

Original Article

Differential microRNA expression in asthma and the role of miR-1248 in regulation of IL-5

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Abstract: Asthma is a chronic inflammatory disease that can be difficult to manage due to a lack of diagnostic biomarkers and an incomplete understanding of the molecular pathogenesis. MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs with increasing importance in regulation of immune function and as biomarkers. We profiled miRNAs in the serum of asthmatics and non-asthmatic controls to identify miRNAs that could serve as diagnostic markers and potential regulators of allergic inflammation. Differential expression of miR-1248, miR-26a, Let-7a, and Let-7d were observed in asthmatic patients compared to controls. Predictive algorithm analyses of these miRNAs revealed their specificity for different Th2 cytokines, including IL-5, which has not previously been shown to be post-transcriptionally regulated. Using multiple approaches, we showed that miR-1248 physically interacts with the IL-5 transcript in the 3' untranslated region and serves as a positive regulator to increase IL-5 expression. Collectively, our results demonstrate a previously uncharacterized mode of regulation of IL-5 expression and potential use for miRNAs in the diagnosis and clinical management of asthma.

Keywords: Asthma, microRNA, cytokines, biomarkers, interleukin-5

Introduction

Allergic asthma is a highly prevalent disease characterized by airway obstruction and bronchial hyper-responsiveness following various stimuli [1]. A complex interplay of the respiratory epithelium, innate immune system, and adaptive immunity produces these characteristics [2]. However, the molecular pathogenesis of asthma is not well defined, and regulatory mechanisms that govern the inflammatory processes are still unknown. In addition, diagnosis of asthma can be challenging, which is due in large part to a lack of objective and non-invasive biomarkers. Our overall goal is to identify biomarkers that may be involved in asthma pathogenesis and can be used in asthma diagnosis.

Central to the pathogenesis of allergic asthma is the Th2-mediated inflammatory response. Th2-predominant T-lymphocytes modulate

allergic disease by secreting a host of pro-inflammatory cytokines. Canonical cytokines involved in the Th2 response are IL-4, IL-5, and IL-13. Physiologic effects of these interleukins result in the majority of the immunologic and histopathologic features of asthma. IL-4 and IL-13 promote class-switching in B-cells thereby upregulating their IgE production, while IL-5 is a strong signaling factor for eosinophil survival [2]. The latter cytokine is emerging as a key player in allergic disease as a target in difficult to control asthma [3, 4] and in eosinophilic diseases such as eosinophilic esophagitis [5]. The factors that regulate the "turning off" of mediators such as IL-5 are poorly characterized.

Regulation of cytokines post-transcriptionally, by the action of microRNAs (miRNAs) and RNA-binding proteins (RBPs) on stability or translation of transcripts, is emerging as a crucial means of regulating the inflammatory response. MiRNAs are short, single-stranded, non-coding

RNA molecules that direct RBPs to multiple mRNA targets via partial complementarity. MiRNAs associate with members of the Argonaute family of proteins (such as Ago2) and form the central component of RNA-induced silencing complex (RISC) [6]. Binding of RISC to their target mRNA transcripts, usually in the 3'untranslated region (3'UTR), leads to mRNA instability or translational repression [6]. In some cases, miRNA-Argonaute complex, possibly in association with a distinct set of regulatory proteins, can actually enhance gene expression [7, 8]. Thus miRNAs may play diverse roles in the regulation of inflammatory mediators.

In addition, miRNAs have been shown to play a significant role in diverse disease processes [9-11]. They have been found to be dysregulated in a number of diseases, either as a consequence of the disease process, or even as a pathogenic factor in disease progression [9-11]. As over 2000 miRNAs have been identified, differential regulation patterns may serve as a molecular fingerprint to diagnose disease. Indeed, multiple studies have also demonstrated their utility as diagnostic or prognostic biomarkers [12, 13]. Given their presence in bodily fluids such as serum and saliva, they carry tremendous diagnostic potential as a non-invasive biomarker [14-16].

In this study, we hypothesized that circulating miRNAs are differentially regulated in asthmatics compared to non-asthmatic controls. We found that several miRNAs are differentially expressed in serum of asthmatic subjects, and these are predicted to regulate Th2 mediators such as IL-5. We confirmed that IL-5 is regulated by miRNA, and identified miR-1248 as a positive regulator of its expression. The implications of these findings are described herein.

Methods

Patient selection

The study was approved by the institutional review board. All participants provided written informed consent. Patients were classified as asthmatic based on history and lung function, including forced expiratory volume in one second (FEV1) reversible by >12% and >200 ml post-bronchodilator, or airway hyper-responsiveness by methacholine (provocating concen-

tration producing a 20% fall in FEV1 of less than 8 mg/ml). Patients were considered allergic if they had a history of aeroallergen sensitivity and at least one positive skin test to a standard panel of 40 relevant aeroallergens, and non-allergic if the skin test panel was negative.

Isolation and characterization of miRNAs

Blood was isolated by venipuncture in a red top tube, left at room temperature for 15-30 minutes, and then centrifuged at 3000 RPM in a clinical centrifuge to isolate serum. For isolation of total RNA, 500 µl of serum was mixed with 5 µl of DNase (Promega) for 30 min at 37°C, then 2 µl of 50 nM Cel-miR-39 (synthesized by Integrated DNA Technologies) was added as a "spike-in" normalization control [17]. Subsequently, 1.5 ml of TRIzol reagent (Life Technologies) was added, total RNA was extracted per the manufacturer's protocol, and RNA concentration was measured by A280/260 using a NanoDrop Lite (Thermo Scientific).

For analysis by quantitative real time PCR (qPCR), 1 µg of RNA was reverse transcribed to cDNA using the miScript Reverse Transcription Kit (Qiagen) to add a universal adapter to the 3' end of miRNAs. Quantitation of miRNAs by qPCR was performed on a MyIQ2 real time thermocycler (Bio-Rad) by using specific primers to miRNAs of interest (250 nM), miScript universal 3' primer (250 nM), 1 µl of cDNA (diluted 1:10), and the IQ SYBR Green Supermix (Bio-Rad,) in a total volume of 10 µl. In addition to unknown samples, varying concentrations of synthetic Cel-miR-39 were run to generate a standard curve for absolute quantitation of samples in copy number/ml. A 2-step program was used, 95°C for 10 sec, 60°C for 30 sec, with 40 cycles. Sample cycle threshold (Ct) values were normalized to Cel-miR-39 expression to control for variability in RNA isolation and reverse transcription.

miRNA binding assays

The co-immunoprecipitation of mRNA with Ago2 was performed as previously described [18, 19]. In short, Jurkat T-cells (10×10^6 cells) were lysed in polysomal lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes pH 7.0, 0.5% NP-40) for 8 min on ice, and then added to protein A sepharose beads (Sigma-Alrich) pre-incubated with 10 µg of either an anti-Ago2 anti-

Table 1. Patient demographics

	Normal n=10	Asthma n=10
Male/female	6/4	4/6
Age (y), mean (range)	38.4 (23-65)	49.2 (22-64)
Allergic	3/10	8/10
FEV1 % predicted, mean (SD)	91.3 (15.7)	80.5 (24.1)
FEV1 (L), mean (SD)	3.41 (1.16)	2.56 (1.10)
Smoker (current, ex)	3,1	4,1
ICS ¹	0	0
ICS/LABA ²	0	5
Anti-leukotriene	0	3
Anti-cholinergic	0	2
Serum RNA concentration (µg/ml), mean (SD)	122.9 (43.3)	96.5 (35.9)

¹ICS, inhaled corticosteroids, ²LABA, long acting beta agonist.

body (Millipore) or an IgG isotype control antibody (Santa Cruz Biotechnology). After 60 min, beads were washed thrice with NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl₂, 1 mM MgCl₂, and 0.05% Nonidet P-40). To confirm successful immunoprecipitation (IP), 10 µl of beads were removed, incubated with SDS-PAGE loading buffer, and analyzed by Western blot to confirm the presence of Ago2. The rest of the beads were subjected to DNase treatment, followed by phenol-chloroform extraction of RNA. Following reverse transcription with the High-Capacity Reverse Transcription Kit (Applied Biosystems), samples were analyzed by qPCR.

Isolation of miRNA bound specifically to IL-5 RNA was performed using a biotin pulldown procedure as previously described [18, 19] with some modifications. Regions of DNA corresponding to the IL-5 3'UTR and CCL2 3'UTR (negative control) were PCR-amplified from total cDNA isolated from primary blood mononuclear cells (PBMCs) using the following primers: IL-5 3'UTR forward: 5'-CAA AGC TTC TAA TAC GAC TCA CTA TAG GGA GAC TAA ACT GGT TTG TTG CAG CC-3'; IL-5 3'UTR reverse: 5'-TCT CCA GAG AAA TGG GGA TG-3'; CCL2 3'UTR forward: 5'-CCA AGC TTC TAA TAC GAC TCA CTA TAG GGA GAA CAC TCA CTC CAC AAC CC-3'; CCL2 3'UTR reverse: 5'-TGT ACA AAA ATA TAT TTA TTT GGT GTA ATA GTT AC-3'. The products, which contain a T7 RNA polymerase initiation site, were reverse transcribed with the MAXIscript T7 kit (Ambion) in the presence of a 4:1 ratio of CTP to biotin-11-CTP (Roche Applied Sciences). The biotinylated RNA was incubated with fresh lysates of 10x10⁶ Jurkat cells, and then purified using streptavidin dynabeads (Life Technologies). The pulldown of Ago2 was

confirmed by Western blot. For miRNA isolation, a phenol-chloroform extraction was performed on the beads, and miRNAs were reverse transcribed and analyzed by qPCR as described above.

Reporter and PBMC assays

To clone IL-5 3'UTR into the pmirGLO vector (Promega), the IL-5 3'UTR was amplified from total cDNA from PBMCs using the following primers: IL-5 3'UTR Dral forward: 5'-AAA TTT AAA AGA CTA AAC TGG TTT GTT GCA GC-3'; IL-5 3'UTR Sall reverse: 5'-AAA GTC GAC GAA CAG TTG TCT ATT TTT GTT TTA TTA GA-3'. The vector and PCR product were digested with Dral and Sall (both from New England Biolabs), gel purified, and ligated with Quick T4 DNA Ligase (New England Biolabs). Following transformation into NEB-5-α cells, samples were plated onto an ampicillin-containing agar plate, and colonies were selected and grown in 5 ml of Luria-Bertani broth. Presence of the correct product was confirmed by Sanger sequencing.

HEK293 cells were used for reporter assays. Cells were grown in DMEM containing 10% fetal bovine serum, 5% penicillin, and 5% streptomycin. The cells were seeded on 6-well tissue culture dishes and incubated at 37°C overnight. Co-transfections with the reporter plasmid and either the miRNA mimic or inhibitor were performed using the Attractene transfection reagent (Qiagen) as per manufacturer's recommendations. For the reporter expression, HEK293 cells were transfected with either 50 ng of pmirGLO containing the IL-5 3'UTR (pmirGLO-IL5-3UTR) or 50 ng of pmirGLO empty vector (pmirGLO). For the miRNA precursor, 150 ng of miR1248 precursor plasmid (GeneCopia) or

Table 2. List of miRNA primers used for qPCR

miRNA Name	Primer Sequence (5'-3')
Let-7a	TGAGGTAGTAGGTTGTATAGTT
Let-7d	AGAGGTAGTAGGTTGCATAGTT
miR-26a	TTCAAGTAATCCAGGATAGGCT
miR-21	TAGCTTATCAGACTGATGTTGA
miR-1248	ACCTTCTTGATAAGCACTGTGCTAAA
miR-328	CTGGCCCTCTCTGCCCTTCCGT
miR-133a	TTTGGTCCCTTCAACCAGCTG
miR-126	TCGTACCGTGAGTAATAATGCG
miR-98	TGAGGTAGTAAGTTGTATTGTT
miR-146a	TGAGAACTGAATTCCATGGGTT
Cel-miR-39	TCACCGGGTGTAATCAGCTTG

its corresponding control vector was used. Alternatively, cells were transfected with 150 ng miR1248 inhibitor plasmid (GeneCopias) or its corresponding control plasmid. The reporter assay was carried out using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

In order to determine whether miRNA-1248 had an effect on endogenous IL-5 levels, PBMCs were isolated from healthy donors by Ficoll isolation. The PBMCs (2×10^6 cells) were transfected with 20 nM miR-1248 mimic or negative miRNA control (both from Qiagen) by nucleofection using the Amaxa system (Lonza) per manufacturer recommendations. Cells were then stimulated with phytohaemagglutinin (1 μ g/ml), cultured for 12 h in AIM-V media, and RNA was isolated by TRIzol.

Results

Patient characteristics

To determine whether miRNAs are differentially expressed in asthma, we isolated serum from 10 asthmatics and 10 control subjects (**Table 1**) for profiling. Patients were selected from our Allergy and Immunology clinic using the criteria defined in the methods section to classify into each group. The asthmatic group was composed of mostly allergic subjects (8/10) as defined by one or more positive skin tests to a panel of 40 aeroallergens. In contrast, the control group contained 3 allergic subjects. Lung function was higher in the healthy group, with a significantly higher percent predicted FEV1 (FEV1%, 91.3 vs. 80.5, $p < 0.05$) and absolute FEV1 (3.41 L vs. 2.56 L, $p < 0.05$). There were similar amounts of smokers in each group. Subjects in the asthma group were classified

as either mild persistent or moderate persistent. In the asthma group, no patients were on an inhaled corticosteroid (ICS) only. Five patients were on a combined ICS and long acting beta agonist (LABA), and three of these were also using an anti-leukotriene (montelukast). Two of the latter patients were also using a long-acting anti-cholinergic inhaled agent (tiotropium bromide). The concentration of total RNA isolated from the serum of each group was not significantly different.

MicroRNA profiling

A qPCR approach was utilized to measure expression of ten miRNAs (Let7a, Let7d, miR-21, miR-133a, miR-1248, miR-26a, miR-328, miR-126, miR-146a, miR-98) in the serum of each group (primers used are listed in **Table 2**). These miRNAs were selected based on either published studies that demonstrated a role in the inflammatory response [20-28] or prediction algorithms that suggested that these miRNAs were involved in the regulation of cytokines. We observed differential expression patterns in four miRNAs: miR-1248, miR-26a, Let7a, and Let7d (**Figure 1**). Expression of miR-1248 was increased in asthmatics (mean \pm SEM: $1.3 \times 10^5 \pm 3.5 \times 10^4$ copies/ml) relative to control ($3.9 \times 10^4 \pm 9.3 \times 10^3$ copies/ml). However, all of the other miRNAs were down-regulated in the asthma group vs. controls as follows: Let7a, $2.4 \times 10^4 \pm 6.1 \times 10^3$ copies/ml vs. $1.7 \times 10^5 \pm 3.9 \times 10^4$ copies/ml; Let7d, $1.9 \times 10^4 \pm 5.4 \times 10^3$ copies/ml vs. $6.7 \times 10^4 \pm 1.9 \times 10^4$ copies/ml; miR-26a, $3.8 \times 10^4 \pm 6.2 \times 10^3$ copies/ml vs. $1.3 \times 10^5 \pm 3.2 \times 10^4$ copies/ml. For miR-328, a trend towards lower expression was observed in the asthma group, but this did not reach significance ($p = 0.1$). MiRNA-21 is shown as a representative example of a miRNA without any difference between groups.

We next sought to determine whether miRNA expression level correlates with lung function. No association was observed for miR-1248 expression and FEV1% or Let7d and FEV1% in either the asthma or control groups (**Figure 2A, 2B**). However, there was a significant negative association observed between miR-26a expression and FEV1% in the asthma group (Pearson Correlation, $R = -0.59$, $p = 0.036$) but not in the control group ($R = -0.075$, $p = 0.42$) (**Figure 2C**). There was also a trend in let7a expression towards a negative correlation in the asthma

MicroRNAs in asthma

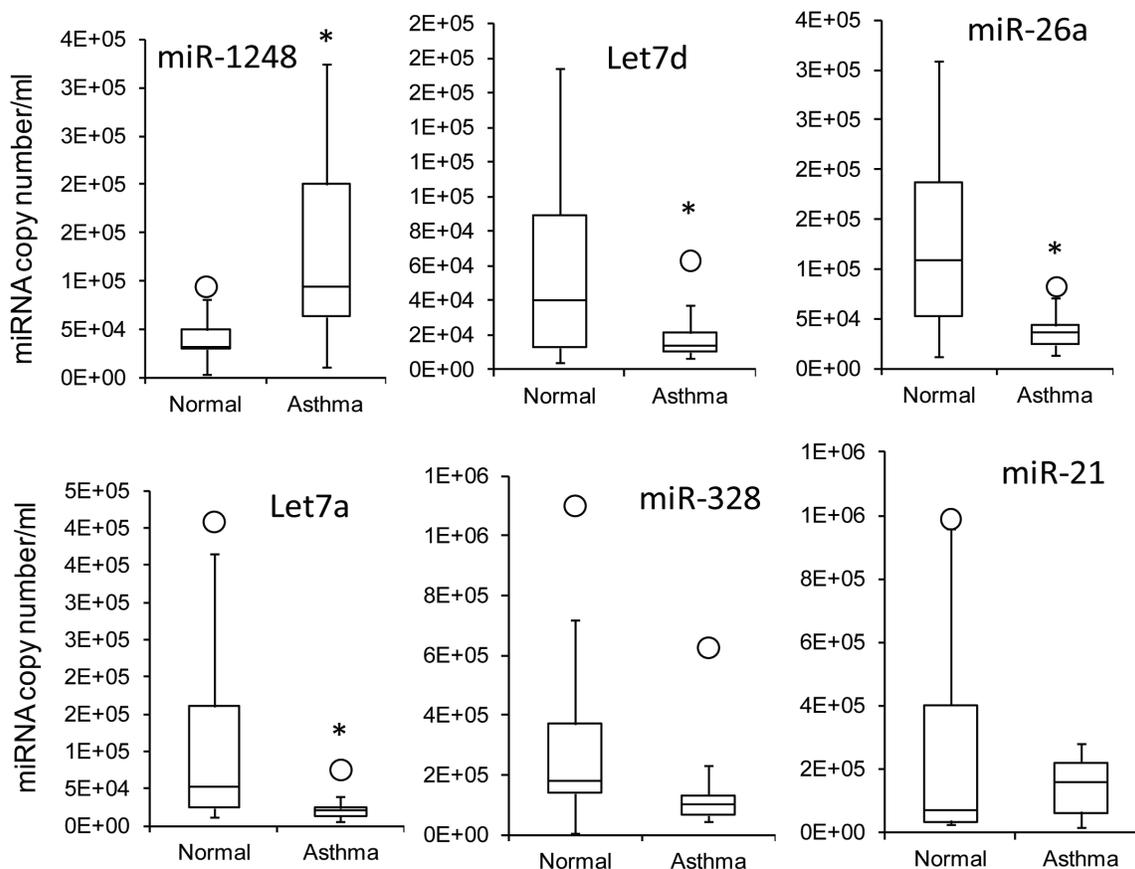


Figure 1. Differential expression of miRNAs in asthma. Box plots demonstrating miRNA expression (copy number/ml) in asthma and control subjects * $p < 0.05$.

group ($R = -.473$, $p = 0.08$) which was not apparent in the control group ($R = -0.02$, $p = 0.476$) (Figure 2D).

In order to determine whether there was functional significance of the miRNA expression patterns, we performed a prediction search for each miRNA to identify putative targets. We performed a search on each miRNA using the following search algorithms (miRanda: <http://www.microrna.org/microrna/home.do>, MirTarget2: <http://mirdb.org/miRDB/>, PITA: <http://genie.weizmann.ac.il/pubs/mir07/index.html>, RNA hybrid: <http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>, and TargetScan: <http://www.targetscan.org>) [29-34]. Targets that were predicted by 3 or more algorithms were selected and subjected to a gene ontology analysis to identify immune targets. In addition, we also utilized the miRecords tool (<http://mirecords.biolead.org/>) and literature searches to identify experimentally validated targets, and the sum-

mary of these searches is summarized in Table 3.

Binding of IL-5 by miRNA

One of the targets predicted to be regulated by miR-1248 is IL-5, a cytokine that has not previously been demonstrated to be regulated by miRNAs. A prediction search indicated that numerous miRNAs can bind to the IL-5 3'UTR, including miR-1248 and miR-328. The predicted binding sites of each using RNA hybrid is depicted in Figure 3A. Given the important role of IL-5 in the allergic inflammatory response and as a stimulator of eosinophils, we sought to determine whether IL-5 is regulated by miRNA, and if miR-1248 plays a role in regulation of the cytokine.

In order to determine if the IL-5 transcript is capable of being bound by miRNAs, an IP was performed using anti-Ago2 to isolate the RISC

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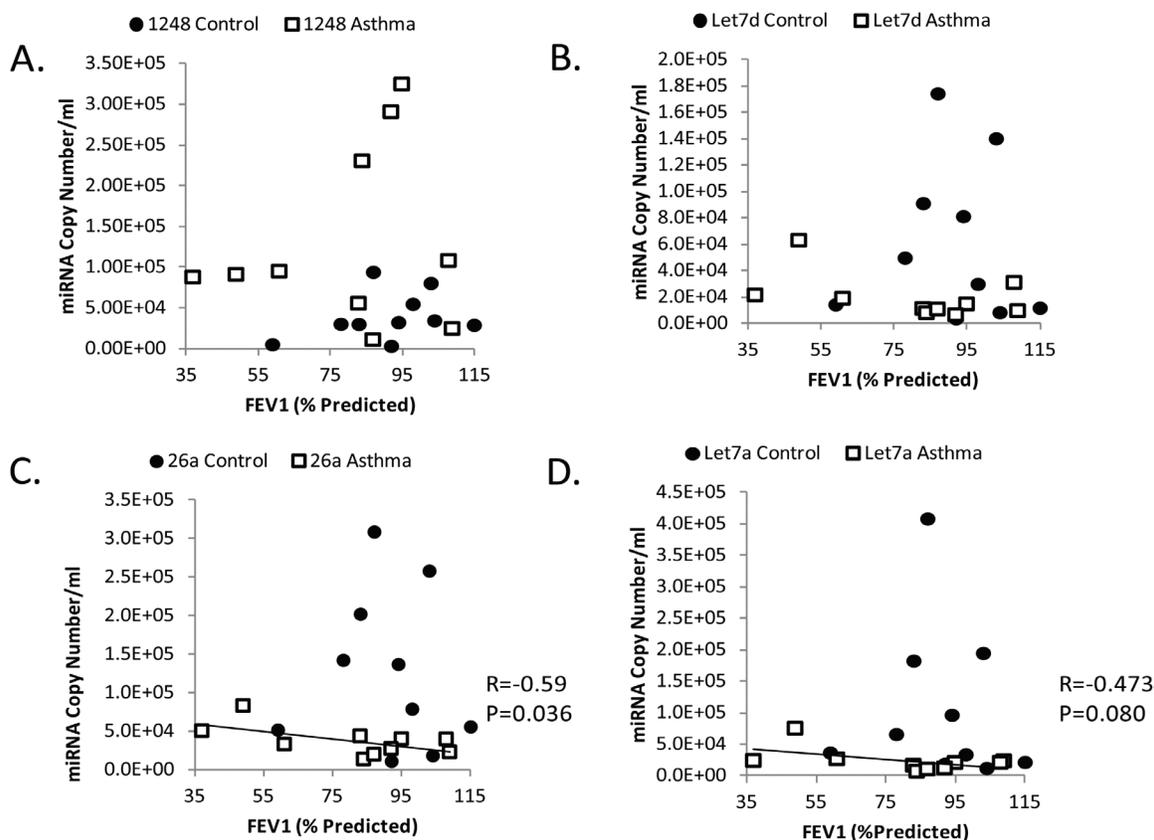


Figure 2. Negative Correlation of serum miRNA expression with lung function. miRNA expression in asthma (open squares) and control subjects are shown for miR-1248 (A), Let-7d (B), miR-26a (C), and Let-7a (D).

Table 3. Prediction of miRNA targets

miRNA	Predicted Targets	p-value
miR-1248	IL-5, IL-13, GATA3, FcεR1β, IL-1β, MMP-1, Mucin-1	0.031
miR-26a	TGF-β receptor (v) ¹ , CCR5, IL-4 Receptor, IL-6, Cox-2, TLR4, IFN-γ, ICOS	0.033
Let-7a, Let-7d	IL-13 (v), TGF-β receptor, TLR4	0.050, 0.040

¹(V): validated experimentally.

complex associated with mRNA. This technique allows the detection of endogenous protein-mRNA complexes formed in the cell by comparing enrichment of mRNA in samples immunoprecipitated (IP'd) with an antibody specific to a protein of interest vs. a non-specific isotype control (IC) as a measure of background. Using Jurkat T-cells, we successfully IP'd Ago2 using an Ago2-specific antibody (**Figure 3B**). An enrichment of IL-5 mRNA was observed by qPCR in the Ago2-IP over the IC-IP indicating that the transcript associates with the RISC (**Figure 3C**). As expected, there was no enrich-

ment seen in GAPDH negative control, indicating no binding.

A complementary biotin-pulldown technique was utilized to confirm binding and to demonstrate miR-1248 association with IL-5 RNA. The 3'UTR of IL-5 was synthesized by in vitro transcription to incorporate biotinylated CTP. The biotinylated RNA was then mixed with a cell lysate of Jurkat cells, and streptavidin beads were used to isolate the RNA and binding partners (**Figure 4A**). Western blot analysis confirmed that Ago2 interacted with the 3'UTR of

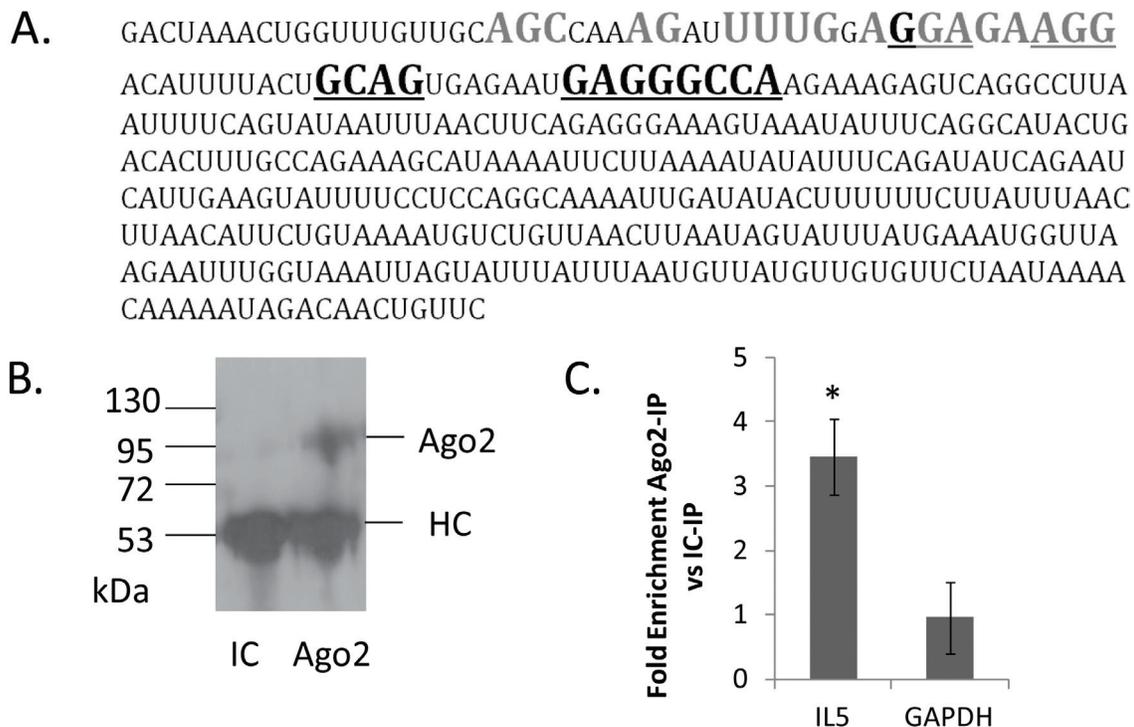


Figure 3. Binding of Ago2 to IL-5 RNA. A: Predicted sites of miRNA binding to IL-5 3'UTR are shown: binding site is shown in larger letters for miR-1248, and underlined for miR-328. B: Western blot demonstrating successful IP of Ago2 using a specific anti-Ago2 antibody but not with an isotype control (IC) antibody. C: IL-5 but not GAPDH mRNA is enriched with Ago2 IP, * $p < 0.05$.

IL-5 (**Figure 4B**). Mixture of the cell lysate with a biotinylated CCL2 3'UTR was used as a negative control, as we previously found that this did region not interact with Ago2 (unpublished data). To ensure that the presence of Ago2 was not due to non-specific retention of proteins from the lysate, the blot was re-probed with β -actin. As expected, The β -actin was present in the lysate, but only found in trace amounts in the pulldown samples.

RNA from pulldown sample was extracted, reverse transcribed, and analyzed by qPCR to assess whether miRNA was enriched in the IL-5 sample vs. the negative control. In the IL-5 pull-down, miR-1248 was readily amplified by qPCR, but undetectable in the negative control sample, indicating binding of the miRNA to IL-5 RNA (**Figure 4C**). The PCR products were then separated on an agarose gel to confirm that there was a single PCR band consistent with the expected size of the miRNA amplicon (~75 nt with the addition of a synthetic poly A tail and 3' adaptor) (**Figure 4D**).

MiR-1248 is a positive regulator of IL-5

To determine whether miR-1248 is capable of regulating IL-5, we transfected a miRNA mimic into PBMCs from healthy donors ($n=4$) and measured the effect on IL-5 mRNA levels. We observed a decrease in Ct values (i.e. increase in gene expression) in each of the samples treated with miR-1248 (**Figure 5A**). Expressing the data as a fold change from a ddCt (difference in IL-5 Ct values normalized to GAPDH Ct values in samples treated with miR-1248 vs. negative control miRNA), this amounted to a 3.55 ± 0.76 fold increase in expression ($p=0.03$), indicating that the miRNA is a positive regulator of IL-5 (**Figure 5B**).

In order to demonstrate that miR-1248 acts directly on the 3'UTR of IL-5, and to confirm the results in the PBMCs, the IL-5 3'UTR was cloned into a dual luciferase reporter. In this system, the IL-5 3'UTR is placed downstream of the firefly luciferase, and the plasmid also contains an independent *Renilla* luciferase. This allows for

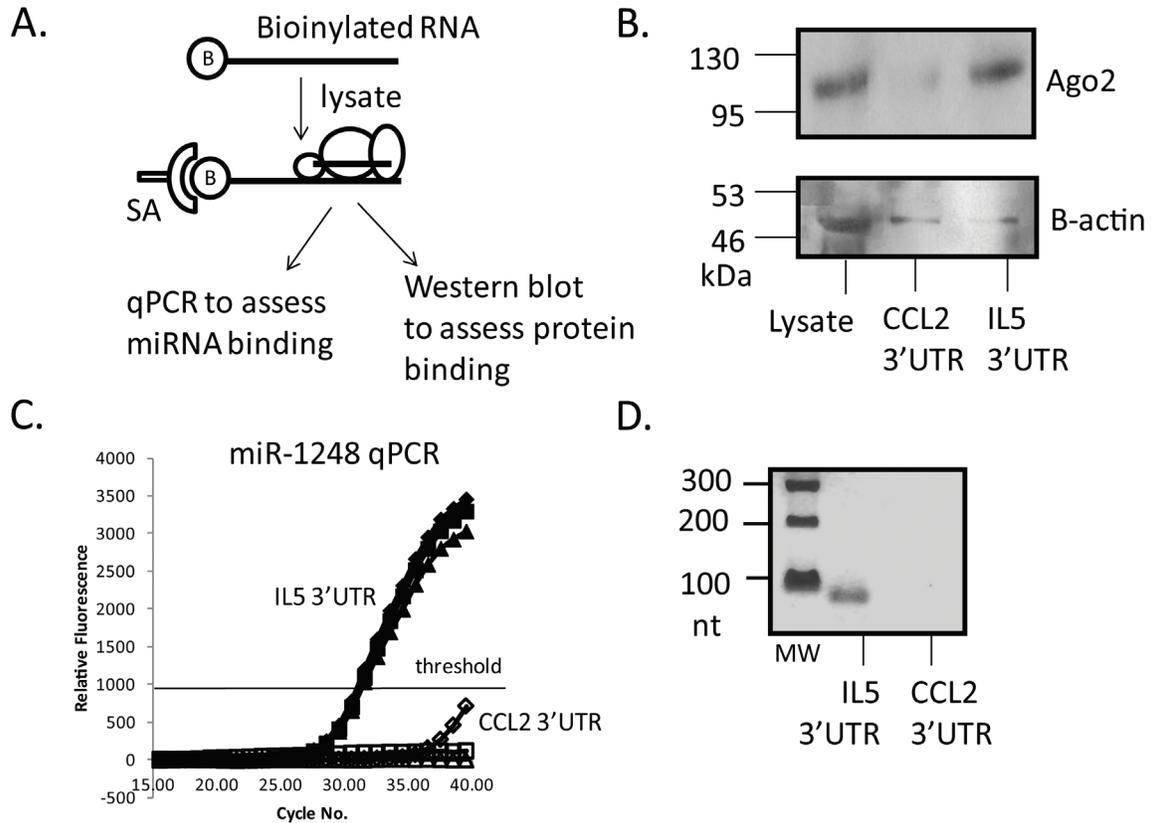


Figure 4. Association of miR-1248 with the IL-5 3'UTR. A: a biotin pull-down assay was used to isolate biotinylated IL-5 RNA (or negative control) bound to Ago2 and miRNA (SA, streptavidin; B, biotin). B: Western blot of the pull-down confirmed that Ago2 was bound to the IL-5 3'UTR. C: qPCR of the pull-down demonstrated enrichment of miR-1248, and separation by gel electrophoresis confirmed an appropriate size product.

normalization of firefly to *Renilla* luciferase signals to control for variability in transfection. Transfection of a miR-1248 mimic led to a 1.32 ± 0.14 fold increase in the pmirGLO-IL-5-3'UTR reporter expression compared to a 1.01 ± 0.09 fold change in the parent plasmid ($p=0.03$) (Figure 5C). Transfection of a miR-1248-antisense inhibitor yielded the opposite effect. Expression of luciferase in the pmirGLO-IL5-3'UTR resulted in a 0.8 ± 0.03 fold change with the inhibitor, compared to the parent vector which showed a 1.0 ± 0.02 fold change ($p=0.01$). Taken together, these data indicate that miR-1248 increases IL-5 expression by acting through the 3'UTR.

Discussion

MiRNAs are emerging as crucial biomarkers in diseases and also as an important regulatory class of molecules that may be involved in the pathogenesis of many illnesses. Diagnosis of

asthma is difficult due to a lack of non-invasive biomarkers, and the regulatory mechanisms that govern cytokine expression are not well defined. As such, we hypothesized that miRNAs are a potential biomarker in serum of asthmatics and that differentially expressed miRNAs regulate inflammatory mediators. In our study, we showed differential serum expression patterns of miR-1248, miR-26a, Let-7a, and Let-7d in asthmatic patients compared to non-asthmatic controls using qPCR analyses, demonstrating the potential of miRNA profiling in the diagnosis and management of asthma. In addition, we showed that miR-1248 regulates IL-5, a key allergic cytokine.

One of the challenges in defining biomarkers in asthma is the difficulty of finding molecules that are present in the blood that reflect the status of lung inflammation. In many cases, sampling of lung tissue by invasive methods such as bronchoscopy is required to assess the

MicroRNAs in asthma

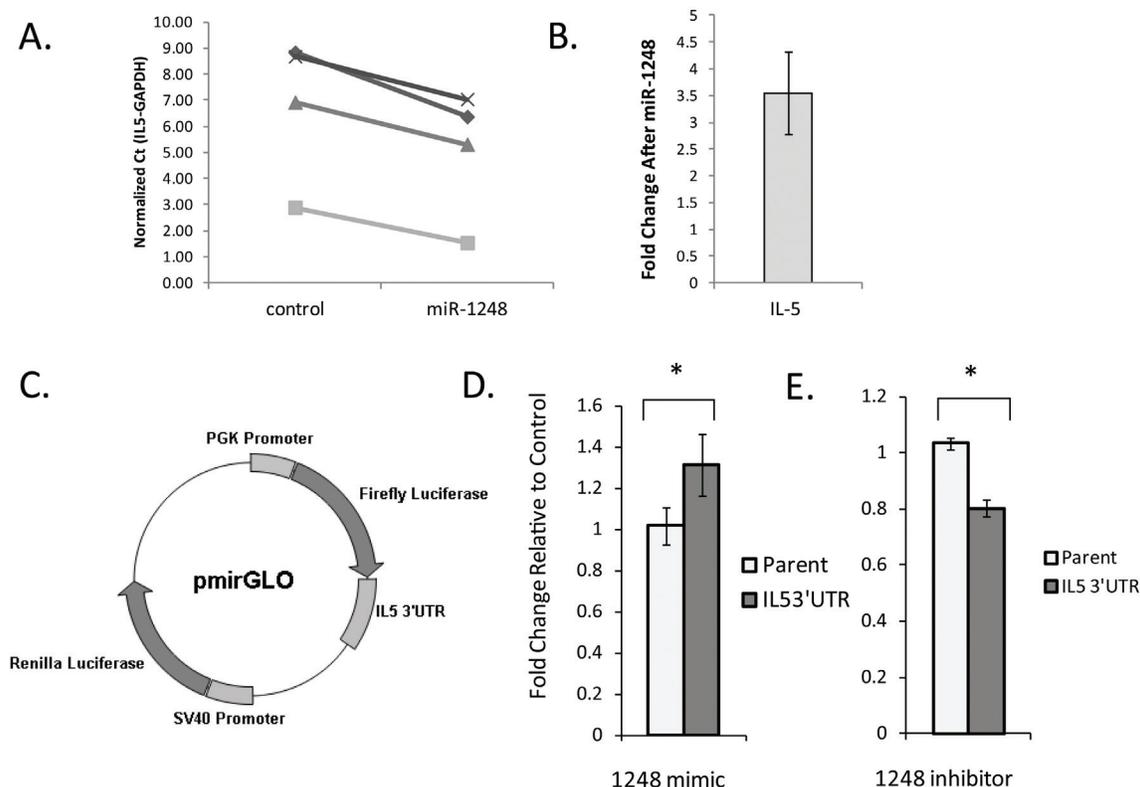


Figure 5. miR-1248 increases expression of IL-5. A: transfection of miR-1248 into PBMCs demonstrated a decrease in normalized Ct as shown, corresponding fold increase shown in panel B, C: the IL-5 3'UTR was cloned into a dual luciferase reporter. D: transfection of miR-1248 increased expression of the IL-5 3'UTR luciferase reporter, while transfection of a miR-1248 antisense inhibitor (Panel E) decreased expression. * $p < 0.05$.

nature of lung inflammation [35]. The difference in miRNA expression we observed in asthmatics is an indication that serum markers have utility in this disease. To determine whether there was any correlation between expression of serum miRNA and lung function, we analyzed the relationship between miRNA expression and FEV1% in our subjects. Interestingly, we observed a negative Pearson correlation for miR-26a and lung function in asthma, but not control subjects. This trend was also observed for Let7a, though it did not reach significance. It was unexpected to find that miRNA expression in asthma decreased with increasing lung function. As expression of these two miRNAs is lower in asthma, we would have predicted the opposite finding, such that miRNA levels would decrease with lower lung function. Consequently, the nature of the relationship between serum miRNA expression and lung function remains unclear. However, we believe that this is an important observation, as it indicates that cellular events occurring in

the lungs are reflected systemically in the blood. It also raises the possibility that profiling of serum miRNA levels may be utilized to assess a patient's disease based on its severity, phenotypic asthma differences, type of inflammation, or response to treatment.

The miRNAs that we found to be differentially expressed in asthma are predicted to regulate Th2 cytokines that play a crucial role in allergic inflammation. In particular, miR-1248 is predicted to regulate numerous cytokines, including IL-5. This cytokine has not previously been shown to be post-transcriptionally regulated. IL-5 is primarily responsible for eosinophilic survival, growth, differentiation, and recruitment [36]. Not surprisingly, it plays a central role in asthma, eosinophilic esophagitis, a variety of other allergic diseases, nasal polyposis, and hypereosinophilic syndromes [36]. As such, IL-5 is an important target, and we aimed to determine whether IL-5 is indeed regulated by miRNA, and miR-1248 in particular.

Using ribonuclear protein immunoprecipitation experiments and biotin pulldown assays, we established that Ago-2 and miR-1248 physically interact with the IL-5 mRNA. These techniques may be a useful screen that can be applied to other systems to determine whether proteins are capable of being regulated by the RISC and miRNA. In addition, the modified biotin pulldown procedure that we developed is a simple tool to identify specific interacting miRNAs in any system. These approaches could be further combined with high throughput screening methods such as mass spectrometry and miRNA microarray/sequencing to globally identify proteins and RNAs bound to transcripts.

The functional effects of miR-1248 were probed to determine whether the miRNA were truly capable of regulating gene expression of IL-5. Transfection of a miRNA-1248 mimic into PBMCs isolated from healthy adults demonstrated that the miRNA actually increased expression of the IL-5 transcript. This was unexpected, as miRNAs typically downregulate gene expression. To validate these findings, demonstrate that there was a direct effect of miR-1248 on IL-5 expression, and show that this was mediated through the 3'UTR of the gene, a luciferase reporter construct was utilized. These assays confirmed the initial observation by showing that miR-1248 increased expression of the reporter, while an antisense inhibitor of miR-1248 decreased expression. Although a few studies have shown different miRNAs to function as positive regulators of their target's expression, such reports are rare in the literature [7, 8, 37]. Also, the mechanism of action of how miRNAs increase expression of IL-5 via its 3'UTR is not clear. There could be interactions with other RBPs, such as the mRNA-stabilizing protein HuR for instance [38], and this is an area that requires further exploration.

It is interesting to note that miR-1248 expression was increased in asthma, while all of the other miRNAs that we found to be differentially expressed were down-regulated. These findings raise the question of whether there might be pathogenic role of miR-1248 in asthma, such that its increased abundance in the disease could serve to elevate Th2 cytokine levels. In addition, it also suggests that antisense inhibitors could be used as potential therapeutic targets. As miRNAs that regulate IL-5 expression might be dysregulated in other diseases

associated with eosinophilia, miR-1248 might also be a useful biomarker/target in these illnesses.

Our findings highlight the potential roles of miRNAs in the diagnosis, pathogenesis and treatment of asthma. We expect that expanding this methodology to profile miRNAs from a larger and more diverse asthma population, and analysis of a larger pool of miRNAs will be a useful technique in the clinical management of asthma. A fingerprint of miRNA expression differences could potentially be used to characterize phenotypic differences in asthma, monitor response to treatment, and personalize patient therapy.

Abbreviations

3'UTR, 3'untranslated region; Ct, cycle threshold; FEV₁, forced expiratory volume in one second; FEV₁%, percent predicted FEV₁; IC, isotype control antibody; ICS, inhaled corticosteroids; IP, immunoprecipitation; IP'd, immunoprecipitated; LABA, long acting beta agonist; miRNAs, microRNAs; PBMCs, peripheral blood mononuclear cells; qPCR, quantitative real time PCR; RBPs, RNA-binding proteins; RISC, RNA-induced silencing complex.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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