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Seasonal Variation in miR-328-3p and let-7d-3p Are Associated With Seasonal Allergies and Asthma Symptoms in Children

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ABSTRACT

Objective: MicroRNAs (miRs) are small non-coding RNA molecules of around 18–22 nucleotides that are key regulators of many biologic processes, particularly inflammation. The purpose of this study was to determine the association of circulating miRs from asthmatic children with seasonal variation in allergic inflammation and asthma symptoms.
Methods: We used available small RNA sequencing on blood serum from 398 children with mild-to-moderate asthma from the Childhood Asthma Management Program. We used seasonal asthma symptom data at the study baseline and allergen affection status from baseline skin prick tests as primary outcomes. We identified differentially expressed (DE) miRs between pairs of seasons using DESeq2. Regression analysis was used to identify associations between allergy status to specific seasonal allergens and DE miRs in 4 seasons and between seasonal asthma symptom data and DE miRs. We performed pathway enrichment analysis for target genes of the DE miRs using DAVID.
Results: After quality control, 398 samples underwent differential analysis between the 4 seasons. We found 52 unique miRs from a total of 81 DE miRs across seasons. Further investigation of the association between these miRs and sensitization to seasonal allergens using skin prick tests revealed that 26 unique miRs from a total of 38 miRs were significantly associated with a same-season allergen. Comparison between seasonal asthma symptom data revealed that 2 of these 26 miRs also had significant associations with asthma symptoms in the same seasons: miR-328-3p ($P < 0.03$) and let-7d-3p ($P < 0.05$). Enrichment analysis showed that the most enriched pathway clusters were Rap1, Ras, and MAPK signaling pathways.
Conclusion: Our results show seasonal variation in miR-328-3p and let-7d-3p are significantly associated with seasonal asthma symptoms and seasonal allergies. These indicate a potentially protective role for let-7d-3p and a deleterious role for miR-328-3p in asthmatics sensitized to mulberry. Further work will determine whether these miRs are drivers or results of the allergic response.

Keywords: RNA-seq; molecular epidemiology; seasons; asthma; pediatrics; circulating microRNAs; allergens

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Disclosure

There are no financial or other issue that might lead to conflict of interest.

INTRODUCTION

Asthma affects more than 300 million people worldwide and results in roughly 250,000 annual deaths.[1] It is expected that by 2025, there could be a further 100 million people with asthma,[2] and as such presents a growing concern. Allergic asthma is the most common type of asthma and a frequent manifestation of indoor and outdoor allergen exposure. Extensive overlap exists between asthma and allergies, and 10%-40% of patients with allergies also have asthma.[3] Allergic asthma itself has also increased in recent years, notably in Westernized countries.[4] Allergic responses are triggered by allergens acting as antigens that react with specific immunoglobulin E (IgE) antibodies. Allergens may be derived from many different animals, insects, mites, plants, or fungi.[5] Pollen allergens are primarily from trees, grasses, and weeds, and their air concentrations differ among locations and atmospheric conditions.[6] More than 150 seasonal pollen allergens have been identified (http://www.allergen.org).

Seasonal variation in exposure to pollen allergens can lead to seasonal increases in asthma exacerbations. Sensitization to the seasonal allergens timothy grass (*Phleum* species [sp.]) or birch (*Betula* sp.) pollens is associated with asthma in children.[7] Tree pollen counts peak in mid spring and exhibit substantive impacts on allergy and asthma exacerbations, particularly in children.[8] An Australian study assessing the role of ambient levels of different pollens on a large time series of child and adolescent asthma hospitalizations found grass and weed pollens to be important triggers of asthma exacerbations in children and adolescents.[9] Another study reported significant associations between airborne grass pollens and asthma attacks requiring physician consultation.[10]

MicroRNAs (miRs) are small non-coding RNA molecules of around 18–22 nucleotides that have emerged as core regulators of inflammatory processes. Many studies have demonstrated a role of miRs in regulating the susceptibility and response to allergies.[11,12,13] In a recent study, Wardzynska *et al.*[14] linked asthma exacerbations to the epigenetic dysregulation of circulating miR changes. In addition, miR expression was associated with clinical symptoms and patterns of T-cell cytokine expression. Others have documented links between miRs and asthma caused by viral respiratory infections.[15] However, there has not yet been work investigating the role of miRs in the seasonality of allergy or asthma exacerbations.

The purpose of this study was to determine whether seasonal variation in circulating miRs concentrations in asthmatic children is associated with seasonal variation in allergen affection status and asthma symptoms.

MATERIALS AND METHODS

The Childhood Asthma Management Program (CAMP)

The CAMP was a randomized, placebo-controlled, four-year trial of inhaled anti-inflammatory therapy for mild-to-moderate persistent childhood asthma. A total of 1,041 participants were enrolled in the trial between 1993 and 1995 at age 5–12 years; follow-up continued to 2012 when participants were aged 22–30 years.[16]

Seasonal asthma symptoms and allergen affection status

Seasonal asthma symptoms and allergen affection status were the primary outcomes. Seasonal symptoms were assessed with baseline questionnaires which asked "During

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Table 1. Tested Allergens

No.	Category/family	Allergen test	Sensitized subjects (%)	Type	Season
1	Fungi	*Penicillium* sp.	74 (18.59%)	Mold	Fall
2		*Aspergillus* sp.	70 (17.58%)	Mold	Fall
3		*Hormodendrum* (now *Cladosporium* sp.)	51 (12.81%)	Mold	Fall/Winter
4		*Alternaria* sp.	121 (30.40%)	Mold	Fall/Winter
5	Grass (*Poaceae* family)	Bermuda grass (*Cynodon* sp.)	76 (19.09%)	Grass	Summer
6		Timothy grass (*Phleum* sp.)	162 (40.70%)	Grass	Summer
7	Aster (*Asteraceae* family)	Ragweed (*Ambrosia* sp.)	133 (33.41%)	Weed	Fall
8		Sagebrush (*Artemisia* sp.)	41 (10.30%)	Weed	Fall
9		→ including Wormwood (*Artemisia* sp.)	2 (0.50%)	Weed	Fall
10	Amaranth (*Amaranthaceae* family)	Kochia, Burning Bush (now *Bassia scoparia*)	17 (4.27%)	Weed	Fall
11		Lambs Quarters, Goosefoot (*Chenopodium* sp.)	9 (2.26%)	Weed	Fall
12		Pigweed (*Amaranthus* sp.)	16 (4.02%)	Weed	Fall
13		Russian thistle, Saltwort (*Salsola* sp.)	41 (10.30%)	Weed	Fall
14	Buckwheat (*Polygonaceae* family)	Yellow Dock, Dock (*Rumex* sp.)	9 (2.26%)	Weed	Summer
15	Plantain (*Plantaginaceae* family)	English Plantain (*Plantago* sp.)	0 (0%)	Weed	Summer
16	Beech (*Fagaceae* family)	White oak (*Quercus alba*)	10 (2.51%)	Tree	Spring
17		Red oak (*Quercus rubra*)	33 (8.29%)	Tree	Spring
18		Oak (*Quercus* sp.)	21 (5.27%)	Tree	Spring
19	Birch (*Betulaceae* family)	Alder (*Alnus* sp.)	10 (2.51%)	Tree	Spring
20		Birch (*Betula* sp.)	23 (5.77%)	Tree	Spring
21	Cypress (*Cupressaceae* family)	Cedar/Juniper (*Juniperus* sp.)	10 (2.51%)	Tree	Spring
22	Elm (*Ulmaceae* family)	Elm (*Ulmus* sp.)	32 (8.04%)	Tree	Spring
23	Mulberry (*Moraceae* family)	Mulberry (*Morus* sp.)	11 (2.76%)	Tree	Spring
24	Olive (*Oleaceae* family)	Olive (*Olea* sp.)	19 (4.77%)	Tree	Spring
		→ including Privet (*Ligustrum vulgare*)		Tree	Spring
		→ including Ash (*Fraxinus* sp.)		Tree	Spring
25	Walnut (*Juglandaceae* family)	Walnut (*Juglans* sp.)	17 (4.27%)	Tree	Spring
25	Willow (*Salicaceae* family)	Cottonwood (*Populus deltoides*)	43 (10.80%)	Tree	Spring
		→ including Poplar (*Populus* sp.)		Tree	Spring
27	Uncategorized	6 tree mix	7 (1.75%)	Tree	Spring
28		Eastern weeds	2 (0.50%)	Weed	Fall

[month], how many days per week did a child have asthma symptoms?" for each of 12 months. Parents or guardians responding with values > 7 were removed from analysis (n = 92). Per-month symptom data was taken from questionnaires and averaged into a seasonal measure of asthma symptoms. Allergen affection was assessed with skin prick tests for 28 common allergens conducted at baseline. A wheal diameter of 3 or more millimeters was considered a positive reaction indicating allergy. Four seasons were defined by groups of 3 calendar months: Spring was March, April and May; Summer was June, July and August; Fall was September, October, and November; and Winter December, January, and February. Allergens were categorized into seasons of primary effect (**Table 1**): Spring (n = 13), summer (n = 5), Fall (n = 10), or Winter (n = 0). Subjects were classified in terms of blood draw dates for miR sequencing into seasons as for allergens.

miR data collection, quality control (QC), filtering, and normalization

We used available small RNA sequencing of blood serum from 398 children with mild-to-moderate persistent asthma from the CAMP. Small RNA-seq libraries were prepared using the Norgen Biotek (Thorond, Canada) Small RNA Library Prep Kit and sequenced on the Illumina (San Diego, CA, USA) NextSeq 500 platform by Norgen Biotek. The exceRpt pipeline was employed for the QC of the RNA-seq data.[17] We excluded miRs with mapped read counts < 5 or with coverage < 50% of all subjects from the study. Using the DESeq2 R package,[18] we normalized reads by relative log expression, which has been shown to be a robust normalization method.[19] The small RNA sequencing was performed in 22 batches, which can

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introduce technical effects due to inconsistencies during preparation and handling. Guided principal components analysis (gPCA)[20] was used to check for batch effects on mapped read counts per sample, which did not detect a significant batch affect after data normalization (P = 0.371, **Supplementary Fig. S1**).

Statistical analysis

Our analysis was composed of 3-steps: 1) we assessed differentially expressed (DE) miRs between pairs of seasons; 2) significantly DE miRs were assessed for the association with seasonal allergen affection status; and 3) miRs significant in step 2 were assessed for the association with seasonal asthma symptoms (**Fig. 1**). In each step, the same season is

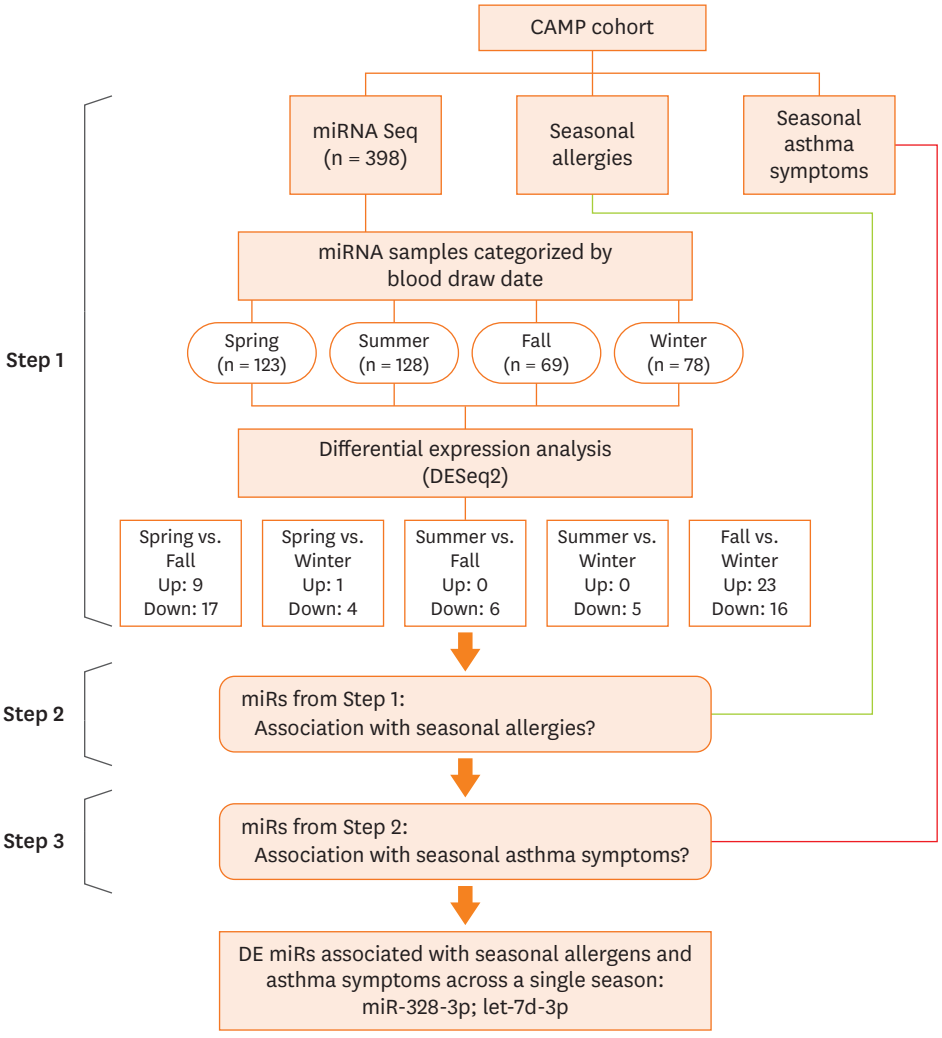

Fig. 1. Analysis plan and step 1. Using data from the CAMP cohort, we conducted 3 analyses using 3 types of data: 1) miR (assessed by season); 2) allergies to seasonal allergens; and 3) seasonal asthma symptoms. This occurred in 3 steps. First, in step 1, we compared expression of miR across seasons. We then categorized blood samples by blood draw date into seasons and used DESeq2 to perform differential expression between pairs of seasons. In step 2, we continued analysis on miRs determined to be DE by season in step 1. For each such miR, we checked it for their association with allergies to allergens prominent in the same seasons demonstrating differential expression for the miR. In step 3, we checked miRs with significant associations in step 2 for association with asthma symptoms in the same season. As a final result, we identified 3 miRs that were over or under expressed, associated with an allergen and asthma symptoms all in the same season.
CAMP, Childhood Asthma Management Program; miR, microRNA; DE, differentially expressed.

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assessed: *e.g.*, if a miR is overexpressed in Spring vs Fall, we check it for the association with Spring allergens and Spring symptoms; then also for Fall allergens and Fall symptoms.

First, we looked for DE miRs through a total of 6 pairwise season comparisons (Spring vs. Fall, Spring vs. Winter, Spring vs. Summer, Summer vs. Fall, Summer vs. Winter, and Fall vs. Winter) using the DESeq2 package in R with a Benjamini-Hochberg false discovery rate (FDR) multiple testing correction (**Fig. 1**).[21] DESeq2 is a method for differential analysis of count data that uses an Empirical Bayes approach for shrinkage estimation for fold changes to improve stability and interpretability of estimates. For each gene, DESeq2 models the read counts using a generalized linear model with a negative binomial distribution. In order to test whether each model coefficient differs significantly from zero, DESeq2 reports the standard error for each shrunken logarithmic fold change (LFC) estimate, which is obtained from the curvature of the coefficient's posterior at its maximum. For significance testing, DESeq2 uses a Wald test where the shrunken estimate of LFC is divided by its standard error.[18] On top of this, we applied an additional significance threshold of 10% FDR. Next, the statistically significant miRs were passed to step 2 (**Fig. 2**). The distributions of miRs among participants sensitized and not sensitized to allergens in the same seasons were compared. We used a logistic regression analysis to identify associations. miRs with significant associations ($P < 0.05$) were passed to step 3 (**Fig. 3**). These miRs were then checked for significant association ($P < 0.05$) with asthma symptoms from the same season using a multivariable linear regression analysis. All statistical computations were made using the R statistical framework.

Subjects were recruited from 8 different North American cities: Albuquerque, Baltimore, Boston, Denver, San Diego, Seattle, St. Louis, and Toronto. Because of environmental differences in pollen types and abundances, all 3 steps were performed with adjustment for city of recruitment. To adjust for non-specific allergy, all three steps were also adjusted for total IgE.

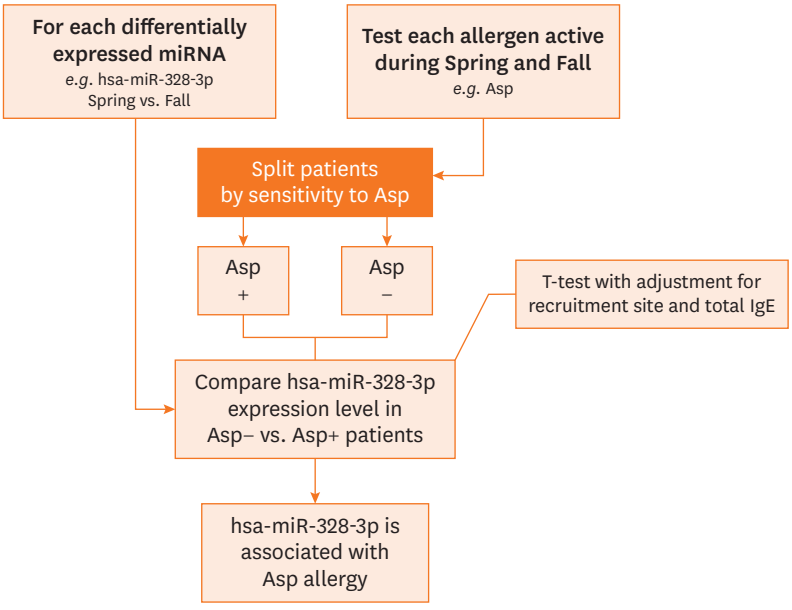

Fig. 2. Step 2. miRs DE between pairs of seasons (step 1) were then checked for their association with allergies to seasonal allergens from the same season. For each allergen primarily active during the season of differential expression, participants were split by their sensitization to that allergen. Levels of the miR were then compared between these 2 groups using the *t*-test.
miR, microRNA; DE, differentially expressed; IgE, immunoglobulin E; Asp, *Aspergillus*.

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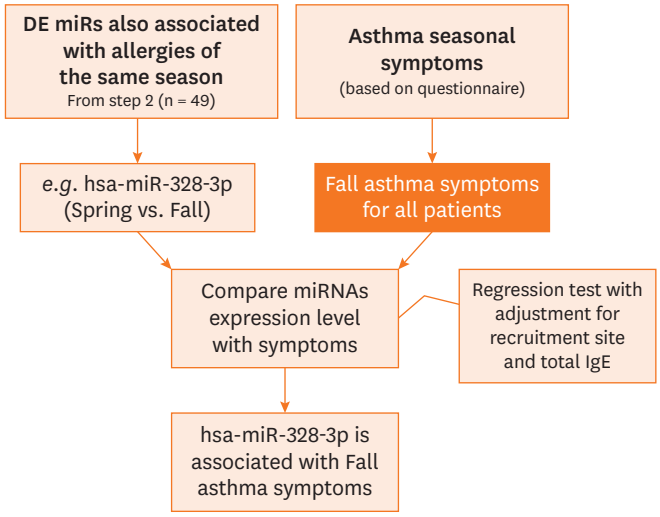

Fig. 3. Step 3. For miRs significantly associated with an allergy of a specific season (from step 2), we check them for associations of miR-328-3pwith asthma symptoms from that season. Seasonal asthma symptoms were reported in a questionnaire, and miRs associated with seasonal asthma symptoms were associated in all 3 steps. miR, microRNA; DE, differentially expressed; IgE, immunoglobulin E.

Identification of miR target and functional analysis

We identified putative mRNA targets for DE miRs from Dianna MicroT-CDS with 0.9 miTG as a threshold.[22,23] Then, we used DAVID 6.8 for the functional enrichment of the identified putative targets.[24] We considered an adjusted *P* value threshold of ≤ 0.05 and a gene count of 3 or more significant enrichment.

RESULTS

Characteristics of cohort

Among 1,041 children with asthma from the CAMP cohort, small RNA sequencing data from baseline blood serum were available on 398 children with seasonal asthma symptom and allergen affection status data (**Table 2**). The majority of subjects had miR assessed in the spring and summer. Subjects were broadly similar across the seasons of blood draw, with only significant differences by recruitment city (**Table 2**). Most patients were from St. Louis (n = 72), then San Diego (n = 55), Denver (n = 54), Boston (n = 54), Toronto (n = 45), Seattle (n = 44), Baltimore (n = 44), and fewest from Albuquerque (n = 30).

Allergen affection to 28 common allergens was assessed with skin prick tests conducted at baseline. Allergens were categorized into seasons of primary effect (Spring, n = 13; Summer, n = 5; Fall, n=10; Winter, n = 0), based on expert analysis. These are categorized in **Table 1**.

Seasonal miR differential expression, allergy, and asthma symptoms

After QC, filtering, and normalization, we had 398 samples and 266 miRs for differential analysis between the 4 seasons (step 1, **Fig. 1**). In Fall vs. Winter, we found the highest number of up-regulated (n = 23) & down-regulated (n = 16) miRs, where up-regulated miRs are more abundant in Fall than in Winter, and *vice versa* for down-regulated (**Supplementary Table S1**). In Spring vs. Fall, 9 miRs were up-regulated and 17 down-regulated. In Spring vs. Winter, 1 miR was up-regulated, and 4 miRs down-regulated. In Summer vs. Fall, 6 miRs were down-regulated, and in Summer vs. Winter, 5 miRs were down-regulated.

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Table 2. Baseline epidemiologic and clinical characteristics

Characteristics	Spring (n = 123)	Summer (n = 128)	Fall (n = 69)	Winter (n = 78)	*P* value
Sex					
Male	75 (61.0)	79 (61.7)	38 (55.1)	54 (69.2)	
Female	48 (39.0)	49 (38.3)	31 (44.9)	24 (30.8)	
Age (year)					0.522
Mean ± SD	9.01 ± 2.26	8.73 ± 2.28	8.91 ± 2.11	8.58 ± 1.75	
Median [Min, Max]	8.96 [5.25, 13.0]	8.61 [5.18, 12.9]	8.70 [5.18, 13.1]	8.62 [5.34, 12.4]	
Race					0.263
White	97 (78.9)	98 (76.6)	58 (84.1)	52 (66.7)	
Black	20 (16.3)	26 (20.3)	9 (13.0)	21 (26.9)	
Hispanic	6 (4.9)	4 (3.1)	2 (2.9)	5 (6.4)	
Clinic					< 0.001
Albuquerque	11 (8.9)	17 (13.3)	1 (1.4)	1 (1.3)	
Baltimore	21 (17.1)	3 (2.3)	6 (8.7)	14 (17.9)	
Boston	10 (8.1)	16 (12.5)	14 (20.3)	14 (17.9)	
Denver	12 (9.8)	20 (15.6)	9 (13.0)	13 (16.7)	
San Diego	25 (20.3)	20 (15.6)	4 (5.8)	6 (7.7)	
Seattle	9 (7.3)	20 (15.6)	11 (15.9)	4 (5.1)	
Saint Louis	22 (17.9)	20 (15.6)	16 (23.2)	14 (17.9)	
Toronto	13 (10.6)	12 (9.4)	8 (11.6)	12 (15.4)	
Height (cm)					0.916
Mean ± SD	133 ± 14.5	133 ± 14.6	134 ± 12.9	132 ± 12.5	
Median [Min, Max]	134 [105, 170]	132 [108, 167]	133 [110, 177]	133 [108, 158]	
BMI					0.169
Mean ± SD	17.8 ± 3.18	17.8 ± 3.20	18.8 ± 3.50	18.3 ± 3.22	
Median [Min, Max]	17.1 [13.3, 34.3]	17.0 [13.0, 28.1]	18.1 [13.4, 30.7]	17.3 [13.7, 29.7]	
Log10 IgE					0.778
Mean ± SD	2.60 ± 0.680	2.62 ± 0.706	2.71 ± 0.589	2.65 ± 0.767	
Median [Min, Max]	2.71 [0.480, 4.15]	2.63 [0.780, 4.61]	2.75 [0.850, 3.72]	2.70 [0.300, 4.13]	
FEV1pp					0.552
Mean ± SD	92.8 ± 14.2	95.2 ± 12.3	94.8 ± 13.7	94.2 ± 13.4	
Median [Min, Max]	94.0 [55.0, 124]	95.0 [61.0, 122]	95.0 [64.0, 122]	93.0 [64.0, 128]	
Missing	0 (0)	1 (0.8)	3 (4.3)	0 (0)	
FVCpp					0.535
Mean ± SD	104 ± 13.1	106 ± 12.2	104 ± 12.2	103 ± 12.2	
Median [Min, Max]	103 [73.0, 132]	105 [73.0, 136]	105 [69.0, 134]	104 [71.0, 132]	
Missing	0 (0)	1 (0.8)	3 (4.3)	0 (0)	
PC20					0.205
Mean ± SD	0.0114 ± 1.08	−0.0205 ± 1.04	0.131 ± 1.12	0.292 ± 1.20	
Median [Min, Max]	0.190 [−2.94, 2.04]	−0.0450 [−2.12, 2.53]	0.170 [−2.28, 2.32]	0.300 [−1.71, 2.48]	

Values are presented as number (%) not otherwise specified.
BMI, body mass index; SD, standard deviation; Min, minimum; Max, maximum; IgE, immunoglobulin E; FEV1pp, forced expiratory volume in the one second percentage predicted; FVCpp, forced vital capacity percentage predicted; PC20, provocative concentration of methacholine causing a 20% drop in FEV1 from baseline.

The DE miRs were assessed for the association with seasonal allergies, from the same seasons, in step 2 (**Fig. 2**). This was accomplished by selecting an allergen primarily associated with one of the seasons wherein the miR was differentially expressed. Next, all patients were split into 2 groups: those sensitized to the allergen and those not sensitized. After that, the levels of the miR among the 2 groups were checked for differences using a logistic regression analysis. We identified a total of 26 unique miRs out of the 38 miRs significantly associated with 1 or more same-season allergen (**Supplementary Table S2**). Finally, miRs were assessed for association with seasonal asthma symptoms of the same season in step 3 (**Fig. 3**). We found that 2 miRs were significantly associated with seasonal asthma symptom data from the same season: miR-328-3p with *Aspergillus* in the Fall ($P = 0.03$; $\beta = -0.089$; 95% confidence interval [CI] = 0.842–0.991), and let-7d-3p with mulberry in the Spring ($P = 0.05$; $\beta = -0.084$; 95% CI= 0.844–1.00) (**Table 3**).

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Table 3. List showing association between differentially expressed miRs and seasonal symptom date

miR	Allergen	Season symptom		
hsa-miR-328-3p	*Aspergillus*	*P* value = 0.052	Fall	*P* value = 0.080
		β = 0.12		β = −0.07
		OR = 1.13		OR = 0.92
		95% CI = 1.003–1.30		95% CI = 0.85–1.01
			Spring	*P* value = 0.030
				β = −0.89
				OR = 0.91
				95% CI = 0.84–0.99
hsa-let-7d-3p	Mulberry	*P* value = 0.044	Spring	*P* value = 0.050
		β = −1.55		β = −0.084
		OR = 0.21		OR = 0.91
		95% CI = 0.03–0.69		95% CI = 0.84–1.00

miR, microRNA; OR, odds ratio; CI, confidence interval.

Table 4. Pathway enrichment analysis of differentially expressed miRs

Annotation cluster	Term	Gene count	%	*P* value	Fold enrichment	Benjamini
KEGG_PAHWAY	hsa04015:Rap1 signaling pathway	22	2.67	5.52E-05	2.69	2.426e-3
KEGG_PAHWAY	hsa04014:Ras signaling pathway	23	2.79	5.53E-05	2.62	2.025e-3
KEGG_PAHWAY	hsa04010:MARK signaling pathway	22	2.67	7.29E-04	2.24	1.4478e-2

Enrichment score: 3.88.
miR, microRNA.

In a sensitivity analysis, we found that self-reported prior inhaled corticosteroid use was not associated with seasonal symptoms (Spring $P = 0.15$, Summer $P = 0.19$, Fall $P = 0.65$, Winter $P = 0.54$).

Identification of putative targets and functional enrichment of DE miRs

We identified 52 unique miRs from a total of 81 DE miRs across seasons (**Supplementary Table S1**), of which 29 miRs were common in the 6 comparisons. We retrieved a total of 1,302 putative gene targets for these 52 DE miRs from the DIANA MicroT-CDS database (miTG score ≥ 0.9, **Supplementary Table S3**). We then performed enrichment analysis of retrieved putative gene targets through DAVID. The most enriched pathway cluster was characterized by Rap1 signaling pathways (KEGG; hsa04015), the Ras signaling pathway (hsa04014), and the mitogen activated protein kinases (MAPK) signaling pathway (hsa04010) (**Table 4**).[25]

DISCUSSION

The occurrence of seasonal allergic asthma has escalated in recent decades and is now a major global health challenge. Seasonal allergic asthma is among the most severe asthma phenotypes and is related to respiratory allergic diseases triggered by pollen.[26-28]

In this manuscript, we identified 2 miRs, miR-328-3p and let-7d-3p, which were significant in 3 related analyses. First, they were DE between pairs of seasons, according to blood draw dates of the CAMP samples. Second, they were associated with allergy to seasonal allergens active in the same seasons *Aspergillus* in the Fall for miR-328-3p and mulberry in the Spring for let-7d-3p). Third, they were associated with rates of asthma symptoms in those seasons. The let-7d-3p is decreased in the spring and decreases with sensitization to mulberry; decreasing let-7d-3p is also associated with increasing asthma symptoms in the spring, indicating a potentially protective role for let-7d-3p. The role of miR-328-3p is potentially

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more complicated, with a decrease in the fall and increase with allergies to *Aspergillus*; increasing miR-328 is also associated with greater occurrences of asthma symptoms during the fall; these effects point to a potentially deleterious effect of miR-328 in people sensitized to *Aspergillus*.

We found a total of 82 miRs DE by season with the highest number of miRs DE between Fall vs. Winter, Spring vs. Fall, and Summer vs. Fall. Many studies have reported seasonal patterns in asthma exacerbations with the highest exacerbation rate in children in the fall and the lowest exacerbation rate in the summer. The seasonal rise in fall exacerbations is highly consistent,[29-33] and may be due to pollen exposure, increased cold air exposure, and viral infections common in the Fall.

Interestingly, we found a total of 14 miRs associated with Fall allergen sensitization out of 26 unique miRs that were associated with the same season's allergen (**Supplementary Table S2**).

We also tested each DE miR for the association with symptoms in those seasons. We found seasonal variations in the circulating miRs miR-328-3p and let-7d-3p to be subsequently associated significantly with both seasonal allergies and asthma symptoms (**Table 3**). Our results suggest a protective role of miR-328-3p in asthmatics sensitized to *Aspergillus*. The miR-32-3p was associated with sensitization to *Aspergillus* and decreased asthma symptoms in the spring and fall. *Aspergillus* is a common indoor and outdoor mold that grows under damp conditions, including wet spring and fall seasons. The miR-328 has a complex role in the lung, and our results first provide the association between miR-328 and allergic asthma. It has been shown to promote wound repair in bronchial epithelial cells yet decrease bacterial clearance and host defense against microbial infection in the lung.[34,35]

Our results also demonstrate a protective role of let-7d in seasonal allergic asthma. The let-7d was inversely associated with both mulberry pollen sensitization and spring season asthma symptoms. Mulberry trees are widely distributed across North America and similar to other trees, bloom in the spring can trigger seasonal allergy and asthma symptoms. Consistent with our results, decreased let-7d expression has been associated with asthma affection status compared to healthy controls.[36] Moreover, allergen desensitization to wasp venom leads to higher let-7d expression further supporting a protective role of let-7d in allergic inflammation.[37]

Gene targets of DE miRs between the seasons were enriched for MAPK and Rap1 signaling pathways. MAPK belong to a large family of proline-directed serine-threonine protein kinases that play a fundamental role in cellular functions. Activation of MAPK via pharmacological or genetic approaches blocks allergic inflammation of airways.[38] A previous study reported that activation of the MAPK signaling pathway can control the production of IgE and interleukin (IL)-4 as well as inhibit inflammatory mediators in asthma.[39,40] Furthermore, the MAPK signaling pathway controls immune responses and inflammation in asthma by regulating the gene expression of inflammatory factors such as TNF-alpha and IL-6.[41,42]

Rap1 (Ras-proximate-1 or Ras-related protein 1) is a small GTPase, which are small cytosolic proteins that act as cellular switches and are essential for efficient signal transduction.[43] The association of the RAP1 pathway with seasonal allergic asthma has not yet been reported.

Our study has several strengths. This is the first study to analyze seasonal variation in circulating miR and report its association with seasonal allergies and asthma symptoms in

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children. Another strength includes the use of small-RNA next-generation sequencing to comprehensively identify miRs in an unbiased way. Our study also has limitations. We were unable to account for actual pollen levels and differences in pollen exposure to each subject. While we did adjust for city of recruitment in an effort to account for regional differences in pollen, there may be remaining effects. We were also unable to tell if the miR levels were a cause or an effect of the allergen exposure. Although we found no associations of miRs with symptoms and ICS use, recall bias may have resulted in inaccuracies in these data. Similarly, the reporting of asthma symptoms may have been inaccurate due to recall bias. It is not clear how much these unmeasured confounders may have altered the findings given the other measured confounders (total IgE and geographic location) that were included as covariates in the model. Future work will involve more careful collection of exposures, pollen counts, or medication, and examine their effect on miR expression by season. Finally, allergies to some of the pollens were rare in our cohort, including to mulberry. In future work, our results with mulberry and miR-328-3p should be investigated in a larger dataset. Future work will also assess miR associations to allergies without considering seasonal symptoms.

In conclusion, our results show seasonal variations in miR-328-3p and let-7d-3p to be significantly associated with seasonal asthma symptoms and seasonal allergies. Further work is needed to determine whether these miRs are drivers or results of allergic response.

SUPPLEMENTARY MATERIALS

Supplementary Table S1
Differentially expressed miRs

Click here to view

Supplementary Table S2
List of associated differentially expressed miRNAs and seasonal allergens

Click here to view

Supplementary Table S3
List of putative target genes of differentially expressed miRNAs from DIANA MicroT-CDS. Shown are genes with predicted score of binding > 0.9

Click here to view

Supplementary Fig. S1
Box plot showing sum of (A) raw read counts per batch (B) normalized read counts per batch. *P* values calculated from gPCA package for the identification of batch effect.

Click here to view

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