Detection of Mitochondrial DNA Mutations in Primary Breast Cancer and Fine-Needle Aspirates

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ABSTRACT

To determine the frequency and distribution of mitochondrial DNA mutations in breast cancer, 18 primary breast tumors were analyzed by direct sequencing. Twelve somatic mutations not present in matched lymphocytes and normal breast tissues were detected in 11 of the tumors (61%). Of these mutations, five (42%) were deletions or insertions in a homopolymeric C-stretch between nucleotides 303–315 (D310) within the D-loop. The remaining seven mutations (58%) were single-base substitutions in the coding (ND1, ND4, ND5, and cytochrome b genes) or noncoding regions (D-loop) of the mitochondrial genome. In three cases (25%), the mutations detected in coding regions led to amino acid substitutions in the protein sequence. We then sequenced an additional 46 primary breast tumors with a rapid PCR-based assay to identify poly-C alterations in D310, and we found seven more cancers with alterations. Using D310 mutations as clonal markers, we detected identical changes in five of five matched fine-needle aspirates and in four of four metastases-positive lymph nodes. The high frequency of D310 alterations in primary breast cancer combined with the high sensitivity of the PCR-based assays provides a new molecular tool for cancer detection.

INTRODUCTION

Complex rearrangements and single-base substitutions in the mitochondrial mtDNA3 are associated with a number of sporadic and maternally inherited degenerative diseases that affect tissues highly reliant on mtDNA bioenergetics (1). Human mtDNA has a mutation rate at least 10 times higher than nuclear DNA (2). Most likely, mutations are linked to the high levels of reactive oxygen species (3) and involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mtDNA heavy-strand replication (4). The homopolymeric C-stretch (CCCCCCC..TCCCCC) is part of the conserved sequence block II located within the regulatory D-loop region and is conserved across vertebrates (5).

In recent years, somatic mutations in the mtDNA have been observed in human neoplasia (6–13). Three studies that extensively analyzed the mitochondrial genome using direct sequencing, approximately two-thirds of the tumors showed mtDNA abnormalities (8, 9, 13). Base substitutions and deletion/insertion mutations were detected in both coding and noncoding regions, but complex rearrangements were not observed. Moreover, we have recently identified a mononucleotide repeat4 between 303 and 315 nucleotides (D310) as a frequent hot spot of deletion/insertion mutations in primary tumors. This homopolymeric C-stretch (CCCCCCC..TCCCCC) is part of the conserved sequence block II located within the regulatory D-loop region and involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mtDNA heavy-strand replication (14, 15).

There are only two previous reports that have analyzed alterations of the mtDNA in breast cancer, and they relied largely on restriction enzyme analysis. The first of these studies was limited by the low number of cases analyzed (16). In the second study (12), a larger number of patients was screened, but just two selected areas of the D-loop (a dinucleotide repeat at 514 bp and the region between 16108 and 16420 bp) were tested.

We determined the frequency and distribution of mitochondrial mutations in primary breast cancer and investigated the potential use of mtDNA mutations as markers for breast cancer detection.

MATERIALS AND METHODS

Specimens and DNA Extraction. Eighteen invasive ductal carcinomas of the breast from 17 patients (1 patient had two synchronous bilateral breast tumors) were screened by direct sequence analysis for mutations in the mtDNA genome. All patients were female except for one case in a male (B35). Paired normal breast and tumor specimens along with blood were collected at the Department of Pathology, University Campus BioMedico, Rome, Italy. Tumor specimens were frozen and microdissected on a cryostat so that the tumors contained at least 70% neoplastic cells. For two cases (B24 and B27), metastatic lymph nodes were also available. DNA from tumor sections was digested with 1% SDS/protease K, extracted by phenol-chloroform, and ethanol precipitated. Control DNA from peripheral lymphocytes and matched normal tissue was processed as described previously (17).

An additional 46 breast tumors were analyzed only for mutations in the D310 repeat by a PCR-based method. Paired normal and tumor samples (paraffin-embedded tissues) obtained from the Department of Pathology, University Campus BioMedico, Rome, Italy, and the Department of Pathology, The Johns Hopkins University, Baltimore, MD. FNA samples from breast lesions and lymph nodes were available for 20 and 15 of the cases, respectively. DNA from paraffin samples (normal, tumor, and lymph node) were processed as described previously (18). Papanicolaou-stained smears from FNA specimens were microdissected, and cells were destained with ethanol 100%. Digestion was performed in 1% SDS/protease K, and DNA was extracted by phenol-chloroform and ethanol precipitated.

PCR Amplification. To exclude the possibility of pseudogene amplification, seven large (1–3 kb each) mtDNA fragments were amplified, and human

mtDNA-less cells (p cells) were used as a negative control. Amplification was performed as described previously by Fliss et al. (9). Briefly, total DNA was subjected to step-down PCR protocol: 95°C for 2 min, 1 cycle; 95°C for 30 s, 64°C for 1 min, 70°C for 2 min 30 s, three cycles; 95°C for 30 s, 58°C for 1 min, 70°C for 2 min 30 s, 2 cycles; 95°C, for 30 s, 57°C for 1 min, 70°C 2 min 30 s, 35 cycles; and a final extension at 70°C for 5 min. PCR products were gel-purified with the Qiagen gel extraction kit (Qiaquick columns; Qiagen, Chatsworth, CA). Tumor DNA and paired lymphocytes and normal tissue were analyzed in all cases.

Radioactive-based Manual Sequencing. The analysis was carried out using Thermosequenase (Perkin-Elmer, Roche Molecular Systems, Inc. Branchburg, NJ) with cycle conditions according to the manufacturer instructions. Sequencing products were separated on a Genomyx electrophoresis apparatus (Beckman Coulter, Inc., Fullerton, CA). For samples with mutations, the analysis was repeated in two independent PCR and sequencing reactions, and both strands were sequenced.

Fluorescence-based Automatic Sequence. DNA templates were processed for the DNA sequencing reaction using ABI-PRISM BigDye Terminator chemistry (Applera) and both forward and reverse sequence-specific primers. Sequence data were generated using the ABI PRISM 3700 DNA Analyzer. To compare variations between normal and tumor samples, sequences were analyzed by Sequencer 3.1.1. Ambiguous peaks were analyzed further by manual sequencing.

RESULTS

Direct Sequence Analysis of the mtDNA. Fig. 1A shows the regions of the mitochondrial genome that have been sequenced in this study. In total, 84% of the mtDNA was analyzed, 55% by radioactive-based manual sequencing and 29% by fluorescence-based automated

![Fig. 1. mtDNA sequence analysis. A, schematic representation of a linearized mitochondrial genome. I, regions sequenced by manual sequencing; [ ], regions sequenced by automatic sequencing; and [ ], region sequenced with both methods. •, positions of the tRNAs; ND1, ND1 dehydrogenase; CO1, cytochrome b oxidase; Cytb, cytochrome b; ATPase, ATP synthase. B, sequence analysis of a case with bilateral breast cancer B27. Na and Nc, normal breast tissues from right and left breasts, respectively. Ta and Tc, tumor tissues from right and left breasts, respectively. Lc, metastatic lymph node draining from the right breast. Left, the G → A transition at position 3918 within the ND1 gene is observed in the tumor from the left breast but not in the tumor from the right breast or in the metastatic lymph node. Right, both the tumor from the right breast and the metastatic lymph node show a 1-bp deletion in the D310 repeat that it is absent in the tumor from the left breast. C, automatic and manual sequence analysis of case B24. P, peripheral lymphocytes; N, normal breast tissue; T, tumor; Lc, metastatic lymph node. Left, fluorescence-based automatic sequence revealed a tumor specific C → T transition at position 13708 within the ND5 gene; Right, the mutation was confirmed by manual sequencing and was also detected in a corresponding metastatic lymph node.](image-url)
sequence analysis. A 623-bp region including part of the ND4 gene, tRNAHis, and part of the ND5 gene was analyzed by both methods.

Of the 122 genetic variants detected, 94 were previously recorded polymorphisms, whereas 16 were new polymorphisms and were submitted to the mtDNA database. The remaining 12 variants, identified in 11 tumors (61%), were somatic mutations not present in the matched lymphocytes (Table 1). To exclude the possibility that the mtDNA mutations were just tissue-specific polymorphisms, we also tested paired normal breast tissues, and in all cases the mutations were only detected in the primary tumor (Fig. 1, B–C).

The highest frequency of mutations (58%) was detected in the D-loop, with five mutations (deletions or insertions) in the D310 repeat and two single base substitutions. The remaining five mutations were single base substitutions within the ND1, ND4, ND5, and cytochrome b genes. In three cases, the base substitution led to an amino acid change in the protein sequence (Table 1). One of the mutations (12344/ND5) was detected by radioactive-based manual sequencing but missed by fluorescence-based automatic sequencing.

Metastatic lymph nodes were available from two patients (B24 and B27), and in both cases they contained the mutant mtDNA derived from the tumor tissue (Fig. 1, B–C). For patient B27, two bilateral synchronous breast tumors were analyzed. Interestingly, the tumor from the right breast (Tb) harbored a 1-bp deletion in the D310 repeat, whereas the tumor from the left breast (Tl) carried a point mutation at position 3918 within ND1 (Fig. 1B). In a metastatic lymph node draining from the right breast, we observed only the mutation detected in the ipsilateral tumor (Fig. 1B). These results indicate that the tumors differ from one to another (either they arise from different clones or diverge early in tumor progression) and clearly indicate that the lymph node metastases originate from the right breast tumor.

**D310 Mutations as Clonal Markers for Cancer Detection.** We extended the analysis for the D310 mutational hot spot to 46 additional breast tumors using a rapid PCR-based method. Including the results from the 18 tumors analyzed by direct sequencing, a total of 12 D310 mutations in 64 tumors (19%) were detected. The majority of the mutations were 1- or 2-bp insertions or deletions with the exception of two cases that showed an 8-bp and a 9-bp deletion (Table 2). Representative results are shown in Fig. 2. The sensitivity of the PCR assay was tested on serial dilutions of normal (lymphocyte) total DNA from two patients showing different D310 polymorphisms (1-bp difference in the length of the first Cs stretch). The assay was still able to detect the alterations at a dilution of 1:500 with as little as 40 pg of starting DNA template (~6 cells).

To investigate the potential use of D310 mutations in breast cancer detection, FNA samples and lymph nodes from patients with and without poly-C alterations in the tumor were analyzed. FNAs were available from five of the patients with D310 alterations, whereas lymph nodes were available from four of those patients. The same deletions/insertions detected in the primary tumor were also observed in all paired FNA samples and metastatic lymph nodes (Table 2). In patient B63 (Fig. 2), one of the two lymph nodes analyzed was classified as disease-free by histopathological examination, but it was found to harbor the identical 2-bp insertion seen in the primary tumor. No D310 alterations were detected in the 15 FNAs and 14 lymph nodes from patients without D310 mutations in the primary tumor (Fig. 2).

**DISCUSSION**

We have sequenced 84% of the mitochondrial genome, uncovering somatic mutations in 61% of the primary breast cancers analyzed. Moreover, we confirmed that the mononucleotide repeat located between 303 and 315 nucleotides within the D-loop (D310) is a hot spot for mutations in breast tumors. Because radioactive-based manual

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of mtDNA mutations detected in primary breast tumors</th>
</tr>
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<tbody>
<tr>
<td>Tumor</td>
<td>Nucleotide position</td>
</tr>
<tr>
<td>B24</td>
<td>13708</td>
</tr>
<tr>
<td>B27Tb</td>
<td>303–315</td>
</tr>
<tr>
<td>B27Tl</td>
<td>3918</td>
</tr>
<tr>
<td>B32</td>
<td>303–315</td>
</tr>
<tr>
<td>B33</td>
<td>12344</td>
</tr>
<tr>
<td>B35</td>
<td>16292</td>
</tr>
<tr>
<td>B38</td>
<td>11900</td>
</tr>
<tr>
<td>B39</td>
<td>303–315</td>
</tr>
<tr>
<td>B41</td>
<td>303–315</td>
</tr>
<tr>
<td>B42</td>
<td>303–315</td>
</tr>
<tr>
<td>B44</td>
<td>14809</td>
</tr>
</tbody>
</table>

* Single-letter abbreviations for the amino acid residues are: A, Ala; E, Glu; L, Leu; K, Lys; M, Met; T, Thr and V, Val.

* These mutations were also detected in matched histopathologically metastatic lymph nodes; del, deletion; ins, insertion.

This mutation has been described as a “secondary” mutation in Leber’s hereditary optic neuropathy (LHON syndrome).

**Table 2 | Mutations detected in the D310 repeat by PCR-based method in primary breast tumors and corresponding FNA and lymph nodes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Molecular analysis</th>
<th>Cytology</th>
<th>FNA</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>B13</td>
<td>1-bp ins</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B20</td>
<td>8-bp del</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B48</td>
<td>1-bp del</td>
<td>Pos</td>
<td>+</td>
<td>L1 Neg</td>
</tr>
<tr>
<td>B55</td>
<td>1-bp ins</td>
<td>Pos</td>
<td>+</td>
<td>L1 Pos</td>
</tr>
<tr>
<td>B58</td>
<td>1-bp ins</td>
<td>Pos</td>
<td>+</td>
<td>L1 Pos</td>
</tr>
<tr>
<td>B63</td>
<td>2-bp del</td>
<td>Pos</td>
<td>+</td>
<td>L1 Pos</td>
</tr>
<tr>
<td>B64</td>
<td>1-bp del</td>
<td>Pos</td>
<td>+</td>
<td>L1 Neg</td>
</tr>
</tbody>
</table>

+ N/A, sample not available for the analysis.

b, positive; and −, negative, for cytologic or histopathologic examination. pos, positive; neg, negative.

N = number of lymph node; L = lymph node.

d, ins, insertion; del, deletion.

**Fig. 2. Detection of D310 mutations by a PCR-based assay.** P, peripheral lymphocytes; N, normal breast tissue; T, tumor; FNA; L+, metastatic lymph node, L−, nonmetastatic lymph node. Arrows, the deletion/insertion detected in the tumor DNA. Mutations are present in the tumor DNA but not in peripheral lymphocytes and normal breast tissues. In cases B63 and B64, abnormalities in D310 are also observed in the cytology specimens. For case B63, both a metastatic and an apparently nonmetastatic lymph node show the 2-bp insertion detected in the tumor. D310 mutations were not detected in FNAs nor all lymph nodes from patients without D310 abnormalities (a representative case, B69, is shown).

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sequencing is laborious and time consuming, we analyzed part of the mtDNA genome by fluorescence-based automatic sequencing. When results from the region sequenced with both methods were compared, we discovered one mutation (12334/ND5) that was missed by the automated sequence analysis. A previous study (19) also suggests that radioactive-based manual sequencing is more sensitive than fluorescence-based automatic sequencing in detecting mutations within a mixed population of mutant and wild-type cells (primary tumors). Thus, in our study, the frequency of mutations in the regions analyzed only by fluorescence-based automatic sequencing is likely to be an underestimate.

An interesting finding of our analysis is that mutations were detected only in the D310 repeat but not in other mitochondrial microsatellites analyzed, including two regions reported previously to be altered in breast cancer (12). These observations argue against the hypothesis of generalized mitochondrial microsatellite instability in breast tumors. The D310 repeat is among the most polymorphic of mitochondrial microsatellites, and heteroplasmy at this site has been described in normal tissues (5). We have recently cloned and sequenced part of the D-loop region containing the D310 in lymphocytes from cancer patients. Interestingly, the level of heteroplasmy was significantly higher in lymphocytes from patients carrying D310 mutations in the tumor as compared with lymphocytes from patients without D310 abnormalities. These observations lead us to hypothesize that in normal tissues, the D310 alterations are already present in a minority of mitochondria and achieve homoplasmy in the tumor because of clonal expansion driven by mutations in the nuclear DNA. Consistent with this model, Jones et al. (13) demonstrated heteroplasmy for the genetic variant detected in the corresponding tumor in the normal tissue from a patient with pancreatic cancer. In the present study, 10 of 12 mutations detected in the D310 were in the normal range of the constitutive polymorphisms (6–9 Cs in the first stretch), and the two base substitutions observed in the D-loop are described as polymorphisms in the normal population. It is possible that the remaining base substitutions may represent rare polymorphisms. However, three of the five missense mutations led to amino acid changes in the protein sequence, and mtDNA missense and frameshift mutations have been described in other tumor types (8, 9, 13). Moreover, the two relatively large deletions observed in D310 almost eliminate the mononucleotide repeat and might interfere with the function of the conserved sequence block II in priming for replication. Thus it is likely that at least a portion of these variants arise as de novo somatic mitochondrial mutations and are likely to have functional relevance in tumor development.

In a previous study (9), we demonstrated that mitochondrial mutations are detectable in diagnostic samples from patients with cancer. The analysis of mtDNA mutations for cancer detection has some advantages as compared with the detection of alterations in the nuclear DNA. Mitochondrial mutations are essentially homoplasmic (8, 9, 13), and there is evidence that tumor cells have a higher mtDNA content than normal cells (9, 13). Thus homoplasmic mtDNA mutations represent a powerful marker of clonality. Here we identified the same D310 alterations in FNAS and lymph nodes from patients with abnormalities in the tumor. Mutations were not detected in metastatic lymph nodes from patients without D310 abnormalities in the tumor, supporting the hypothesis that mtDNA mutations occur in the early stages of the cancer development. Remarkably, in one of the breast tumors analyzed in this study, both a metastatic and an apparently nonmetastatic lymph node from the patient showed the 2-bp insertion identified in the tumor. Conventional cytomorphological and histopathological examination have high sensitivity and specificity in detecting tumor cells with a clearly malignant phenotype. However, the accuracy of the analysis decreases when the morphological abnormalities are slight or the number of tumor cells is much lower as compared with normal cells (e.g., low cellularity for cytology, micrometastases in lymph node; Refs. 20, 21). We determined that the PCR-based assay for the D310 is able to detect the specific altered fragment from an amount of total DNA as little as 40 pg. Thus, the analysis of the mtDNA might allow the detection of tumor cells in cases in which the sensitivity of conventional methods is low (e.g., nipple aspirates).

Our observations suggest that only a fraction of the mtDNA mutations present in breast tumors can be detected by the PCR-based assay for the D310. However, gene-chip technologies similar to those developed for detecting mutations in nuclear genes (17) could be applied to develop high-throughput analysis of the entire mitochondrial genome with sufficient sensitivity and specificity to detect tumor cells in FNAS and micrometastases in lymph nodes.

REFERENCES

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