High-Throughput Mitochondrial Genome Screening Method for Nonmelanoma Skin Cancer Using Multiplexed Temperature Gradient Capillary Electrophoresis

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Background: We explored the utility of multiplexed temperature gradient capillary electrophoresis (TGCE) as a screening tool for identifying genetic changes in the human mitochondrial genome. We examined changes in mitochondrial DNA (mtDNA) in nonmelanoma skin cancers (NMSCs) using TGCE to resolve genetic differences contained within the tumors compared with the control DNA.

Methods: The entire mtDNA from NMSC tissue samples was amplified in 17 overlapping amplicons averaging 1.1 kb in size. Fourteen of these amplicons were digested with restriction endonucleases into as many as five smaller analyzable fragments. Digested tumor mtDNA amplicons were annealed with digested amplicons from the control DNA to form heteroduplexes in regions of DNA mismatch. TGCE was performed in a 96-well parallel format to detect mtDNA changes in a high-throughput fashion.

Results: TGCE resolved heteroduplexes from homoduplexes in singlet reactions and in multiplexed assays. Using a single programmed temperature gradient, we detected 18 of 20 mtDNA changes contained within the specimens. This system was also able to detect a single nucleotide change in a fragment as large as 2 kb.

Conclusion: Multiplexed TGCE is a sensitive and high-throughput screening tool for identifying mtDNA variations.

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Many techniques have been developed to study genomic variation, including those based on electrophoretic conformational changes, enzymatic or chemical cleavage reactions, microarray analysis, heteroduplex detection, and HPLC fractionation. The goal of these screening techniques is to greatly reduce the need for DNA sequencing. This goal is easier to achieve when scoring a specific number of predetermined mutant alleles is the experimental objective rather than mutation discovery of unknown genomic variation, especially as the size of the genomic locus increases. Even DNA sequencing itself may not be dependable in some cases of heterozygosity, in cases of pooled specimens, or in cases of mitochondrial DNA (mtDNA) heteroplasmy. DNA microarrays can be used to resequence entire genes or even smaller genomes on a single array, but this approach is not easily adaptable for the needs of many laboratories and remains costly. Currently, the gel-based assays offer an alternative for genomic variation screening but are limited in their capacity by the number of wells and available manpower.

Human mtDNA is a circular molecule consisting of 16,569 bp encoding 2 ribosomal RNAs, 22 tRNAs, and 13 polypeptides involved in oxidative phosphorylation (1–3). Mutations in mtDNA can cause a variety of human diseases typically characterized by neurologic, muscular, or endocrine phenotypes. Recent publications reported a high frequency of homoplasmic mtDNA point mutations in a variety of human tumors (4–6). A possible explanation for mutant mtDNA homoplasmy in tumor cells is that mutant mtDNAs might alter mitochondrially mediated apoptotic pathways to escape cell death. Alternatively, the mtDNA changes may endow the tumor cell
with a selective growth advantage directly or in combination with acquired nuclear-encoded mutations.

Denaturing gradient gel electrophoresis (7), single-strand conformation polymorphism analysis (8,9), conformation-sensitive gel electrophoresis (10,11), thermal gradient gel electrophoresis (12), enzymatic cleavage (13–15), and denaturing HPLC (DHPLC) (16) are examples of presequencing screening methods that have been developed to detect DNA variants. DHPLC (17,18), oligonucleotide sequencing arrays (19), and temporal temperature gradient gel electrophoresis (TTGE) have been used successfully as screening tools for mtDNA mutations (20–24). In the case of denaturing gradient gel electrophoresis, single-strand conformational polymorphism analysis, and conformation-sensitive gel electrophoresis, the detection efficiency is ~80%. Some studies have reported that DHPLC has a sensitivity close to 100% when performed at the optimized temperature for each sample (25). However, melting domains must be precisely determined, and DNA molecules with multiple melting domains may need to be run on the column at multiple temperatures. In addition, DHPLC has a throughput limited by the serial nature of injecting DNA samples onto a single column, although this technique can be amenable to multiplexed assays (17,26).

Temperature gradient capillary electrophoresis (TGCE) is a high-throughput method for detection of DNA variation with a high sensitivity, rapid separation time, and a small DNA concentration (~200 pg/μL) requirement (27). TGCE identifies DNA variation in ethidium bromide-stained heteroduplex strands by use of capillary gel electrophoresis in a parallel array run through a programmable thermal gradient in combination with a laser-induced fluorescence detection system (28,29). We report the application of this new high-throughput method for detection of mtDNA sequence variations in cutaneous tumors and the first use of multiplexed TGCE mutation detection without the requirement for fluorescently tagged DNAs.

**Materials and Methods**

**Patient Population and Collection of Clinical Skin Samples**

Skin was obtained from patients with nonmelanoma skin cancer (NMSC) undergoing skin cancer excision in our Dermatology Clinic. Samples were collected under a protocol approved by the Vanderbilt University Institutional Review Board.

**DNA Isolation and PCR**

Genomic DNA was isolated from squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) samples by use of the DNeasy Tissue Kit (Qiagen Inc.). DNA from four tumor samples (two SCC and two BCC) was screened with use of 17 overlapping PCR amplicons. Oligonucleotide primers (Sigma Genosys) were used for amplification of the entire mtDNA (Table 1). The PCR amplicons were visualized by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. Reactions were performed in a 50-μL volume containing 10 ng of total genomic DNA as template, 0.2 mM each deoxynucleotide triphosphate (New England BioLabs), 0.5 μM each of the forward and reverse primers, and 2.5 U of Optimase polymerase (Transgenomic Company) in 1× Optimase Mg2+-free buffer supplemented with 1.5 mM magnesium sulfate. PCR conditions for all amplicons were 30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2 min, followed by cooling to 4°C. The initial denaturation was at 95°C for 5 min, and the final elongation was at 72°C for 5 min.

**Digestion of Amplicons**

For multiplex analysis, PCR products were digested with restriction endonucleases (Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue2/). The digestions using DpnII, DdeI, Mspl, AluI, and HaeIII (New England BioLabs) were performed in Optimase buffer at 37°C for 2 h in a total volume of 25 μL. Results of these digestions were visualized by gel electrophoresis on a 2% agarose gel stained with ethidium bromide.

**Sample Preparation for TGCE**

Our intended use of this screening technique is for the detection of somatic mtDNA mutations in tumors. However, for purposes of validating this method, we allowed heteroduplex species to form by mixing the tumor DNA with our laboratory reference DNA isolated from the skin of a healthy 30-year-old volunteer in a 1:1 ratio. The samples were denatured for 3 min at 95°C and annealed in a thermal cycler via a stepwise reduction in temperature as follows: decrease from 95°C to 80°C at 3°C/min, decrease from 80°C to 55°C at 1°C/min, hold at 55°C for 20 min, decrease from 55°C to 45°C at 1°C/min, and decrease from 45°C to 25°C at 2°C/min. Samples were stored at 4°C until TGCE analysis was performed.

TGCE analysis was performed on a Revealed Discovery System (SpectruMedix). This system performs temperature gradient electrophoresis for DNA variant detection on an automated 96-capillary array instrument. DNA samples consisting of homoduplexes and heteroduplexes were separated by capillary electrophoresis, during which a thermal ramp from 50°C to 60°C was applied over 25 min. The injection conditions were 6 kV for 90 s, and the capillary length was 70 cm. The gel in the capillary was 20 g/L 7 × 106 molecular weight polyethylene oxide dissolved in 1× Tris-borate-EDTA buffer with ethidium bromide added at a final concentration of 0.5 mg/L. SpectruMedix Check Mate Software was used for instrument control and data acquisition with a data acquisition time of 65 min. Data were analyzed by use of the SpectruMedix Revealed Mutation Software, which generated mutation scoring results for all samples in a 96-well tray. The system generated the electropherograms for our
reference sequence, the wild-type plus mutant (mixed) samples, and the mutant unmixed samples. Mutations were identified by automated detection of peak patterns where the presence of two peaks on an electropherogram indicated a nucleotide change. All mutant DNA sequences were confirmed by use of the ABI 377 automated DNA sequencer (Applied Biosystems) in the Vanderbilt DNA sequencing core laboratory.

Results
Oligonucleotide primers were designed to amplify the entire mitochondrial genome in 17 amplicons, at a single annealing temperature, and with a mean amplicon size of 1.1 kb (range, 418-2002 bp; Table 1 in the online Data Supplement). mtDNA from four NMSC patients with known nucleotide changes previously identified by sequencing was screened by TGCE. Samples were prepared for singlet or multiplexed TGCE by restriction enzyme digestions (Table 1). The targets for detection were in 20 NMSC mtDNA variants: 8 from BCC and 12 from SCC tumors (Table 1).

The corresponding mixed (tumor plus control), control, and tumor DNAs are shown in the electropherograms designated with letters M, C, and T, respectively, in Figs. 1 and 2 of the online Data Supplement. Amplicon length is given in parentheses for each panel. Single peaks are expected in homoduplex species, whereas multiple peaks are expected in heteroduplex species. Examples of the TGCE output showing the detection of heteroduplexes in mixed specimens are presented in Figs. 1, 2, and 3 and are displayed in tabular format in Table 1. Fig. 1 shows examples of the successful detection of the T5004C, G9123A, and A15326G mutations from SCC tumors and A9448G, A13032G, and A16051G mutations from BCC tumors. In this analysis, the sensitivity was 70% (14 of 20) for the detection of these mutations in singlet reactions despite the generally large sizes of the amplicons.

For multiplexed analysis, each large PCR product (0.8–2 kb; Fig. 3 in the online Data Supplement) was digested with the appropriate restriction enzyme (Table 1 in the online Data Supplement) to generate smaller fragments that could be individually resolved by TGCE (Fig. 4 in the online Data Supplement). DNA digests from amplicons 5, 6, 11, 12, and 15 generated DNA fragments <50 bp, which are not analyzable in our assay because DNA oligonucleotide primers are not removed before TGCE loading. These small fragments are generated at the ends of the amplicons, and the regions containing these smaller fragments are screened by larger adjacent overlapping amplicons. Examples of successful multiplexed screening for amplicon fragment 11 (digested with Ddel to generate three analyzable products of 187, 198, and 390 bp) are shown in Fig. 2A. As shown in the electropherogram in Fig. 2A, the G9123A mutation was detected the 390-bp digestion product of the mtDNA of SCC tumor S3T. Fig. 2B shows the electropherogram produced by multiplexed TGCE analysis of fragment 17. Alul digestion of the 1029-bp amplicon from BCC tumor specimen B8T yielded fragments of 218, 352, and 459 bp, which were resolved by TGCE, and enabled successful detection of the A16051G mtDNA mutation in the 459-bp fragment (Fig. 2B).

A 2002-bp amplicon was the largest fragment analyzed in singlet and multiplexed reactions. Two mutations were

| Tumor | Amplicon no. | Nucleotide change | Gene | Detected in uniplex? | Detected in multiplex? | Homoplasy or Heteroplasmy |
|-------|--------------|-------------------|------|----------------------|------------------------|---------------------------|
| B1T   | 5            | G3010A            | RNR2 | No                    | No                     | Homoplasmy                |
| B1T   | 7            | A4769G            | ND2  | Yes                  | Yes                    | Homoplasmy                |
| B1T   | 11           | A9448G            | CO3  | Yes                  | Yes                    | Homoplasmy                |
| B1T   | 16           | A15326G           | Cytb | No                    | Yes                    | Homoplasmy                |
| S2T   | 3            | C476T             | RNR1 | Yes                  | Yes                    | Homoplasmy                |
| S2T   | 8            | G5979A            | CO1  | No                    | No                     | Homoplasmy                |
| S2T   | 10           | C7956T            | CO2  | Yes                  | Yes                    | Heteroplasmy              |
| S2T   | 12           | C10527T           | ND4L | Yes                  | Yes                    | Homoplasmy                |
| S2T   | 13           | C12465T           | ND5  | No                    | Yes                    | Homoplasmy                |
| S2T   | 1            | C16520T           | D-loop | Yes                       | NA<sup>a</sup>                  | Heteroplasmy              |
| B8T   | 2            | A263G             | R sequence | Yes                               | NA                      | Homoplasmy                |
| B8T   | 14           | A13032G           | ND5  | Yes                  | ND                     | Homoplasmy                |
| B8T   | 16           | C15409T           | Cytb | No                    | Yes                    | Homoplasmy                |
| B8T   | 17           | A16051G           | D-loop | Yes                       | Yes                    | Homoplasmy                |
| S3T   | 6            | C3992T            | ND1  | Yes                  | Yes                    | Homoplasmy                |
| S3T   | 7            | T5004C            | ND2  | Yes                  | Yes                    | Homoplasmy                |
| S3T   | 10           | G8269A            | CO2  | Yes                  | ND                     | Homoplasmy                |
| S3T   | 11           | G9123A            | ATP6 | Yes                  | Yes                    | Homoplasmy                |
| S3T   | 12           | A10044G           | tG   | No                    | Yes                    | Homoplasmy                |
| S3T   | 16           | A15326G           | Cytb | Yes                  | ND                     | Homoplasmy                |

<sup>a</sup> NA, not applicable (no multiplex analysis for small fragments); ND, not done.
detected by multiplexed analysis in tumor specimens S3T and S2T in this region of the mtDNA, and one of these two mutations (C10527T in S2T) was also detected in a singlet reaction despite the large amplicon size. Fig. 3A shows an electropherogram for the successful detection of the C10527T mutation in a uniplex reaction from fragment 12 of SCC tumor specimen S2T. The results of multiplexed analysis of the same fragment are shown in Fig. 3B. Digestion with AluI generated five analyzable products of 247, 312, 366, 447, 588 bp and two nonanalyzable products of 12 and 17 bp. An extra peak representing a heteroduplex species is present in the 588-bp fragment and corresponds to the location of the C10527T mutation.

Both heteroplasmic and homoplasmic mtDNA mutations were detected in these experiments (Table 1). The application of multiplexing by digestion of the larger amplicons with restriction endonucleases improved the sensitivity of the approach. Analysis of smaller DNA fragments improved the overall sensitivity of the approach to 90% (18 of 20 mutations detected). There were no false positives identified in this TGCE protocol when the Optimase DNA polymerase was used for amplification of the PCR products.

Discussion
Detection of DNA variations has become a focus in many areas of genetics, particularly in the study of genes associated with human disease. For mutation screening of a limited number of specific alleles, assays such as sequencing, real-time PCR with molecular beacons, and pyrosequencing have advantages, including automation and high-throughput capabilities in a properly equipped laboratory. The task of screening DNA sequences for unknown mutations or sequence variations is considerably more complex. Although sequencing and DNA microarray chips are capable of generating these data, they generate enormous amounts of data at a high cost. Gel-based screening techniques are often more cost-effective...
and certainly diminish the need for sequencing, but gel-based methods are limited in their potential throughput. Two techniques have emerged that combine high-sensitivity heteroduplex detection with automated liquid handling for the process of mutation discovery: DHPLC and TGCE.

In our study, we evaluated the use of TGCE to screen for the presence of mtDNA sequence variations in human NMSC samples. We developed a set of 17 oligonucleotide primers to amplify the entire mitochondrial genome in overlapping segments by PCR using a single annealing temperature. The amplicons were then digested with restriction endonucleases to generate a series of smaller fragments with sizes sufficiently different to allow for multiplexed TGCE analysis without an additional need to fluorescently label each product to be resolved in the capillary. The use of the smaller DNA fragments generated by the multiplexing also improved the sensitivity of the approach over uniplex reactions with larger amplicons. Like other heteroduplex detection methods, the amplified DNA must be mixed with control DNA, and the combined sample must be denatured and cooled slowly to allow for the formation of heteroduplexes. Mixing with a control DNA is not always necessary if screening for only heteroplasmic mutations is desired. We have performed TGCE analysis with serial dilutions of samples with known mtDNA mutations to determine the sensitivity of TGCE in detecting low degrees of heteroplasmy. We have observed successful mutation detection when the altered base is present in concentrations as low as 10% of the total mtDNA species (data not shown). TGCE was also particularly amenable to multiplexing because the programmable thermal gradient allows for the analysis of DNA variations in multiple thermal domains. Indeed, a single reaction condition was capable of detecting 90% of our previously known mtDNA sequence variations in these tumors. The mtDNA has an overall GC content of 44.3%. The two amplicons with mutations that were not detected under these TGCE conditions had GC contents of 47.1% and 47.7%, respectively, and were among the four amplicons with the highest GC content. It may be possible to achieve even better results with refinement of the thermal gradient conditions, such as use of a narrower temperature ramp (27) or with the use of techniques such as the addition of a GC clamp to the end of the amplicons to change the melting profiles.

A variety of methods for mtDNA mutation screening have been published, such as DHPLC (17) and TTGE (20). TGCE in the 96- or 384-well format offers substantial advantages over these techniques in terms of sample capability, which is limited by the number of wells and manual gel preparation for TTGE and by the serial nature of running DNA on a DHPLC column. Furthermore, the melting profiles of the DNA fragments generated by

![Fig. 2. Overview of TGCE multiplex analysis of amplicons from tumor samples. (A, left), overview of TGCE multiplex analysis of amplicon 11 of the S3T tumor sample. The three vertical arrows indicate the peaks for each fragment generated after digestion with DdeI. Amplicon 11 (817 bp) is cleaved into fragments of 187, 198, and 390 bp (magnified in middle and right-hand panels). Peak 3 contains the G9123A mutation (horizontal arrow in the right-hand panel). (B, left), overview of TGCE multiplex analysis of amplicon 17 of the B8T tumor sample. The three vertical arrows indicate the peaks for each fragment generated after digestion with AluI. Amplicon 17 (1029 bp) is cleaved into fragments of 218, 352, and 459 bp (magnified in middle and right-hand panels). Peak 3 contains the mutation A16051G (diagonal arrow in the right-hand panel).](image-url)
Multiplexed assays often require multiple injections on the DHPLC column at various temperatures to ensure optimum sensitivity, which further limits the maximum capacity of the system. With TGCE, the throughput of the device is limited by the number of capillaries in the array. However, the parallel nature of the analysis combined with a short running time and automated plate handling makes the maximum throughput higher than existing methods for mtDNA mutation screening. In terms of the sensitivity and cost, TGCE required low sample concentrations and has a relatively low cost for sample preparation because the unpurified PCR product is used to perform the analysis. This screening method could be useful for detecting inherited germline mutations in mtDNA in patients with mitochondrial disease or to detect acquired somatic mutations of mtDNA. The sensitivity of the assay could also be useful in population studies using pooled mtDNA. The limitations of this procedure are its inability to distinguish between mutant and wild-type homoduplexes without mixing and the lack of separation of multiple distinct heteroduplexes species such as can be seen in DHPLC or TTGE. As in other heteroduplex-based mutation detection methods, incomplete restriction enzyme digestion may interfere with the detection of DNA variants, and small restriction fragments may be lost. Furthermore, detection of unknown mtDNA mutations still requires sequencing in the region to identify the specific nucleotide change.

In conclusion, we found that TGCE could be a useful tool for mutation discovery in mtDNA and also offers several advantages over existing methodologies. TGCE combines high-sensitivity heteroduplex detection with high-throughput capability. This screening method was 90% sensitive and 100% specific in our assay to detect predetermined mtDNA mutations. The system offers high flexibility for multiplexing (without a need for additional fluorescent tagging of DNA) and mutation detection in a broad range of DNA melting domains. This method could facilitate the rapid detection of mtDNA sequence variations and be applicable to wide variety of mutation discovery applications.

This work was supported by a VA Medical Research Service Advanced Career Development Award, by a Vanderbilt Skin Diseases Research Center Pilot and Feasibility Award (NIH P30AR41943), by an American Cancer Society Institutional Research Grant, and by an Ellison Medical Foundation New Scholar Award to Dr. Sligh. We thank Dr. Michel McDonald and Dr. Thomas Stasko for generously providing patient materials.

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