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

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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.

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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.

Keywords

otitis media; miR-146a; miR-146b; IRAK1; TRAF6; toll-like receptor

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, 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

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: 200ng/ml tumor necrosis factor-alpha (TNF- α) or 100ng/ml interleukin 1 beta (IL-1 α ; R&D Systems, Minneapolis, MN) or normal growth media alone (control) for 2, 4, 6, 8, 16 and 24 hours, harvested and stored at –80°C. All treatment conditions and time points were performed in triplicate.

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.

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.

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 (75ng) 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 minutes, 95°C for 10 minutes, and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Reactions were performed in triplicate. Expression of targets were normalized to internal control/housekeeping genes and relative expression differences determined using the $2^{-\text{CT}}$ 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.

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 $6\mu\text{m}$ 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 $400\times$ (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 regression model with generalized estimating equations was used for correlation of miRNA expression with ME thickness in number of cells.

3. RESULTS

In vitro cell culture studies demonstrated that treatment of HMEECs with pro-inflammatory cytokines, TNF- α and IL-1 α , resulted in a significant increase in the expression of

miR-146a and miR-146b over time. TNF- α induced elevation of miR-146a was observed at 8 hours (3-fold, $p=0.022$), 16 hours (3.4-fold, $p=0.046$) and 24 hours (5-fold, $p=0.003$) relative to control (Figure 1A). TNF- α elevation of miR-146b was detected at 16 hours (1.5-fold, $p=0.018$) and 24 hours (1.5-fold, $p=0.014$; Figure 1B). Elevation of miR-146a and miR-146b by IL-1 β treated was observed in all time points with maximal increase at 4 hours (miR-146a: >5-fold, $p=0.028$; miR-146b: 3-fold, $p=0.045$; Figure 1 C–D).

Expression of miR-146a and miR-146b were compared in ME biopsies from patients with OME and ROM relative to CI patients (see patient demographics, Table 1). Significant elevation in miR-146a expression was observed in OME and ROM patients relative to CI controls (Table 2). miR-146b expression was elevated in ROM relative to CI patients; elevation in OME relative to CI patients was not significant (Table 2). miR-146a and miR-146b expression were not significantly different in OME relative to ROM patients ($p=-0.5402$ and 0.3715 , respectively). miR-146a and miR-146b expression correlated with ME thickness (both μm and number of cells; Table 3). In Table 3, the negative coefficient of ME thickness (μm) illustrates that higher Ct (lower miRNA expression) is associated with a decrease in thickness. The odds ratio for ME thickness (in cells) quantifies the effect of a one unit increase in delta C_t (2-fold increase in miRNA) on odds of a lower value outcome, i.e., a 2-fold increase in miR-146a expression was associated with 1.4-fold increased odds of a higher category of ME epithelial thickness. Figure 2 depicts representative specimens with high miR-146 expression and ME thickness (A) and low miR-146 expression and ME thickness (B).

Gene expression analysis of miR-146 targets, IRAK1 and TRAF6, revealed association of a one unit increase in delta C_t of miR-146a with a 0.10 unit decrease in the delta C_t of TRAF6 with marginal significance ($p=0.05$) and an inverse association trending between miR-146b and TRAF6; no significant association was observed between miRNA and IRAK1 expression (Table 4).

4. DISCUSSION

The requirement for proper functioning of the TLR signaling cascade in middle ear health has been demonstrated in both in animal models and clinical populations: deletion or mutation of TLR2 and TLR4 in mice results in transition from the self-limiting characteristic of acute OM to persistent middle ear disease [17], mutation of TLR signaling intermediaries results in abnormal OM recovery [19], multiple TLR4 single-nucleotide polymorphisms are associated with OM [20,21], and TLR4 is depleted in patients with chronic ME disease compared to control subjects [22]. TLR signaling initiates miR-146a/b expression which is in turn crucial for finely tuning the immune response by targeting key intermediaries in the TLR signaling cascade, tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), and to a lesser extent cytokines, cytokine receptors, myeloid differentiation primary response gene 88 (MyD88) and TLRs. [23–26] While Song *et al.* previously investigated miRNA expression in lipopolysaccharide (LPS)-stimulated HMEEC, and did not report a change in miR-146 expression,[4] the authors used a shorter treatment time (two hours) and different stimuli than those herein.

In the study herein, involvement of miR-146a/b in the pathophysiology of OM was supported by cytokine-responsive expression of miR-146a and miR-146b in HMEEC *in vitro*, elevated expression in OM patients relative to a control CI population, and association of miR-146a and miR-146b expression with ME epithelial thickness. The observed inverse correlation trending between miR-146a or miR-146b and TRAF6 expression in OM subjects and controls supports the physiological relevance of elevated miR-146a and miR-146b expression in OM.

While no significant difference in miR-146 expression was observed between patients diagnosed as ROM and OME, this may be due to overlap in OM diagnoses and molecular and microbiologic similarities in the ME of patients clinically characterized as ROM and OME.[27] miR-146a and miR-146b expression significantly correlated with an increase in ME epithelial thickness measured in standardized units as well as cell number, suggestive of both inflammation and hyperplasia. The downstream effector of TLR signaling, nuclear factor kappa B (NF- κ B), is an established cancer marker that promotes cell survival and proliferation, however miR-146 and miR-16b expression has been associated with reduced or increased cell proliferation given different cell types and stimuli [28–30]. The role of these miRNAs in cell proliferation appears to be complex and further experiments are warranted to characterize the effect of miR-146 on ME epithelial cell proliferation during bacterial challenge.

In the current study, miR-146a demonstrated a greater response to cytokine stimulation *in vitro* and more significant correlation with OM and ME hyperplasia than miR-146b, suggesting that the mechanisms employed to control miR-146a and miR-146b transcription during OM are not redundant. While mature miR-146a and miR-146b differ by only two nucleotides and therefore share a seed sequence, target profiles and overlapping functions, they arise from different genomic loci and routes of processing. Previous investigation in other cell types has revealed that miR-146a is induced immediately by TLR signaling via NF- κ B while induction of miR-146b occurs secondarily via STAT-3 dependent mechanism as a consequence of TLR-induced IL-10 production.[24, 31] Further studies will be necessary to determine the signaling events leading to miR-146a and miR-146b expression in OM and the relevance of any difference to their roles in the disease process.

While elevation of middle ear miR-146a and miR-146b expression in OM patients and correlation with middle ear epithelial thickness implicates their involvement in OM, further studies are required to establish a causal relationship. In this study we observed a marginal negative association of miR-146a or miR-146b expression with TRAF6 mRNA levels. Given limited patient material to assay corresponding target protein expression which may be independently affected by miR-146 via translational inhibition, we plan to perform further mechanistic analysis *in vitro*, utilizing miRNA knockdown and expression tools to investigate miR-146 target expression and the requirement for miR-146 in middle ear epithelial cell response to cytokine, LPS or bacterial challenge. Sequencing and annotation of the chinchilla genome have recently made such functional studies feasible in this model as well (<http://ngc.mcw.edu/>) [32].

Limitations of this study include differences in the male/female ratio of OM and CI control populations and use of an unhealthy population as a control; children undergoing cochlear implantation may have genetic causes of hearing loss that affect the innate immune system in unknown ways. Further, biopsy sampling of the ME mucosa in the CI and OM patients may not represent an accurate picture of the entire ME. A limitation of our *in vitro* work is the adult origin of the single cell line used in our experiments. As the only ME epithelial cell line currently available, HMEEC are a commonly used model for pediatric OM. However, we are currently in the process of generating additional ME epithelial cell lines from children with ROM, OME and no OM history in effort to provide more diverse and representative cell culture models for study of pediatric OM.

5. CONCLUSION

In summary, this report is the first to assess miRNA expression in OM patients and the first to implicate miR-146 in the pathophysiology of OM. Given its conserved function in other tissues and cell types and findings presented herein, miR-146 has the potential to play a key role in many aspects of otitis media pathogenesis including the host response to repeated exposure to OM pathogens, disease recurrence and chronicity. Future studies are warranted to further elucidate the role of miR-146 in OM pathogenesis.

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Figure 1. Pro-inflammatory cytokine induced expression of miR-146a and miR-146b in HMEEC
 The human middle ear epithelial culture was exposed to 200ng/ml TNF- α at various time points. Both miR-146a (A) and miR-146b (B) were upregulated. Cells exposed to 100ng/ml IL-1 β demonstrated significant upregulation of both miR-146a (C) and miR-146b (D) at every time point tested. Bar represents mean $\pm$ SEM. *= $p < 0.05$ vs control.

Figure 2. Representative specimens demonstrate miR-146 expression correlation with middle ear epithelial thickness

Representative human middle ear epithelial specimens are shown with greater miR-146 gene expression and thicker epithelium (A; miR-146a $C_t = -2.68226$, miR-146b $C_t = -5.85374$, epithelial thickness = $30.4\mu\text{m}$), and lower miR-146 gene expression and thinner epithelium (B; miR-146a $C_t = 5.07923$, miR-146b $C_t = 3.44399$, epithelial thickness = $13.7\mu\text{m}$).

Sections were stained with hematoxylin and eosin. Green bar depicts region measured. Scale bar (black) = $100\mu\text{m}$. *Note to the editor: This figure is to be printed in color.*

Table 1

Patient Demographics

	CI (n=10)	OME (n=20)	ROM (n=9)
<i>Sex</i>			
Female	7 (70%)	6 (30%)	4 (44.4%)
Male	3 (30%)	14 (70%)	5 (55.6%)
<i>Ethnicity</i>			
Caucasian	8 (80%)	13 (65%)	5 (55.6%)
African American	0 (0%)	4 (20%)	1 (11.1%)
Hispanic	0 (0%)	2 (10%)	2 (22.2%)
Asian	2 (20%)	0 (0%)	0 (0%)
Unknown	0 (0%)	1 (5%)	1 (11.1%)
<i>Age at Tti (days)</i>	899.8±563.5	1849.7±969.9	620.8±430.2

Table 2

miR-146 expression relative to CI controls.

Group	Ratio	Lower CL	Upper CL	p-value
miR-146a				
OME or ROM				0.0082
OME	4.066	1.531	10.794	0.0061
ROM	5.454	1.773	16.775	0.0041
CI	1	—	—	—
miR-146b				
OME or ROM				0.1167
OME	2.302	0.773	6.86	0.1301
ROM	3.711	1.057	13.037	0.0412
CI	1	—	—	—

Table 3

Correlation of miR-146 expression with ME thickness.

ME Thickness (μm)				
	<i>Estimate</i>	<i>Lower CL</i>	<i>Upper CL</i>	<i>p-value</i>
miR-146a	-2.179	-3.791	-0.567	0.008
miR-146b	-1.490	-2.611	-0.369	0.009
ME Thickness (number of cells)				
	<i>Odds Ratio</i>	<i>Lower CL</i>	<i>Upper CL</i>	<i>p-value</i>
miR-146a	1.445	1.074	1.945	0.015
miR-146b	1.210	1.018	1.439	0.031

Table 4

Correlation of miR-146 with IRAK1 and TRAF6 gene expression

	Estimate	Lower CL	Upper CL	p-value
miR-146a				
IRAK1	0.127	-0.217	0.470	0.471
TRAF6	-0.101	-0.203	0.000	0.050
miR-146b				
IRAK1	0.131	-0.181	0.443	0.410
TRAF6	-0.073	-0.162	0.016	0.107