tr>
<td>T146C</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>C150T</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>T152C</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>T16189C</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>C16223T</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T16362C</td>
<td>57</td>
<td>65</td>
</tr>
<tr>
<td>T16519C</td>
<td>34</td>
<td>47</td>
</tr>
</tbody>
</table>
corresponding to 77% of all tumor cell lines. Although the germ cell lines from the same individuals could not be obtained, the database (GiiB-JST mtSNP; human mitochondrial genome single nucleotide polymorphism) indicates that no such nucleotide variation was found at this site among 99 Japanese. Similarly, at the 152 np site, only ~18% among the 99 Japanese have this nucleotide change. On the other hand, 46% of liver tumor cell lines and 33% of stomach cell lines had changes from T to C at this site.

To examine whether site-specific nucleotide changes can be detected in patient tumor tissue, 10 pairs of noncancerous/cancerous parts from stomach and liver tumor tissues were studied. The D-loop DNAs were recovered from microdissected, formalin-fixed, and paraffin-embedded specimens. Because the cell numbers were very small at less than several hundreds and the mtDNAs might have been attached to nonhistone proteins, the greatest difficulty was obtaining sufficient and qualified DNA from the specimens. Furthermore, the DNA double helix may be broken down into smaller fragments or be separated into single strands. It is more difficult to amplify the longer DNA fragments by eliminating several obstacles. However, we successfully amplified the D-loop region with nested PCR. Many nucleotide variations were detected in the cancerous and noncancerous parts of the tumor tissues. Unexpectedly, the nucleotide sequences detected in both cancerous and noncancerous parts from tumor tissue samples appeared to be heterogeneous. Particularly in liver tumor samples, the D-loop region in the noncancerous part exhibited slightly higher heterogeneity than in the corresponding cancerous part (Fig. 5). The data indicate that mtDNA is heteroplasmic in tumor tissues and that the homoplasmity of mtDNA from cultured tumor cells is acquired in the process of cloning a single cell line from the tumor tissue.

It is generally accepted that most cases of chronic hepatitis are caused by hepatitis B and/or C virus infection, which finally results in a high frequency of liver tumor development. Some biochemical or physiologic changes in the mitochondria may induce instability in the mtDNA D-loop regions in both noncancerous and cancerous parts of tumor tissue samples. Several site-specific nucleotide changes detected in the tumor cell lines were found more often in the cancerous and noncancerous parts from stomach and liver tumor tissues. Although it is necessary to investigate whether these nucleotide substitutions have some deleterious effect on the interaction between proteins coded by nuclear genes or affect both mtDNA replication and transcription, the present data strongly suggest that site-specific nucleotide changes might not be directly involved in the mechanism of tumorigenesis.

Finally, it should be mentioned that we could not completely exclude the possibility of DNA slippage during DNA synthesis when performing PCR cloning and sequencing. However, this experimental error is unlikely, as cloning-sequence analysis was conducted first with the 15 independent clones obtained from each of the 76 cell lines and no sequence variation was found, thereby indicating the absence of any such error of DNA synthesis and sequencing. Furthermore, cell lines were established in the past and a detailed background of cell lines could not be followed up retrospectively any further. Therefore, neither normal tissue samples nor blood could be obtained from the original individuals as controls for each respective analysis.

Materials and Methods

Isolation of DNA from Cell Lines

Cells were derived from 76 human cell lines, including stomach (n = 21), liver (n = 13), breast (n = 12), lung (n = 8), brain (n = 6), colon (n = 5), ovary (n = 5), renal (n = 2), prostate (n = 2), melanoma (n = 1), and osteosarcoma (n = 1) lines. The cells were grown in the DMEM supplemented with 10% fetal bovine serum. Cells (~10^6) of each tumor cell line were harvested and total genomic DNAs were isolated with a column as shown in the Qiagen Blood and Body Fluid Spin Protocol (Qiagen, Inc., Valencia, CA).

Tissue DNA Samples

Tissue DNA samples were prepared from microdissected, formalin-fixed, and paraffin-embedded tissues obtained from the patients. None had received radiation therapy or chemotherapy before the operation. Cancerous and noncancerous parts of tumor tissue sectioned into 10 μm slices on plain glass slides were selected manually and transferred into a 0.5 mL tube containing 350 μL ethanol. After centrifugation at 13,000 × g for 10 minutes, the ethanol was removed and 350 μL xylene were added followed by incubation at 55°C overnight. This step was repeated twice. The xylene was removed by centrifugation at 13,000 × g for 10 minutes and 350 μL ethanol were added followed by incubation at 37°C for 1 hour. The ethanol was removed by centrifugation at 13,000 × g for 10 minutes. This step also was repeated twice. The treated samples were desiccated for 20 minutes, suspended in proteinase K buffer [1 mol/L Tris (pH 8.0-8.5), 0.5 mmol/L EDTA, 0.5% Tween 20], and subjected to incubation at 55°C overnight. Finally, the samples were heat treated at 95°C for 5 minutes and stored at 4°C.

PCR of the mtDNA D-Loop Region

The D-loop DNAs derived from the cell lines were amplified by PCR. PCR was done in the final volume of 50 μL with primers of F15971 (TTAACTCCACCATAGCAC) and R594 (GAGTGAAGCTGATTAAC) by nested PCR with the inner primers of F16031 (ATGGGGAG). The PCR conditions were the same as those used to prepare the tumor cell lines.

Cloning and Sequencing of the mtDNA D-Loop Region

The mixed PCR products derived from three independent amplifications were cloned into the TOPO TA-PCR cloning vector (Invitrogen, Carlsbad, CA). The cloned D-loop DNAs were sequenced with the ALF express II TM DNA sequencer (version 2.1, Amersham Pharmacia Biotech, Inc., Piscataway, NJ).
using the Thermo Sequence CyTM5 Dye Terminator kit (Amersham Pharmacia Biotech). Thermal cycling was done in the Perkin-Elmer model 9600 and the PCR conditions were as follows. First, one cycle for 1 minute at 96°C followed by 30 cycles of 30 seconds at 96°C, 10 seconds at 50°C, and 120 seconds at 60°C. The PCR products were purified by an Auto SeqTM G-50 spin column (Amersham Pharmacia Biotech) before sequencing. Polyacrylamide gel of the ReproGel Set (Amersham Pharmacia Biotech) was used for electrophoresis.

References
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