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Label-free visualization of cholesteatoma in the mastoid and tympanic membrane using CARS microscopy ☆

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Abstract

Objective: The present study aimed to evaluate the possibility of using coherent anti-Stokes Raman spectroscopy (CARS) microscopy to determine the specific molecular morphology of cholesteatoma by detecting the natural vibrational contrast of the chemical bonds without any staining.
Materials and methods: Specimens from the mastoid and tympanic membrane with and without cholesteatoma were analyzed using CARS microscopy, two-photon excited fluorescence (TPEF) microscopy, and the second harmonic generation (SHG) microscopy.
Results: In cholesteatoma tissues from the mastoid, a strong resonant signal at 2845 cm^{-1} was observed by CARS, which indicated the detection of the CH_2 hydro-carbon lipid bonds that do not generate visible signals at 2940 cm^{-1} suggestive of CH_3 bonds in amino acids. A strong resonant signal at 2940 cm^{-1} appeared in an area of the same specimen, which also generated abundant signals by TPEF and SHG microscopy at 817 nm, which was suggestive of collagen. In the tympanic membrane specimen with cholesteatoma, a strong resonant signal with corrugated morphology was detected, which indicated the presence of lipids. A strong signal was detected in the tympanic membrane with chronic otitis media using TPEF/SHG at 817 nm, which indicated collagen enrichment. The CARS and TPEF/SHG images were in accordance with the histology results.
Conclusion: These results suggest the need to develop a novel CARS microendoscope that can be used in combination with TPEF/SHG to distinguish cholesteatoma from inflammatory tissues.

Keywords: Cholesteatoma; Imaging; Label-free; Raman spectroscopy; High resolution

1. Introduction

Cholesteatoma is a benign, gradually expanding destructive cystic epithelial lesion of the temporal bone that is generated from keratinizing stratified squamous epithelium (Semaan and Megerian, 2006). Surgical removal of the pathological tissue is the only effective therapy for cholesteatoma, but some cases of cholesteatoma can recur after this procedure (Tomlin et al., 2013; Neudert et al., 2014). Indeed, the rate of residual

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cholesteatoma following surgery can reach as high as 35%, and surgery is the major contribution to recurrence in the clinic (Wilson et al., 2013). Even the application of endoscope during surgery in combination with operating microscope only provides an incremental benefit for prevention of residual cholesteatoma (James et al., 2016). Our hypothesis is that the conventional endoscope has a limitation in detecting focal cholesteatoma due to its lower spatial resolution and poorer depth sensation than the operating microscope. Therefore, there is a need to improve the visualization by developing a novel endoscopic approach that is capable of detecting the specific chemical structures within the cholesteatoma to assure a clear surgical margin during surgery. This potential novel endoscope may also be useful to predict which retraction pocket on the tympanic membrane will develop into cholesteatoma and guide the treatment strategy in the clinic.

Raman spectroscopy is a powerful tool capable of generating a characteristic signature of the sample material and operates by detecting energy associated with the molecular bond vibrations. The process in which the incident photons scatter inelastically upon interaction with matter is also known as Raman scattering (Wachsmann-Hogiu et al., 2009). Pandey et al. reported that Raman spectroscopy is capable of discerning the molecular pathology of differential proliferative middle ear lesions and may aid in the assessment of pathological borders to improve surgical outcomes in cases of middle ear disease (Pandey et al., 2015). However, the Raman spectroscopy signal is faint and insufficient for clinical application during surgery. High-resolution coherent anti-Stokes Raman spectroscopy (CARS) is an advanced molecular imaging technique that has recently been applied to label-free imaging of cells and tissues using the natural vibrational contrast and has significantly boosted and enhanced Raman signal levels (Pezacki et al., 2011; Moura et al., 2016). CARS occurs when a target molecule is simultaneously irradiated by two laser beams at different frequencies, a pump beam ω_P and a Stokes beam ω_S. When the difference between the higher frequency (pump beam) and the lower frequency (Stokes beam) equals the vibrational frequency of the target bond of the molecule, a CARS signal is generated (Pezacki et al., 2011; Folick et al., 2011; Rodriguez et al., 2006).

We aimed to evaluate the feasibility of using CARS microscopy to display the specific molecular morphology of cholesteatoma and the potential of CARS to be integrated as a novel endoscope for cholesteatoma imaging in the clinic. We focused on detecting the vibrations of CH_2 bonds in the lipids accumulated in the cholesteatoma and the CH_3 bonds in the peptides of proteins that are enriched in the tissues of chronic suppurative otitis media attributed to the inflammatory infiltrations and biofilm formation, among others (Fig. 1) (Bloksgaard et al., 2012; Gu et al., 2014; Knutsson et al., 2011; Uno and Saito, 1995). Two-photon excited fluorescence (TPEF) and potentially the second harmonic generation (SHG) from fibrillar collagen structures caused by chronic inflammation was also evaluated (Knutsson et al., 2011; Uno and Saito, 1995; Jiang et al., 2011).

2. Materials and methods

2.1. Specimens

The following 4 types of specimens were collected from 3 patients undergoing middle ear surgeries at the Helsinki University Central Hospital: cholesteatoma tissues from the mastoid, the mucosa of the temporal bone air cell without visible cholesteatoma in the same mastoid that was 10 mm away from the identified cholesteatoma, the tympanic membrane with cholesteatoma on the mucosa layer, and the residual membrane near the perforation of the tympanic membrane of chronic otitis media. After surgical removal, all specimens were immediately fixed using 4% formaldehyde overnight and maintained in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) until CARS microscopy and histology study. No additional procedures were added to the standard surgery when the specimens were obtained, and no personal information was exposed. The study protocol was approved by the ethics committee of the Helsinki University Central Hospital and followed the rules of the Declaration of Helsinki, which was developed by the World Medical Association and was updated at the 64th WMA General Assembly in Fortaleza, Brazil, in 2013 (WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2014).

2.2. CARS microscopy

The images were acquired with a commercial Leica TCS SP8 CARS confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany), which consists of an inverted microscope equipped with an ultra-short pulsed light source (picoEmerald, APE, Berlin, Germany) that produces the two synchronous beams needed for CARS microscopy. The Stokes beam at 1064 nm was emitted from a neodymium-doped yttrium orthovanadate (Nd:YVO_4) laser while a tunable pump/probe beam at 780–940 nm was generated by an optical parametric oscillator (OPO). The pulse width was 5–7 ps with a repetition rate of 80 MHz corresponding to the Raman line width of 2–3 cm^{-1}. The pulses from the two sources were temporally and spatially overlapped on the focal plane of the microscope. Up to 100 mW of average power from both the pump and the Stokes source was delivered to the specimen with an identical laser intensity for each measurement. The laser generating wavelength of 817 nm was used for the CARS modality simultaneously with the Stokes beam at 1064 nm to excite the symmetric vibrational resonance of the CH_2 hydro-carbon bonds in the lipids at 2845 cm^{-1} and that of the CH_3 bonds in amino acids of the proteins at 2940 cm^{-1} (Pirhonen et al., 2016; Surmacki et al., 2013). Meanwhile, the TPEF and SHG was measured using excitation at 817 nm and broad-band detection at 380 nm – 550 nm according to the literature (Jiang et al., 2011). The detected TPEF was compared with the conventional autofluorescent microscopy measured with 488 nm excitation. The generated two-photon excited and

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