Hearing Research 244 (2008) 1–6

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Methodological paper

Technical report: Laser microdissection of cochlear structures from celloidin embedded human temporal bone tissues and detection of the mitochondrial DNA common deletion using real time PCR

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ARTICLE INFO

Article history:
Received 20 May 2008
Received in revised form 23 July 2008
Accepted 24 July 2008
Available online 31 July 2008

Keywords:
Laser microdissection
Human temporal bones
Mitochondrial DNA deletions

ABSTRACT

Laser microdissection (LMD) has been used to isolate groups of cells and single cells from numerous tissues. In this study, we describe a technique for isolating cochlear structures and individual spiral ganglion cells from archival celloidin embedded human temporal bone sections. The specimens isolated are suitable for quantifying the mitochondrial DNA (mtDNA) common deletion (CD) within these tissues using a real time polymerase chain reaction (PCR) assay. The results presented in this manuscript demonstrate the feasibility of using this LMD technique to study the accumulation of mtDNA deletions in diseases of the ear. To our knowledge, this approach to analyzing archival human temporal bone tissues has not been previously reported.

1. Introduction

LMD was developed over 10 years ago to isolate groups of cells and single cells in tissue sections (Emmert-Buck et al., 1996). This methodology has proven superior to mechanical dissection techniques for the study of nucleic acids and proteins in single cells (Sirivatanauksorn et al., 1999). More recently (Kimura et al., 2005) have described a laser microdissection and pressure catapulting (LMPC) technique to isolate the cochlear elements from archival human temporal bone tissues. In their report, tissue from the organ of Corti, spiral ganglion, stria vascularis, and spiral ligament were selected for study and native sequences from the mtDNA ND1 region of the minor arc were identified using a real time polymerase chain reaction (PCR) assay. However, the copy number of the reference mtDNA sequence was not measured and the exact amount of mtDNA in each cochlear element was not determined. In addition, identification of mtDNA mutations was not attempted. Subsequently, Markaryan et al. (2008a) have identified mtDNA deletions in cochlear elements isolated by LMD from archival human temporal bone tissues with presbycusis. This study utilized nested and long range PCR to identify three unique mtDNA deletions and the previously reported CD.

In this manuscript, a technique for isolating the cochlear elements and single spiral ganglion cells from archival celloidin embedded human temporal bones using LMD is described in detail. In addition, a method for quantifying the CD level in these samples using real time PCR is reported. To our knowledge, this approach to analyzing archival human temporal bone tissues has not been previously reported.

2. Materials and methods

2.1. Temporal bone processing and case selection

The methodologies used for archiving human temporal bone specimens in our laboratory have been previously described (Nelson and Hinojosa, 2006) and are summarized in this paragraph. Following acquisition, the bones were placed in cold 20% formalin for 24 h and then in cold 10% formalin for an additional 10 days. After decalcification with 1% nitric acid for 35 days and dehydration in graded concentrations of ethanol, the specimens were embedded in celloidin. Serial sections of the temporal bones were cut in the horizontal plane at a thickness of 20 μm. Temporal bone sections from individuals with normal hearing and presbycusis were selected from the archival collection in the Bloom Temporal Bone Laboratory for this study. Individuals with presbycusis had bilateral sensorineural hearing loss which was symmetrical

Abbreviations: LMD, laser microdissection; mtDNA, mitochondrial deoxyribonucleic acid; CD, common deletion; PCR, polymerase chain reaction; LMPC, laser microdissection and pressure catapulting; MGB, minor groove binder; LCM, laser capture microdissection; SG, spiral ganglion; OC, organ of Corti; SV, stria vascularis; SL, spiral ligament; BT, basal turn; AT, apical turn; GC, ganglion cell

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doi:10.1016/j.heares.2008.07.007

within 10 dB at each octave. All individuals had a minimum progression of decreased hearing acuity of 10 dB difference between each successive octave in the downward sloping high frequency component of the audiogram. Hearing loss was defined as a sensorineural threshold greater than or equal to 30 dB. Medical histories of the individuals were reviewed. Those with medical conditions or histopathologic findings of other disorders that may have contributed to their hearing loss were not included in the study.

2.2. Section mounting and LMD technique

The isolation of individual cochlear elements and single cells was performed by LMD. Midmodiolar temporal bone sections were transferred from an 80% ethanol storage solution and mounted on director laser microdissection slides (Expression Pathology, Inc., Gaithersburg, MD). The celloidin was removed using a solution of equal parts ethanol and ether (Wackym et al., 1993) and the sections were allowed to dry at room temperature. Areas where the otic capsule bone separated from the slide were trimmed manually with a scissors. Membranous structures of the inner ear maintained a firm attachment to the slide. LMD was performed with the Leica AS LMD instrument (Leica Microsystems, Wetzlar, Germany). The 337 nm pulse nitrogen laser was used with a pulse duration of 4 ns, a pulse energy of 300 μJ, and a repetition rate of 1–30 Hz, generating an average power of 6 mW at 20 Hz. Following proper laser calibration, tissue dissection was performed at X200 magnification with an aperture setting of 6, a power intensity of 45, and a laser speed of 5. Areas of interest were isolated by directing the laser beam along the contours of individual cells or tissue structures. Additional laser pulses were applied to the samples as needed to dislodge them from the surface of the slide.

2.3. DNA isolation

Reagents from the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA) were used to isolate DNA from the LMD samples. Each LMD sample was collected in a 0.2 ml tube and treated with 20 μl of tissue lysis buffer. The samples were stored at room temperature until the DNA isolation was performed. Ten microliters of proteinase K was added to the sample in buffer and mixed by vortexing for 15 s. The tube was incubated at 56° C for 1 h with occasional agitation and then an additional 25 μl of buffer was added. Fifty microliters of buffer containing 1 μg of carrier RNA was added and mixed for 15 s. Fifty microliters of absolute ethanol was added, mixed thoroughly by vortexing for 15 s, and incubated for 5 min at room temperature. The entire lysate was transferred to the QIAamp MinElute Column and centrifuged at 8000 rpm for 1 min. The column was washed with 500 μl of buffer AW1 followed by an additional wash with 500 μl of buffer AW2. The DNA was eluted from the column with 20 μl of water and stored at −20° C.

2.4. Real time PCR assay

We have developed a duplex PCR methodology for quantifying both total mtDNA and mtDNA containing the CD in a single reaction well. The D-loop region copy number was used as a measure of the total amount of mtDNA in a tissue sample. The D-loop contains the light-strand and one of the heavy-strand promoter regions as well as the initiation site of heavy-strand replication. Therefore, this region must be present in replicating mtDNA molecules. The primers and FAM labeled probe for the D-loop region representing the total amount of mtDNA present and the primers and VIC labeled probe for the CD have been described previously (Sabunciyan et al., 2007). The VIC probe was modified at nucleotide position 2 based on our observed sequence of the CD PCR product fragment and appears in bold type below. The use of 3′ MGB (minor groove binder) quenchers increases the probe melting temperature without increasing probe length and therefore allows the design of probes that are shorter and more specific than traditional TAMRA probes. In addition, this nonfluorescent quencher offers the advantage of a lower background signal, which results in improved precision.

The TaqMan PCR assay primers and probe for the D-loop region were:

Forward: 5′-GCT TTC CAC ACA GAC ATC ATA ACA A (nt 263–287).
Reverse: 5′-GTT TAA GTG CTG TGG CCA GAA G (nt 338–317).
Probe: 5′-FAM-AAT TTC CAC CAA ACC CC-MGB (nt 290–306).

The TaqMan PCR assay primers and probe for the CD were:

Forward: 5′-CTT ACA CTA TTC CTC ATC ACC CAA CTA AAA A (nt 8417–8447).
Reverse: 5′-GGA GTA GAA ACC TGT GAG GAA AGG (nt 13509–13486).
Probe: 5′-VIC-CAT TGG CAG CCT AGC ATT-MGB (nt 8481–8482, 13460–13475).

The amplicons, 76 bp for the D-loop and 116 bp for the CD, are within the range of 50–150 bp which is optimal for PCR efficiency. The TaqMan Gene Expression master mix (Applied Biosystems, Foster City, CA) and optimized primer and probe concentrations, 300 nM and 100 nM, respectively, were used in this protocol. A 417 bp mtDNA sequence of the D-loop was amplified from human blood by standard PCR using the following primers: forward – 5′-CTA TCA CCC TAT TAA CCA CT (nt 11–30), reverse – GTT AAA AGT GCA TAC CGC CA (nt 427–408). A 316 bp sequence representing the CD was amplified from cochlear tissue as previously described (Markaryan et al., 2008a). Both fragments were cloned into the 3956-bp PCR4-TOPO vector (Invitrogen, Carlsbad, CA). Purified plasmid DNA preparations were sequenced to confirm the specificity of the inserts and used as calibration standards for the real time PCR assay. Real time PCR amplifications were performed over a copy number range of 3×10^1 to 3×10^6 for the D-loop and 2.7×10^1 to 2.7×10^6 for the CD mtDNA on an ABI 7900HT instrument (Applied Biosystems, Foster City, CA). The real time PCR data was analyzed using Sequence Detection Software version 2.3 (Applied Biosystems, Foster City, CA) to calculate the D-loop and deleted mtDNA copy numbers. The cochlear samples were run in duplicate in two independent experiments. The mean of these values was used to calculate the CD level by dividing the CD copy number by the D-loop copy number and the level was reported as a percentage.

Approval for this research was obtained from the Institutional Review Board at the University of Chicago.

3. Results

Photomicrographs of a midmodiolar temporal bone section from an individual 17 years of age with normal hearing are shown before and following LMD of a single ganglion cell and the cochlear elements including the spiral ganglion, organ of Corti, stria vascularis, and spiral ligament in the middle turn of the cochlea (Figs. 1–5). DNA was isolated from each of these cochlear structures separately and not pooled with samples from other sections. The mtDNA copy numbers for the D-loop and CD sequences in these samples were measured using the real time PCR assay. The CD level in each of these structures was calculated as a percentage by dividing the CD copy number by the D-loop copy number. The results are depicted in Fig. 6. The CD level was observed to vary between each of the cochlear elements and ranged from 3% to 9%.

The LMD technique was also used to isolate the spiral ganglion tissue from the basal and apical turns of the cochlea in a single