International Journal of Pediatric Otorhinolaryngology 88 (2016) 104–108

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: http://www.ijporlonline.com/

Association of microRNA 146 with middle ear hyperplasia in pediatric otitis media☆

Tina L. Samuels [a], Justin Yan [a], Pawjai Khampang [a], Alexander MacKinnon [b], Wenzhou Hong [a], Nikki Johnston [a, c], Joseph E. Kerschner [a, *]

[a] Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, Milwaukee, WI, USA
[b] Department of Pathology, Medical College of Wisconsin, Milwaukee, WI, USA
[c] Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, USA

ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form
28 June 2016
Accepted 30 June 2016
Available online 2 July 2016

Keywords:
Otitis media
miR-146a
miR-146b
IRAK1
TRAF6
Toll-like receptor

ABSTRACT

Objective: Toll-like receptor signaling activated by bacterial otitis media pathogens in the middle ear has been shown to play a key role in OM susceptibility, pathogenesis and recovery. Recent studies implicate microRNA 146 (miR-146) in regulation of inflammation via negative feedback of toll-like receptor signaling (TLR) in a wide variety of tissues, however its involvement in otitis media is unknown.
Methods: Human middle ear epithelial cells were stimulated with proinflammatory cytokines, interleukin 1 beta or tumor necrosis factor alpha, for two to twenty-four hours. Middle ear biopsies were collected from children with otitis media with effusion (n = 20), recurrent otitis media (n = 9), and control subjects undergoing cochlear implantation (n = 10). miR-146a, miR-146b expression was assayed by quantitative PCR (qPCR). Expression of miR-146 targets involved in TLR signaling, IRAK1 and TRAF6, was assayed by qPCR in middle ear biopsies. Middle ear biopsies were cryosectioned and epithelial thickness measured by a certified pathologist.
Results: Proinflammatory cytokines induced expression of miR-146 in middle ear epithelial cells *in vitro*. Middle ear miR-146a and miR-146b expression was elevated in otitis media patients relative to control subjects and correlated with middle ear epithelial thickness. A trend towards inverse correlation was observed between miR-146 and TRAF6 expression in the clinical population.
Conclusions: This report is the first to assess miRNA expression in a clinical population with OM. Findings herein suggest miR-146 may play a role in OM.

1. Introduction

Otitis media (OM) is a group of infective and inflammatory conditions affecting the middle ear with varied presentation, complications and treatment. OM is a leading cause of health care visits worldwide and of preventable hearing loss in the developing world [1]. OM is one of the commonest reasons for childhood antibiotic use, and treatment of OM with ventilation tube (VT) insertion the commonest cause for surgery in children in the developed world. However emergence of resistance and side effects are an implicit risk of common and widespread antibiotic use, VT insertion requires anesthesia, and children frequently require repeat VT insertion. Improved treatment of AOM and OME are therefore welcome. Molecular signaling pathways involved in the pathophysiology of disease may offer novel alternatives for targeted therapy, yet a paucity of information exists regarding the molecular events that contribute to OM pathogenesis.

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression by binding to target mRNAs, directing their degradation or translational inhibition. Over half of the human transcriptome is predicted to be under miRNA regulation [6]. miRNAs have been shown to be involved nearly every biological process and have recently been implicated in a variety of otologic pathologies including OM [2–5].

Discovered in a screen for response to microbial infection,

☆ This study was supported by funds from the National Institutes of Health (NIDCD DC007903) and the Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin. Data herein was presented before the International Society for Otitis Media at National Harbor, MD on June 10, 2015.

* Corresponding author. Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226, USA.
E-mail address: jkerschner@mcw.edu (J.E. Kerschner).

http://dx.doi.org/10.1016/j.ijporl.2016.06.056
0165-5876/

miRNA 146 (miR-146) is now one of the three most widely studied miRNA families in inflammation. Mature products of the miR-146 family, miR-146a and miR-146b, are expressed in a variety of cell types where they play a conserved role in negative feedback of toll-like receptors (TLRs) [6]. Infection with non-typeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and/or *Moraxella catarrhalis* is a dominant etiology of OM [7,8]; these pathogens are recognized by TLRs which in turn elicit cytokine and mucin gene expression, inflammation, mucosal hyperplasia, effusion, and leukocytic infiltration of the middle ear [9–14]. TLR signaling has been shown to play a vital role in OM susceptibility, pathogenesis and recovery [15–17], however, no study to date has assessed the involvement of key TLR signaling modulator, miR-146, in OM.

The objective of this study was to determine expression of miR-146a and miR-146b in an *in vitro* model of OM, and to assess the correlation of middle ear miR-146a and miR-146b expression with OM diagnosis, middle ear inflammation and hypertrophy, and expression of TLR signaling molecules in a pediatric population with OM relative to an OM-free control population.

2. Materials and methods

2.1. In vitro cell culture and cytokine treatment

Human ME epithelial cells (HMEEC) were provided by Dr. David Lim (House Ear Institute). Normal growth media consisted of 1:1 mixture of Bronchial Epithelial Cell Growth Medium (BEBM; Cambrex, East Rutherford, NJ) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with final concentrations of 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (ThermoFisher, Carlsbad, CA). Cells were grown in a humidified chamber at 37 °C with 5% carbon dioxide and media changed every three days. Cells were grown to 70–80% confluency and serum-starved for two hours prior to exposure to pro-inflammatory cytokines: 200 ng/ml tumor necrosis factor-alpha (TNF-α) or 100 ng/ml interleukin 1 beta (IL-1β; R&D Systems, Minneapolis, MN) or normal growth media alone (control) for 2, 4, 6, 8, 16 and 24 h, harvested and stored at −80° C. All treatment conditions and time points were performed in triplicate.

2.2. Human specimens

Approval was obtained from the Children's Hospital of Wisconsin Institutional Review Board (protocol 369996-9) for collection of middle ear (ME) biopsies from pediatric patients aged 6 months to 12 years with diagnosis of otitis media with effusion (OME) or recurrent OM (ROM) undergoing surgery for tympanostomy tube (TT) placement at the Children's Hospital of Wisconsin. Approval was obtained from the Children's Hospital of Wisconsin and Sick Kids Hospital in Toronto Research Ethics Board (protocol 1000012606) for collection of ME biopsies from patients aged one to ten years undergoing cochlear implantation (CI) as a control population. OME was defined as persistent, unremitting ME effusion for greater than three months duration. ROM was defined as three or more episodes of acute OM over six months with resolution of acute infection and ME fluid between episodes. Strict criteria were used to exclude patients in the CI control group having had one or more episodes of OM in the previous twelve months of life. Additional exclusion criteria for all patients included history of immunologic, intrinsic or pharmacologic abnormality, anatomic or physiologic defect of the ear, syndrome associated with OM (i.e. Down syndrome, cleft palate), chronic mastoiditis, and history of cholesteatoma. Informed consent was obtained prior to surgery. For OME and ROM patients, after performance of the myringotomy incision, fluid within the ME space was collected. Two to three small (<1 mm) ME mucosal biopsies were taken from the ME with a cup forceps near the Eustachian tube orifice and the TT was then placed as per routine. From each patient, one specimen was placed in RNAlater (ThermoFisher) and the other in Hank's buffered saline solution (HBSS; ThermoFisher) prior to immediate transport to the laboratory. Samples in RNALater were stored at −80 °C prior to RNA extraction. Samples in HBSS were immediately embedded for cryosectioning as below. Mucosal biopsies were obtained from a similar location of the ME of CI patients during cochlear implantation. From each patient, one specimen was placed in RNAlater and the other in 10% formalin. Samples in RNALater were stored at −80 °C prior to RNA extraction. Samples in formalin were stored at 4 °C prior to embedding for cryosectioning as below.

2.3. RNA isolation

RNA was isolated from treated cells and ME biopsies using TRIzol according to a manufacturer instruction (ThermoFisher). RNA integrity was analyzed by gel electrophoresis. Spectrophotometry was performed to assess RNA purity and concentration and RNA was stored at −80 °C until further use.

2.4. Quantitative polymerase chain reaction (PCR)

The expression of mature miR-146a and miR-146b was examined using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assays for miR-146a, miR-146b and RNU6B (internal control; ThermoFisher), as per manufacturer instruction. Additionally, ME RNA (75 ng) was reverse transcribed using Superscript VILO (ThermoFisher) and expression of IRAK1, TRAF6 and HPRT1 (housekeeping gene) were assessed in twelve high- and nine low-miR146-expressing specimens from ten and nine patients, respectively, using TaqMan Gene Expression Assays. Amplification was performed on the Applied Biosystems ViiA7 (ThermoFisher) at 52 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in triplicate. Expression of targets were normalized to internal control/housekeeping genes and relative expression differences determined using the $2^{-\Delta\Delta CT}$ method [18]. Student's t-test was employed to determine statistical significance of relative fold changes in miR-146 expression in cytokine-stimulated cells *in vitro*. Repeated measures ANOVA analysis was used to compare adjusted mean miR-146a and miR-146b expression across the three patient groups (OME, ROM, CI). A linear regression model with generalized estimating equations was used for correlation of miRNA expression with IRAK1 and TRAF6 expression.

2.5. Analysis of ME epithelial thickness

Following transport to the laboratory in HBSS, one ME biopsy was embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA), placed on dry ice and cryosectioned to 6 μm or held at −80 °C until use. Sections were mounted on glass slides and stained with hematoxylin and eosin via automated stainer. Slides were scanned at 400× (Nanozoomer; Hamamatsu Photonics, Japan). Images were analyzed using NDP.view2 software by a board certified pathologist. Average values from up to 13 distinct regions of tissue per patient were used as a representative sampling of the ME. Areas in which the epithelium was detached or disrupted or areas with underlying specialized structures were not analyzed. Epithelial thickness in number of cells was counted and recorded as a range (0–1, 2–5 and 5 + cells) and/or assessed by the software (in μm) and verified by a micrometer. A linear regression model with generalized estimating equations was used for correlation of miRNA expression with ME thickness in microns. Proportional odds