MicroRNA Regulation of Proinflammatory Response in X-linked Adrenoleukodystrophy

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Abstract

The underlying mechanism(s) for development of the inflammatory response in inherited, fatal neurometabolic disease X-linked adrenoleukodystrophy (X-ALD) remain completely unknown. Genetic defect (ABCD1 mutation/deletion), common to all phenotypes of X-ALD, has failed to explain the development of inflammation only in a subset of patients. In this study we document the novel role of microRNAs (miRNAs) in the development of the inflammatory response in unstimulated ALD patient-derived lymphocytes and Abcd1-knockout (Abcd1-KO) mice mixed glial cells. The levels of proinflammatory cytokine gene expression (inducible nitric oxide synthase [iNOS]) were increased in X-ALD patient-derived lymphocytes. Predictions via the use of online bioinformatics algorithms and confirmed by using miRNA mimic of inhibitor-transfection method (gain- and loss-of-function) revealed the role of miR-323-5p in regulating iNOS expression in X-ALD patient-derived lymphocytes. Functional confirmation of the targets was obtained by using the dual-luciferase assay and western blot analysis. Abcd1-KO mice do not develop the inflammatory response characteristic of the fatal X-ALD phenotype. We recently reported that AMP-activated protein kinase (AMPKα1) deletion induced spontaneous iNOS expression in Abcd1-KO mice mixed glial cells. Here we discover the novel role of miR-323-5p regulating the iNOS response in AMPKα1-deleted Abcd1-KO mice mixed glial cells. This study demonstrated the novel role of miR-323-5p in regulating the inflammatory response in unstimulated X-ALD patient-derived cells and mixed glial cells from Abcd1-KO mice suggesting that these miRNA could function as promising novel therapeutic targets for the treatment of X-ALD.

Keywords: X-ALD; microRNA; iNOS; AMPKα1; Abcd1-KO; Glial cells

Introduction

X-linked adrenoleukodystrophy (X-ALD) is a fatal inherited disorder of childhood [1]. The primary defect is the mutation and/or deletion of gene ABCD1 that encodes a peroxisomal integral membrane protein-adrenoleukodystrophy protein (ALDP) [1]. An enduring question in X-ALD has been the unknown etiology of disease progression [1]. Similar gene mutation/deletions (even within families) result in strikingly variable disease phenotypes ranging from relatively mild adrenomyeloneuropathy (AMN) to severe, fatal cerebral adrenoleukodystrophy (ALD) [1]. Intriguingly, more than 1500 mutations/deletions in the ABCD1 gene have been reported with no genotype-phenotype correlation. Roles for epigenetic factors/genes have been proposed though none have been identified [1,2].

Recent studies have established the role of small RNA sequences-microRNAs (miRNA) in regulating gene expression in genetic, metabolic and neuroinflammatory diseases [3,4]. In multiple sclerosis (MS), a disease that shares neurodegeneration and immune response with ALD phenotype, differential miRNA expression has been reported for the variable phenotypes of the disease [5]. Yet the neuroinflammatory mechanism may be different between the two since the infiltrating lymphocytes accumulate behind the demyelinating edge in ALD [6], whereas in MS brain they accumulate at the demyelinating edge [7]. No miRNA(s) related to any phenotype(s) or pathway of X-ALD has been reported to date. In this novel report we document the first evidence of miRNA-regulation of the inflammatory response in X-ALD. X-ALD patient-derived unstimulated lymphocytes developed a spontaneous inflammatory response [8,9]. We used these patient-derived lymphocytes to test and validate the expression of a selected set of miRNAs predicted to be involved in the inflammatory iNOS gene expression. Furthermore, the X-ALD mouse model, a classical knockout of Abcd1 gene (Abcd1-KO), only accumulates VLCFA in body fluids and tissues but does not develop the inflammation and demyelination in the brain that is characteristic of the fatal ALD phenotype [10-12]. We recently documented the novel role of the anti-inflammatory protein AMP-activated protein kinase (AMPKα1) in induction of the proinflammatory response in unstimulated Abcd1-KO mice mixed glial cells [13]. In this study we also document the novel role of miR-323-5p in mediating the AMPKα1 deletion-induced proinflammatory response in Abcd1-KO mice mixed glial cells. This report provides a novel opportunity to unravel the mechanism of disease progression in X-ALD. Since no satisfactory therapy exists for the variable X-ALD phenotypes, the identified miRNA can also be the targets of novel anti-miRNA or miRNA replacement therapies.

Materials and Methods

Reagents

Dulbecco’s Modified Eagle’s Medium (4.5 g/L) and RPMI-1640 was purchased from HyClone (Logan, UT), bovine serum (FBS) was purchased from BioAbChem Inc. (Ladson, SC). Antibodies were purchased from Cell Signaling Technology Inc (Danvers, MA) unless purchased from Cell Signaling Technology Inc (Danvers, MA) unless
otherwise mentioned. ECL and nitrocellulose membranes were purchased from BioRad (Hercules, CA).

Cell culture
All patient-derived cells were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (ccr.coriell.org).

Lymphocytes
Human lymphocytes derived from normal (control; GM03798) and ALD (ALD; GM04673) patients were cultured in RPMI-1640 containing 10% FBS and antibiotic/antimycotic solution.

Primary mixed glial cells
Mouse primary mixed glial cells were prepared from 2-day-old wild type (WT) and Abcd1-KO pups, as described previously [13].

Lentiviral vector mediated knockdown of AMPKα1 in Abcd1-KO mice mixed glial cells
Transduction-ready mouse shRNA lentiviral particles (106 TU/ml) for AMPKα1 (consisting of a pool of 3–5 constructs and puromycin selection gene; sc-29674-V) and control scrambled shRNA lentiviral particles (Scr) (106 TU/ml, sc-108080) were purchased from Santa Cruz Biotechnology (Dallas, TX). Abcd1-KO mixed glial cells were transduced with viral particles (AMPKα1 and control) as described previously [13].

miRNA mimic and inhibitor transfections
X-ALD patient-derived lymphocytes (1 × 10⁶/10 ml) and Abcd1-KO mice mixed glial cells (5 × 10⁵ cells per well in a 6-well plate) were transfected with 5 nM miR-323-5p mimic (Qiagen MSY0004696 [human]; MSY0004638 [mouse], Qiagen, Valencia, CA), 50nM miR-323-5p inhibitor (Qiagen, MIN0004696 [human]; MIN0004638 [mouse]), AllStar Negative Control siRNA (Qiagen SI03650318) or transfection media/reagent (TM) alone using HiPerFect Transfection Reagent (Qiagen 301705) according to the manufacturer's instructions. Transfection efficiency was checked using Syn-hsa-miR-1 mimic (Qiagen MSY000416). Cells were harvested after 72 h for RNA isolation, cDNA conversion and real time PCR.

miRNA-enriched RNA extraction and cDNA synthesis
miRNA fractions from patient-derived lymphocytes and Abcd1-KO mice mixed glial cells were extracted using miRNeasy Mini Kit (Qiagen). miScript cDNA synthesis kit (Qiagen) was used followed by quantitative RT-PCR analyses of selected miRNAs using the miScript SYBR Green PCR kit (Qiagen). Fold change of miRNAs was determined using the ΔΔct method. For analysis of individual miRNAs, human and mouse-specific small nucleolar RNA U6 (RNU6) was selected as a reference miRNA.

RT-PCR
RT-PCR was conducted using CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, CA). Single-stranded cDNA was synthesized from total RNA as described previously [13]. The primer sets were purchased from IDT.

Assay for nitric oxide synthesis
Production of nitric oxide (NO) was determined by assaying culture supernatants for nitrite using Griess reagent as described previously [14].

Immunoblot analysis
Healthy control and ALD patient-derived lymphocytes and ALD-KO mouse mixed glial cells were processed for immunoblot analysis as described previously [8,13].

Luciferase reporter assay
X-ALD patient-derived lymphocytes were transfected with human iNOS-3'UTR (SC206494) fused with luciferase reporter vector or a Renilla control vector (OriGene, Rockville, MD) using FuGENE 6 following the manufacturer's instructions (Roche Applied Science). At 72 h post-transfection, under various treatment conditions, the cells were processed for luciferase assay using a luciferase reporter assay kit (Promega).

Prediction of miRNA targets
The target genes of the respective miRNAs were predicted using algorithms, including miranda, Targetscan via miRwalk suite (http://www.unm.edu/keithlab/apps/zmf/mirwalk) [15]. Search criteria used was- minimum miRNA seed length of 7 nucleotides and binding sites on the 3' UTR of target mRNA. Targets predicted by both the algorithms were identified as predicted targets. The experimentally validated targets were identified by a literature search [16,17].

Statistical analysis
Using the Student-Newman-Keuls test and analysis of variance, p-values were determined for the respective experiments using GraphPad software (GraphPad Software Inc, San Diego, CA).

Results and Discussion
Expression of iNOS is increased and miR-323-5p expression decreased in X-ALD patient-derived lymphocytes
X-ALD patient-derived lymphocytes spontaneously produce NO in vitro [9]. We found that NO levels were significantly increased (p<0.001) time-dependently over 72 h in X-ALD lymphocytes compared with healthy control lymphocytes when maintained in serum-free media (Figure 1A). NO levels were mirrored by a significant increase in iNOS mRNA in X-ALD patient-derived lymphocytes compared to healthy controls (Figure 1B). While iNOS levels have been documented in X-ALD postmortem brains [18] and in patient-derived unstimulated cultured cells in vitro [8,9], the mechanism of induction of iNOS in X-ALD remains unknown. As yet unknown epigenetic factors have been proposed to mediate the progression of inflammation and pathology in X-ALD [2]. Emerging studies suggest that miRNAs provide an added layer in orchestrating inflammatory gene expression [19]. miRNAs regulate cytokine production via mRNA degradation and/or by blocking translation. As miRNAs play a critical role in the regulation of the inflammatory response [19], failure of miRNA regulation is associated with several human inflammatory neurodegenerative disorders, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic...
lateral sclerosis [4]. miRNAs have also been implicated in inflammatory responses in metabolic syndromes [3]. This makes miRNAs, which have never been explored in X-ALD, attractive molecules for their ability to function as regulators of inflammatory cytokine gene expression in X-ALD.

To explore whether miRNA expression changes during the time course of increased iNOS expression in X-ALD lymphocytes, we selected the top 10 miRNAs reported to regulate human iNOS expression [16] (Supplementary Figure 1). Expression of 6 of these predicted miRNAs (miR-146b, miR-657, miR-766, miR-149, miR-323-5p and miR-637) was decreased in X-ALD patient-derived lymphocytes (Supplementary Figures 1A-1F) while 3 miRNAs (miR-487-1, miR-939 and miR-1226) were either increased or
remained unchanged in X-ALD lymphocytes compared to healthy control lymphocytes (Supplementary Figure 1G-1I). As a general rule, miRNA negatively regulate the mRNA expression, hence the iNOS-targeting miRNA that were decreased in X-ALD patient-derived lymphocytes were of interest to us. Interestingly, only miR-323-5p showed a consistent time-dependent decrease in expression (Figure 1C) that paralleled the time-dependent increase in iNOS expression (Figure 1B) in X-ALD patient-derived lymphocytes. Also, iNOS was predicted as a target of miR-323-5p by TargetScan and miRanda databases using miRWalk (Figure 1C). We, therefore, provide the first evidence of dysregulation of miRNA regulating the human iNOS gene expression in X-ALD.

miR-323-5p regulates iNOS expression in X-ALD lymphocytes

To investigate whether the expression of miR-323-5p was functionally involved in iNOS expression in X-ALD lymphocytes, we overexpressed or inhibited miR-323-5p expression by transiently transfecting the X-ALD lymphocytes with miR-323-5p mimics (MIM) or inhibitors (INB) or scrambled negative control miRNA (ALD-NGC) or TM alone (Figure 2). Mimic was used to promote miR-323-5p expression; the inhibitor has the opposite function. Transfected lymphocytes were maintained in serum free media for 72 h. miRNA mimics significantly increased the inhibitors significantly decreased the miR-323-5p expression in X-ALD lymphocytes (Supplementary Figure 2A). There was no significant change in miR-323-5p expression in lymphocytes transfected with ALD-NGC or TM alone (Supplementary Figure 2A). Transfection efficiency was evaluated by transfecting X-ALD lymphocytes with miR-1. miR-1 is reported to be expressed only in muscle cells [20] and hence can be used as a positive control to validate the transfection efficiency in non-muscle cells. Accordingly, we did not observe the expression of Syn-hsa-miR-1 in healthy control or X-ALD patient-derived lymphocytes (Supplementary Figure 3). Transfection with miR-1 mimic significantly increased (p<0.001) miR-1 expression at 72 h in X-ALD lymphocytes (Supplementary Figure 3). HDAC4 is the target gene of miR-1 [21]. Lymphocytes express HDAC4 protein [22] and western blot analysis showed a significant decrease in HDAC4 protein levels in X-ALD lymphocytes that had Syn-hsa-miR-1 overexpression (Supplementary Figure 3).

RT-PCR analysis showed a significant decrease (p<0.001) in iNOS mRNA expression in ALD patient-derived lymphocytes transfected with miR-323-5p mimics (Figure 2A). miR-323-5p inhibitor transfection significantly increased (p<0.01) iNOS expression compared with untransfected ALD lymphocytes at 72 h (Figure 2A). Transfection with negative control (ALD-NGC) or transfection reagent alone (ALD-TM) did not significantly change the iNOS expression in ALD patient-derived lymphocytes at 72 h (Figure 2A). Furthermore, western blot analysis showed that under the same conditions, the iNOS mRNA expression was paralleled by comparable changes in iNOS protein levels in X-ALD lymphocytes (Figure 2B). To validate the interaction between miR-323-5p and iNOS 3'-UTR, we performed a dual luciferase reporter assay. X-ALD lymphocytes were co-transfected with reporter constructs containing luciferase gene fused to iNOS 3'-UTR (iNOS-Luc) and miR-323-5p mimics or inhibitors. Luciferase activity was measured at 72 h post-transfection. The results showed that overexpressing miR-323-5p significantly decreased (p<0.001) the luciferase reporter activity (Figure 2C), whereas the luciferase reporter activity was significantly increased (p<0.01) in cells co-transfected with miR-323-5p inhibitors (Figure 2C) compared to control ALD lymphocytes transfected with iNOS-Luc alone. Cells transfected with empty vector without luciferase reporter (ALD-EV) had negligible reporter activity.

**Figure 2:** iNOS levels are modulated by miRNA gain or loss of function. iNOS mRNA (A) and protein levels (B) decreased in miR-323-5p mimic-transfection group and increased in miR-323-5p-inhibitor transfection group. (C) The iNOS 3'UTR-luciferase construct was cotransfected in X-ALD patient-derived lymphocytes with miR-323-5p mimic or inhibitor or negative control (NGC) miRNA. Relative renilla luciferase activity is expressed as mean ± SD. Data are represented as the mean ± SD of three different experiments. Luciferase activity in mimic (MIM) and inhibitor (INB) were compared to NGC-transfected cells. ALD-C was compared to healthy controls (CTL). *p<0.05, **p<0.01, ***p<0.001. CTL: healthy control lymphocytes; ALD: X-ALD patient-derived lymphocytes.

AMPKα1 deletion–induced iNOS expression in Abcd1-KO mixed glial cells is amenable to miR-323-5p expression

Abcd1-KO mice do not develop the central nervous system inflammatory response characteristic of human cerebral ALD phenotype [10-12]. We recently reported the novel differential loss of anti-inflammatory protein AMPKα1 in ALD patient-derived cells [8]. AMPK activation is coupled to negative regulation of proinflammatory signaling and AMPK knockout mouse models demonstrate spontaneous proinflammatory response [23-25]. More severe inflammatory response is reported in the AMPKα1-knockout mice induced with experimental autoimmune encephalomyelitis [26].

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Abcd1-KO mice glial cells additionally deleted for another peroxisomal transporter ALD-related protein (ALDRP or ABCD2) developed the proinflammatory response [14]. However, ABCD2 level is unchanged in ALD patients’ central nervous system [27] and is, therefore, unlikely to be the causal gene for the proinflammatory response in X-ALD patients’ central nervous systems in vivo. We recently reported that the deletion of AMPKα1 in Abcd1-KO mice mixed glial cells induced spontaneous proinflammatory (iNOS) levels [13]. TargetScan and miRanda algorithms predicted the miR-323-5p binding site on the mouse iNOS 3’-UTR (Figure 3). As reported by us recently, lentiviral vector-mediated AMPKα1 deletion in Abcd1-KO mice mixed glial cells (Figure 3A) significantly induced (p<0.001) iNOS mRNA expression (Figure 3B). To explore whether miR-323-5p is involved in regulating the iNOS levels in AMPKα1-deleted Abcd1-KO mice mixed glial cells, we analyzed the expression of miR-323-5p by quantitative PCR. The results showed that miR-323-5p expression was comparable between wild type and Abcd1-KO mice mixed glial cells (Figure 3C). Interestingly, the expression of miR-323-5p was also similar in vivo between the wild type and Abcd1-KO mice brain cortex (Supplementary Figure 4A). miR-323-5p expression was significantly decreased (p<0.001) in AMPKα1-deleted Abcd1-KO mice mixed glial cells (Figure 3C). To validate the role of miR-323-5p in AMPKα1-deletion induced iNOS expression in Abcd1-KO mice mixed glial cells, we transfected these cells with miR-323-5p mimic and inhibitor. miR-323-5p expression was significantly increased (p<0.001) or decreased (p<0.001) by transfection with miR-323-5p mimic or inhibitor respectively, compared with negative control (ALD-NGC) (Supplementary figure 4B). iNOS mRNA expression was significantly decreased (p<0.001) by miR-323-5p mimics and significantly increased (p<0.01) by miR-323-5p inhibitors (Figure 3D). Western blot analysis confirmed that the mRNA changes were translated into differences in iNOS protein levels (Figure 3E). miR-323-5p mimics significantly decreased iNOS protein levels, and miR-323-5p inhibitors further increased iNOS protein levels in AMPKα1-deleted Abcd1-KO mice mixed glial cells (Figure 3E).

Our results provide the first mechanistic insight into the development of the inflammatory response in X-ALD. Furthermore, we provide the first evidence of the role of miRNA in regulating this inflammatory response. Development of inflammation in the X-ALD central nervous system shortens the life span leading to early death. Novel identification of a mechanistic role for miR-323-5p in mediating/initiating the inflammatory response in ALD phenotype is of considerable interest and might help in the development of miRNA-targeting therapies for the severe form of X-ALD.

Figure 3: miR-323-5p mediates AMPKα1 deletion-induced iNOS expression in Abcd1-KO mice mixed glial cells. (A) Abcd1-KO mixed glial cells were silenced for scrambled control (Scr) or AMPKα1 as described in section 2. iNOS mRNA expression (B) was induced and miR-323-5p expression decreased (C) in Abcd1-KO mixed glial cells deleted for AMPKα1 using lentiviral particles. Abcd1-KO mixed glial cells deleted or AMPKα1 and co-transfected with miR-323-5p mimic (MIM) or inhibitors (INB) modulated iNOS mRNA (D) and protein (E) levels. Mimic and inhibitor transfected cells were compared with negative control (NGC) transfected cells. Results represent the mean ± SD of triplicates from three different experiments. *** p<0.001.
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