In vivo targeting of miR-223 in experimental eosinophilic oesophagitis

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Abstract

Objectives. Eosinophilic oesophagitis (EoE) is characterised by oesophageal inflammation, fibrosis and dysfunction. Micro (mi)-RNAs interfere with pro-inflammatory and pro-fibrotic transcriptional programs, and miR-223 was upregulated in oesophageal mucosal biopsy specimens from EoE patients. The therapeutic potential of modulating miR-223 expression has not been determined. We aimed to elucidate the relevance of oesophageal miR-223 expression in an in vivo model of EoE by inhibiting miR-223 tissue expression. Methods. The expression of miR-223 and the validated miR-223 target insulin-like growth factor receptor 1 (IGF1R) protein was determined in our paediatric cohort of EoE patients. A murine model of Aspergillus fumigatus-induced EoE was employed, and oesophagi were assessed for miR-223, IGF1R, T lymphocyte type 2 (T2) cytokine expression and eosinophil infiltration. Mice were treated with antagonirs targeting miR-223 or resveratrol targeting its upstream regulator Midline-1(MID-1). Results. There was an inverse relationship between an increased expression of miR-223 and a decreased IGF1R protein concentration in biopsy specimens from EoE patients. TNF-related apoptosis-inducing ligand deficiency, MID-1 inhibition and resveratrol treatment suppressed miR-223 expression. Furthermore, inhibition of miR-223 and treatment with resveratrol in the oesophagus resulted in an amelioration of EoE hallmark features including eosinophil infiltration, oesophageal circumference and a reduction in T2 cytokine expression. Conclusion. miR-223 has a key role in the perpetuation of EoE hallmark features downstream of TNF-related apoptosis-inducing ligand and MID-1 in an experimental model. These studies highlight a potentially critical role of miRNA function in EoE aetiology. miR-223 expression in the oesophagus may be therapeutically modulated by resveratrol,
providing a potential new therapeutic option to be explored in EoE patients for this increasingly prevalent condition.

**Keywords:** eosinophilic oesophagitis, microRNA, Midline-1, resveratrol

**INTRODUCTION**

Eosinophilic oesophagitis (EoE) is a chronic inflammatory disorder characterised by symptoms of oesophageal dysfunction and eosinophilic infiltration of the oesophagus, with rising incidence and prevalence. There is an increasing body of evidence that suggests that EoE is driven by food and aero-allergen exposure, resulting in the activation of interleukins (IL)-5, IL-13 and IL-33, subsequently promoting eosinophil migration into and activation in the oesophagus, ultimately leading to oesophageal remodelling. Current therapeutic options are limited to long-term use of proton pump inhibitors, swallowed corticosteroids, elimination diets or oesophageal balloon dilatation, which offer varying success and risks of adverse effects or negative impact on quality of life. There is a need for new, effective and safe therapies for EoE.

Analysis of the EoE transcriptome suggests that, in addition to the dysregulation of EoE associated genes, there could be a significant role for non-coding RNA influence in the pathogenesis of EoE in the form of long-coding RNAs and micro (mi-) RNAs. miRNAs belong to a class of interfering RNAs, typically 19–25 nucleotide bases in length, responsible for the manipulation of gene translation. Epigenetic modulation of such genes occurs through the formation of the RNA-induced silencing complex (RISC), which will bind to target mRNAs resulting in degradation of the target mRNA sequence. Experimentally, specific miRNAs can be inhibited with cholesterol-conjugated complimentary sequences known as ‘antagomirs’, which have been used to highlight key miRNA-mRNA interactions in disorders such as allergic asthma. miRNA array analysis of EoE has demonstrated a profile of miRNAs that could be in the perpetuation of EoE-associated inflammation and remodelling. A miRNA signature was observed in EoE patients consisting of an upregulation of miR-21, miR-223, miR-206 and let-7, as well as a downregulation of miR-375. While such studies provided key insights on the epigenetic miRNA signature of EoE patients, the effect of miRNA modulation on the EoE phenotype has not been thoroughly investigated.

Our previous study focusing on TNF-related apoptosis-inducing ligand (TRAIL) signalling in EoE employed silencing (si)-RNA technology to downregulate the expression of Midline (MID)-1 in the oesophagi of Aspergillus fumigatus (A. fumigatus)-challenged mice. Here, we demonstrate a functional effect of miR-223 in an A. fumigatus model of EoE in regard to eosinophil inflammation, remodelling and T2 cytokine expression. Recently, the stilbenoid resveratrol has identified as a direct small molecule inhibitor of MID-1. Here for the first time – we exploit this novel property to inhibit MID-1 protein in vivo upstream of miR-223 in the oesophagus to ameliorate EoE hallmark features.

**RESULTS**

**miR-223 is upregulated in EoE patient tissue and inversely correlated with IGF1R**

RNA extracted from oesophageal biopsies, from children histologically confirmed to have active EoE and subjects that were controls, was used to determine the expression of miR-223 in our paediatric EoE cohort through the use of TaqMan™ RT and qPCR primers. Supporting published findings, miR-223 was found to be significantly upregulated in EoE-active patients when compared to controls (Figure 1a).

Additionally, we explored the expression of the previously confirmed miR-223 target insulin-like growth factor receptor 1 (IGF1R) in patient biopsy samples. We found reduced levels of IGF1R when compared to controls (Figure 1b) and the expression of miR-223 inversely correlated with IGF1R in confirmed active EoE cases (Figure 1c).

**Upregulated miR-223 in A. fumigatus-exposed murine oesophagi is associated with IGF1R, TRAIL and MID-1**

We continued to explore the role of miR-223 by employing an in vivo model of A. fumigatus-driven...
EoE. Mice exposed to *A. fumigatus* demonstrated a significant increase in miR-223 expression, which was successfully knocked down to baseline levels upon intranasal administrations of antagomir targeting miR-223 compared to mice that received a scrambled control (SCR) antagomir (Figure 2a). Following miR-223 knockdown with antagomir treatment, IGF1R expression was increased compared to SCR control-treated mice (Figure 2b). As in the patient samples, there was a negative correlation between miR-223 and IGF1R in *A. fumigatus*-sensitised and challenged mice (Figure 2c).

TRAIL-deficient mice have previously been shown to be largely protected from the EoE phenotype.⁷ Gene expression analysis of WT and TRAIL⁻/⁻ mice exposed to *A. fumigatus* revealed that TRAIL deficiency was associated with reduced levels of miR-223 expression in the oesophagus (Figure 2d). Likewise, mice intranasally treated with an siRNA targeting MID-1 – that has a pro-inflammatory signalling role downstream of TRAIL⁷,¹⁹ – also reduced the expression of miR-223 in the oesophagus of *A. fumigatus*-treated mice (Figure 2e).

**Eosinophilia, remodelling and T2 cytokine expression are dependent on miR-223 expression in *A. fumigatus*-driven EoE**

Next, the impact miR-223 on eosinophilic inflammation of the oesophagus was assessed...
miR-223 expression is upregulated in *Aspergillus fumigatus* (Asp F)-exposed murine oesophagi, is associated with IGF1R downregulation and is downstream of TNF-related apoptosis-inducing ligand (TRAIL) and MID-1. Relative expression of oesophageal miR-223 in vivo in (a) wild-type BALB/c mice treated with scrambled (SCR) antagomir or antagomir targeting miR-223 (A.223). (b) Expression of IGF1R in BALB/c oesophagi exposed to *A. fumigatus* and treated with SCR or A.223. (c) Spearman’s correlation of oesophageal miR-223 and IGF1R in BALB/c mice. (d, e) miR-223 expression of (d) *A. fumigatus*-exposed wild-type BALB/c (WT) and TRAIL-deficient *+/C0/C0* mice on a BALB/c background and (e) wild-type BALB/c mice treated with *A. fumigatus* and administered either nonsense scrambled control sequence (NONc) siRNA or siRNA targeting Midline-1 (MID-1). Expression is normalised to Sno202 and saline (SAL) control group for miR-223, and β-actin and SAL control group for IGF1R (n = 5–7). Data are from a single technical replicate experiment, expressed as mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.
through the enumeration of eosinophil infiltrates in transverse, H&E-stained oesophageal sections under light microscopy (Figure 3a). As expected, A. fumigatus-exposed mice given scrambled control antagonir had an elevated number of eosinophils within the squamous-epithelial region of the oesophagus. In mice that were receiving antagonir-223 therapy, however, there was a marked reduction of eosinophil infiltration when compared to the scrambled control group (Figure 3a–d).

We then assessed the impact of miR-223 inhibition on key remodelling features seen in murine EoE, subepithelial fibrosis and oesophageal enlargement. Performing image analysis, we found that miR-223 antagonir treatment reduced A. fumigatus-induced subepithelial fibrosis (Figure 3e–h) and enlargement of the oesophageal circumference when compared to the appropriate controls (Figure 3i).

To further understand the pro-inflammatory role of miR-223 in A. fumigatus-driven EoE, the eosinophil-associated T2 cytokines (IL-5 and IL-13) were assessed in protein-extracted oesophageal homogenates through the use of ELISA. Here, we demonstrate that mice with diminished miR-223 expression through antagonir-223 had a reduction of IL-5 and IL-13 protein expression despite A. fumigatus exposure (Figure 3j and k).

Administration of resveratrol in A. fumigatus-driven EoE results in both miR-223 downregulation in addition to EoE inflammatory features

Oral administration of the stilbenoid resveratrol (RES) throughout the model effectively reduced the expression of MID1 in the oesophagus from 24hrs following A. fumigatus administration (Figure 4a). Additionally, we determined that resveratrol treatment effectively reduced the expression of oesophageal miR-223 (Figure 4b), similar to what occurs in MID-1 siRNA-treated mice. We further explored the ability of resveratrol to modulate EoE through MID-1/miR-223 by exploring disease features previously investigated. Here, we demonstrate that resveratrol reduces eosinophilic inflammation (Figure 4c), oesophageal enlargement (Figure 4d) and oesophageal expression of IL-5 (Figure 4e) and IL-13 (Figure 4f), similar to what occurs in antagonir-223-treated mice.

DISCUSSION

It has been established that dysregulation of miRNA occurs in the oesophagus of EoE patients; however, until now there have not been any studies that have investigated the function of specific miRNAs and the consequences of inhibiting miRNAs of interest either directly such as with antagonirs, or through blocking of upstream pathways thought to be associated with miRNA expression. miR-223 is one of the most upregulated miRNAs found in EoE patient biopsies, which has been demonstrated in multiple previous studies in addition to our cohort of EoE paediatric patients. Here, we further explore the role of this miRNA through in vivo studies, where we demonstrate for the first time that antagonir targeting therapy in the oesophagus can effectively downregulate the expression of miR-223 and reduce hallmark features associated with EoE. This study is the first of its kind to highlight the therapeutic effectiveness of silencing miRNA and their upstream signalling pathways in the oesophagus in mouse models of EoE.

Inhibition of miR-223 resulted in a reduction of the T2 cytokines IL-5 and IL-13. Both cytokines have been shown to play an essential role in the perpetuation of EoE, with IL-5 being involved in eosinophil maturation and recruitment to the oesophagus while IL-13 elicits local allergenic effects and the activation of the STAT6 pathway.

Hallmark histological EoE features of inflammation and remodelling were measured in antagonir- and resveratrol-treated mice challenged with A. fumigatus. It was found that miR-223 promoted eosinophilic infiltration in the oesophagus and increased oesophageal circumference and subepithelial fibrosis. While the clinical relevance of the observed reduction in eosinophilic inflammation remains elusive, these results provide evidence for the proposal that miR-223 inhibition or associated pathways may be a potential therapeutic option for EoE. Given the regulatory effect of miR-223 on IL-5 and IL-13, we can infer that miR-223 promotes inflammation through T2 cytokines. The effect of miRNAs on T2 cytokines in EoE has been previously documented in paediatric EoE cohorts where it was found that miR-146a and miR-146b were upregulated in patient serum: both miR-146a and miR-146b were found to have a
potential role in the promotion of T2 responses through the selective downregulation of T1-associated genes.\textsuperscript{10} As miR-223 was found to be upregulated both in serum and oesophageal biopsies,\textsuperscript{10} it is plausible that miR-223 is manipulating the T helper cell phenotype by exploiting similar mechanisms. Although it should be noted that the upregulation of miR-223 in oesophageal biopsies has been shown consistently, serum miR-223 has not always been found to correlate with this.\textsuperscript{18} Future studies will need to elucidate this further.

The transmembrane receptor IGF1R has previously been identified \textit{in vitro} as a target of miR-223 and confirmed using luciferase assays by multiple research groups.\textsuperscript{22–24} Lu \textit{et al.}\textsuperscript{25} previously identified that IGF1R was upregulated in cultured eosinophil progenitor cells from miR-223\textsuperscript{−/−} mice.

Figure 3. Inhibition of miR-223 in the oesophagus results in reduced eosinophilic infiltration of the oesophagus and oesophageal enlargement with antagomir-223. (a) Enumeration of eosinophils present in the oesophageal squamous-epithelial cell layer as determined by light microscopy analysis of H&E-stained BALB/c oesophageal transverse sections (n = 4 or 5). (b–d) Representative images of (b) SAL, (c) AspF/SCR or (d) AspF/A.223 with eosinophils circled in yellow. (e) Oesophageal fibrosis analysis with representative images of (f) SAL, (g) AspF/Scr and (h) AspF/A223-treated BALB/c mice. (i) Oesophageal circumference analysis of oesophageal tissue determined using image analysis (n = 5–8). (j, k) Protein expression of (j) IL-5 and (k) IL-13 determined by ELISA and normalised to tissue BSA (n = 5–8). Data are from a single technical replicate experiment, expressed as mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.001.
It remains untested, but is highly unlikely, that miR-223 effects on IGF1R can account entirely for the amelioration of all EoE hallmark features observed after miR-223 inhibition. The lower magnitude of increased miR-223 expression in the EoE mouse model versus in EoE patients (1.5–2.0 fold, \( P = 0.03 \) versus 10 fold, \( P < 0.001 \); Figures 1a and 2a) has resulted in a lower magnitude of change in IGF1R expression in the Aspergillus fumigatus EoE mouse model (Figure 2b) versus in EoE.

**Figure 4.** Resveratrol reduces MID-1 and miR-223 expression while reducing eosinophilic oesophagitis (EoE) features. (a) The E3 ubiquitin ligase Midline-1 (MID-1) is upregulated in Aspergillus fumigatus (Asp F)-exposed C57Bl/6 mice and inhibited by oral resveratrol (RES; \( n = 3–6 \)). (b) Resveratrol treatment decreased miR-223 expression \(( n = 5 \) or 6). (c) Enumeration of eosinophils present in the oesophageal squamous-epithelial cell layer \(( \text{per mm}^2)\) as determined by light microscopy analysis of H&E-stained C57Bl/6 oesophageal transverse sections \(( n = 6 \) or 7). (d) Oesophageal circumference analysis of oesophageal tissue determined using image analysis \(( n = 6 \) or 7) in mice treated with RES. (e, f) Protein expression of (e) IL-5 and (f) IL-13 determined by ELISA and normalised to tissue BSA \(( n = 5 \) or 6). Data are from a single technical replicate experiment, expressed as mean ± SEM. *\( P < 0.05 \), **\( P < 0.01 \).
patients (Figure 1b). However, we detected a comparable inverse correlation between miR-223 and IGF1R expression in A. fumigatus-treated mice \((r = -0.67, P = 0.039, \text{Figure 2c})\) versus EoE patients \((r = -0.65, P = 0.0059, \text{Figure 1c})\).

Causally linking the effects of a specific miRNA on their \textit{in vitro} validated mRNA targets to aspects of disease pathogenesis \textit{in vivo} is challenging. In addition, targeting miR-223 in a tissue-specific manner in patients with EoE will require significant efforts and ultimately may be unsuccessful. In contrast, resveratrol has a good safety profile, is readily available, and taken orally may have sufficient local uptake and bioavailability to suppress disease hallmark features in the oesophagus of EoE patients. This is the first demonstration \textit{in vivo} that resveratrol can inhibit MID-1 in the oesophagus and extends previous \textit{in vitro} studies that identified resveratrol as a direct small molecule inhibitor of MID-1.\textsuperscript{17} Here, we show for the first time the ability of resveratrol to also regulate downstream miRNA-223 signalling which is likely to be via MID-1 inhibition. Resveratrol has also been shown to reduce calpain activity in models of allergic asthma.\textsuperscript{26} Interestingly, calpain 14 is an oesophagus-specific calpain identified as upregulated in EoE GWAS\textsuperscript{27} and more recently has been shown to play a key role in STAT6-mediated inflammatory IL-13 signalling in EoE.\textsuperscript{28} Calpains do not contribute to murine EoE models as shown in mice lack calpain 14.\textsuperscript{29} Thus, the effect of resveratrol reported here is likely to occur independent of modulating calpain activity. Resveratrol’s inhibitory action upon calpain activity is complemented by our data showing additional calpain-independent mechanisms, at a moderate dose range with oral delivery which provide a strong rationale for human trials of resveratrol for the treatment of EoE.

Overall, we report a functional role for miR-223 in the perpetuation of hallmark EoE features including T2 cytokines and confirm that miR-223 is a downstream effector of inflammatory MID-1 signalling in this setting. Resveratrol holds promise to be trialled as a potential future therapeutic intervention in EoE, an increasingly prevalent condition with limited current treatment options.

**METHODS**

**Patient cohorts and analysis**

Patient cohorts and methods for RNA and protein extraction and analysis have been described previously.\textsuperscript{30} In brief, biopsies collected from the distal third of the oesophagus in paediatric patients with active EoE \((n = 17)\) and non-EoE control children \((n = 21)\) underwent either RNA or protein extraction. Extracted RNA was utilised in this study for TaqMan\textsuperscript{TM} analysis to determine miR-223 expression. Written informed consent was obtained from all parents/guardians and assent from children \((\text{where age-appropriate})\) prior to collection of additional biopsy samples for research purposes during an oesophago-gastroscopy that was clinically indicated for diagnostic purposes by an appropriately qualified paediatric gastroenterologist. The study was approved by the Hunter New England Local Health District \((12/04/18/4.04)\) and The University of Newcastle Human Research Ethics Committees.

**Aspergillus fumigatus model of EoE**

Wild-type \((\text{WT})\) and TRAIL-deficient \((\text{TRAIL}^{-/-})\) BALB/c and WT C57BL/6 mice \((\text{male, 8–12 weeks of age})\) were obtained from Australian BioResources \((\text{Moss Vale, NSW, Australia})\) under a material transfer agreement with Amgen \((\text{Macquarie Park, NSW, Australia})\). All experiments were approved by the Animal Care and Ethics Committee of the University of Newcastle.

Mice were intranasally challenged with 100 \(\mu\)g of \(\text{A. fumigatus}\) extract \((\text{Greer Laboratories, Lenoir, NC, USA})\) in 50 \(\mu\)L of sterile saline three times a week for three weeks after administration of isoflurane anaesthetic. Control mice received 50 \(\mu\)L of saline only. Oesophageal samples were collected 24 h after the first or final \(\text{A. fumigatus}\) challenge.

**Antagomir knockdown of miR-223**

Antagomirs targeting miR-223 and scrambled control antagomirs designed not to target any known miRNA sequence were purchased from Dharmacon \((\text{Millennium Science, Mulgrave, VIC, Australia})\). Mice were administered 50 \(\mu\)g of antagomirs intranasally in 50 \(\mu\)L of sterile saline, 24 h prior to the initial \(\text{A. fumigatus}\) challenged. This dose was repeated daily throughout the course of the model.

**Resveratrol treatment**

Resveratrol was administered orally to anaesthetised mice at a dose of 25 mg kg\(^{-1}\) suspended in 50 \(\mu\)L with a thickening agent in sterile saline while vehicle controls received thickening agent alone in sterile saline. Drinking water was withheld for 60 min post-administration. Mice received the initial dose 24 h prior to the initial \(\text{A. fumigatus}\) challenge. This dose was selected based on previous reports of topical application to the lungs in models of allergic airways disease\textsuperscript{31,32} and was repeated daily throughout the course of the model.

**RNA extraction and PCR**

Collected oesophageal tissue from mice was immersed in RNAlater\textsuperscript{®} \((\text{Ambion, Life Technologies, Mulgrave, VIC, Australia})\).
Australia) prior to being frozen at −80°C. RNA was extracted using TRIzol® (Invitrogen, Life Technologies, Mulgrave, VIC, Australia) as per the manufacturer’s instructions. miR-223 expression was quantified from RNA extracted from mouse and human tissue using TaqMan™ primers and chemistry (Life Technologies, Mulgrave, Australia). miR-223 expression was normalised to U6 in human samples and Sno202 in mouse samples. qPCR was performed using the Eppendorf Realplex PCR system (Hamburg, Germany).

Protein extraction

Snap-frozen oesophageal samples underwent protein extraction and were quantified as previously described. Briefly, samples were weighed prior to being homogenised (Tissue-Tearor, BioSpec Products, Bartlesville, OK, USA) and protein levels for IL-5, IL-13, MID-1 or IgF1R were determined by ELISA (R&D systems, Minneapolis, MN, USA). Protein extraction was performed using the Eppendorf Realplex PCR system (Hamburg, Germany).

Histological analysis of mouse oesophagi

Mid-sections of oesophageal tissue were fixed in 10% formalin for 24 h before routine processing to paraffin wax, sectioning at 5 μm and underwent H&E staining to enumerate eosinophils. Eosinophil infiltration into the oesophagus was determined by blinded personnel counting the number of eosinophils within 1 mm² of transverse oesophageal section stained with Masson trichrome. The degree of oesophageal fibrosis was determined as the area per micrometer (in μm² per μm) by using Image-Pro Plus 6 software (Media Cybernetics, Rockville, MD, USA).

Oesophageal circumference analysis

Oesophageal sections were incised longitudinally and flattened on sections of blotting paper. Photographs were taken of the excised oesophagus, and oesophageal circumference was measured using Image-Pro Plus 6 software (Media Cybernetics) as previously described.

Statistical analysis

Statistical significance was determined between experimental groups using ANOVA with Dunnett’s correction for multiple testing (Mann-Whitney and Spearman’s correlation for human data) in GraphPad Prism 6 (La Jolla, CA, USA). Data are presented as mean ± SEM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

AMC and JM conceived and designed research. AMC and LAS performed experiments. SN coordinated collection of clinical samples. AMC, LAS, and JM analysed data. AMC, LAS, SN, EP ALF, JM, SK, PSF, and JM interpreted results of experiments. AMC and LAS prepared figures and drafted manuscript. All authors edited and revised manuscript and approved final version.

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