

RESEARCH LETTER

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Unique basophil microRNA signature in chronic spontaneous urticaria patients who respond to omalizumab

To the Editor,

Involvement of basophils in chronic spontaneous urticaria (CSU) immunopathogenesis is suggested by the blood basopenia that correlates with disease activity and may result from circulating basophils moving to cutaneous sites of wheals.¹ The humanized IgG₁k monoclonal antibody omalizumab binds to the Cε3 domain of Fc on the IgE heavy chain leading to rapid reduction in free IgE levels and subsequent decrease in FcεRI expression on basophils and mast cells. Whilst omalizumab has transformed the treatment approach of patients with H₁-antihistamine-resistant (refractory) CSU,² approximately one-third of CSU patients fail to respond to anti-IgE treatment spurring a search for biomarkers predictive of response. Serum autoreactivity (determined by serum-induced basophil histamine release assay) is associated with a slower response to omalizumab than CSU patients without autoantibodies.³ However, in the ASTERIA I study, baseline thyroperoxidase antibody levels or positive chronic urticaria index test failed to predict omalizumab response in CSU.² Although lower IgE levels are associated with non-response to omalizumab in CSU, sub-analysis of ASTERIA I and ASTERIA II studies of omalizumab treatment in CSU patients found that IgE levels do not impact omalizumab efficacy.^{4,5} Thus, additional biomarkers are needed to predict response to omalizumab. MicroRNAs are small non-coding RNAs that suppress gene expression at the post-transcriptional level by binding to the 3' untranslated segment of target messenger RNA(s) to inhibit protein synthesis and thereby regulate innate and adaptive immune responses. We recently reported that in refractory CSU patients, a unique set of differentially expressed extracellular vesicle microRNAs may predict complete response to omalizumab.⁶ Given the potential role of basophils in CSU immunopathogenesis, an exploratory aim of this Institutional Review Board-approved observational study (written informed consent obtained) presented here was whether basophil microRNAs could serve as omalizumab response biomarkers in CSU.

Patients with refractory CSU received 3-monthly doses of omalizumab and disease activity was captured using weekly Urticaria Activity Score (UAS7). Nineteen adult subjects whose baseline characteristics were previously described⁶ completed UAS7 scores over a 12-week study period. From these 19 subjects, we focussed on subjects with the most discordant response to omalizumab by quantitating the area under the curve for each subject's weekly UAS7 values over the 12-weeks. We identified six complete responders (CR)

and four non-responders (NR). Response classification details were previously published.⁶ Basophils (CD45⁺CD123⁺IgE⁺) were isolated from nucleated peripheral blood cells by negative selection using STEMCELL Technologies EasySep™ Human Basophil Enrichment Kit (Vancouver, Canada). The purity of the basophil isolates was $94 \pm 2.5\%$ (mean $\pm$ SD) per manufacturer's flow cytometry data. Basophil total RNA (including microRNAs) was isolated in T.K.B.'s laboratory using QIAGEN miRNeasy Micro extraction kit (QIAGEN Sciences, Germantown, MD). Integrity of the total RNA samples was evaluated using a 2100 Bioanalyzer instrument (Agilent Technologies, Inc.). Purity of RNA samples was determined by measuring OD_{260/280} and OD_{260/230} ratios. Samples with an RNA Integrity Number (RIN) of ≥ 7 and OD_{260/280} and OD_{260/230} ratios >1.8 were used for further analysis (median RIN = 7.8). Total RNA concentrations were determined using Thermo Fisher Scientific Qubit RNA HS Assay Kit (median total RNA quantity = 175.4 ng). These total RNA samples from six CR and four NR subjects were sent for miR-Seq analysis to Qiagen Genomic Services, which repeated quality and quantity assessments. Total basophil RNA (100 ng) was used to synthesize cDNA using QIAseq microRNA Library Kit. Specially designed 3' and 5' adapters were ligated to mature microRNAs, and ligated microRNAs were subsequently reverse transcribed to cDNA using a reverse transcription primer containing an integrated Unique Molecular Index (UMI). Following cDNA clean-up, polymerase chain reaction library amplification (19–22 cycles) was performed with a universal forward primer and indexing reverse primers. The quality of the microRNA libraries was assessed using Agilent TapeStation 4200. Samples were prepared for sequencing using QIAseq miRNA Library Kit that incorporates a UMI barcode into individual microRNA transcripts; UMI counts (ie counts of unique miRNA transcripts) were generated using Qiagen's GeneGlobe software. The microRNA sequencing libraries were sequenced using a NextSeq 500 instrument (Illumina, Inc.). Raw reads were de-multiplexed and FASTQ files for each sample were generated using Illumina bcl2fastq software. FASTQ data were checked using the FastQC tool. Reads and UMI counts were generated using the QIAGEN GeneGlobe pipeline. During the QIAseq microRNA Library Kit construction process, each microRNA molecule was tagged with a UMI. Following sequencing and trimming, reads were analysed for presence of UMIs. All reads containing identical insert sequence and UMI sequence (insert-UMI pair) were collapsed into a single read. These reads were passed into the analysis pipeline.

Additionally, reads containing partial UMIs were also passed into the analysis pipeline for true microRNA quantification by eliminating library amplification bias. Cutadapt (version 1.11) was used to remove adapter sequences and output adapter-free sequences. UMI counts for each microRNA were read into R (r-project.org) using Bioconductor edgeR package and filtered to remove microRNAs that were either unexpressed or expressed at a consistent, unreliably low level. Samples were normalized using a trimmed mean of M-values with counts converted to log counts/million (logCPM) using voomWithQualityWeights function from Bioconductor limma package.⁷ This function converts counts to logCPM and estimates observation and sample-level weights for use in a conventional linear model to control for heteroscedasticity. For each subject, there were repeated measurements; within-subject correlations were estimated using limma duplicateCorrelation function and controlled for using a linear mixed model when making comparisons. Comparisons between groups were made using empirical Bayes adjusted contrasts.⁸ Multiplicity was controlled at a false discovery rate (FDR; Benjamini-Hochberg correction) of .05 (ie the expected maximum proportion of false positives is estimated to be 5%). Because, comparisons between CR and NR subjects at baseline have no repeated measures, a conventional weighted ANOVA model was used to make comparisons. In total, 1785 microRNAs were used for this analysis. To test for a different temporal outcome of UAS7 score over time, we used a linear mixed model that included dichotomized microRNA expression (high or low at baseline), a time coefficient (treatment week), and an interaction between the two. We tested for a significant interaction (ie changes in UAS7 at

Key Messages

- In chronic spontaneous urticaria, a unique basophil microRNA signature associates with complete response to omalizumab.
- Basophil microRNA profiling may prove a useful omalizumab response biomarker in chronic spontaneous urticaria.
- Validation of these findings in a second cohort of chronic spontaneous urticaria patients is indicated.

any time, dependent on baseline expression) using a likelihood ratio test (LRT). Statistical analysis was performed using R Statistical Software (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria). A p -value < .05 was used to indicate statistical significance.

We found three differentially expressed basophil microRNAs at baseline (immediately prior to omalizumab treatment) associated with complete response to omalizumab (FDR < .05) (Figure 1A). CR ($N = 6$) compared to NR ($N = 4$) had higher expression of miR-1236-3p (logFC, 4.63; 95% CI, 0.89 to 8.37; FDR = 0.024), miR-1200 (logFC, 4.68; 95% CI, 0.90 to 8.37; FDR = 0.024) and miR-4664-3p (logFC, 4.34; 95% CI, 0.65 to 8.03; FDR = 0.033) (Figure 1B-D, upper panels). Participants were dichotomized into high and low expression groups (defined as those with an expression level above or below the geometric mean expression level

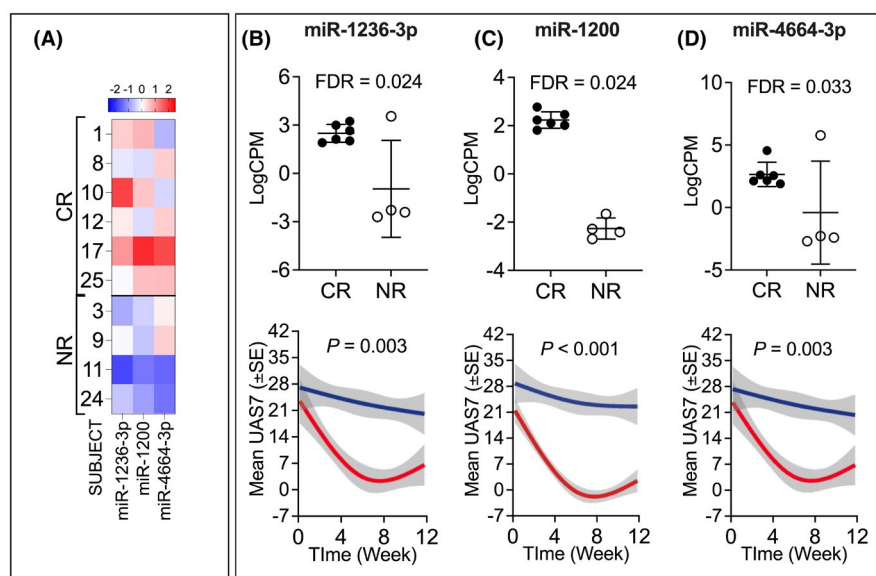
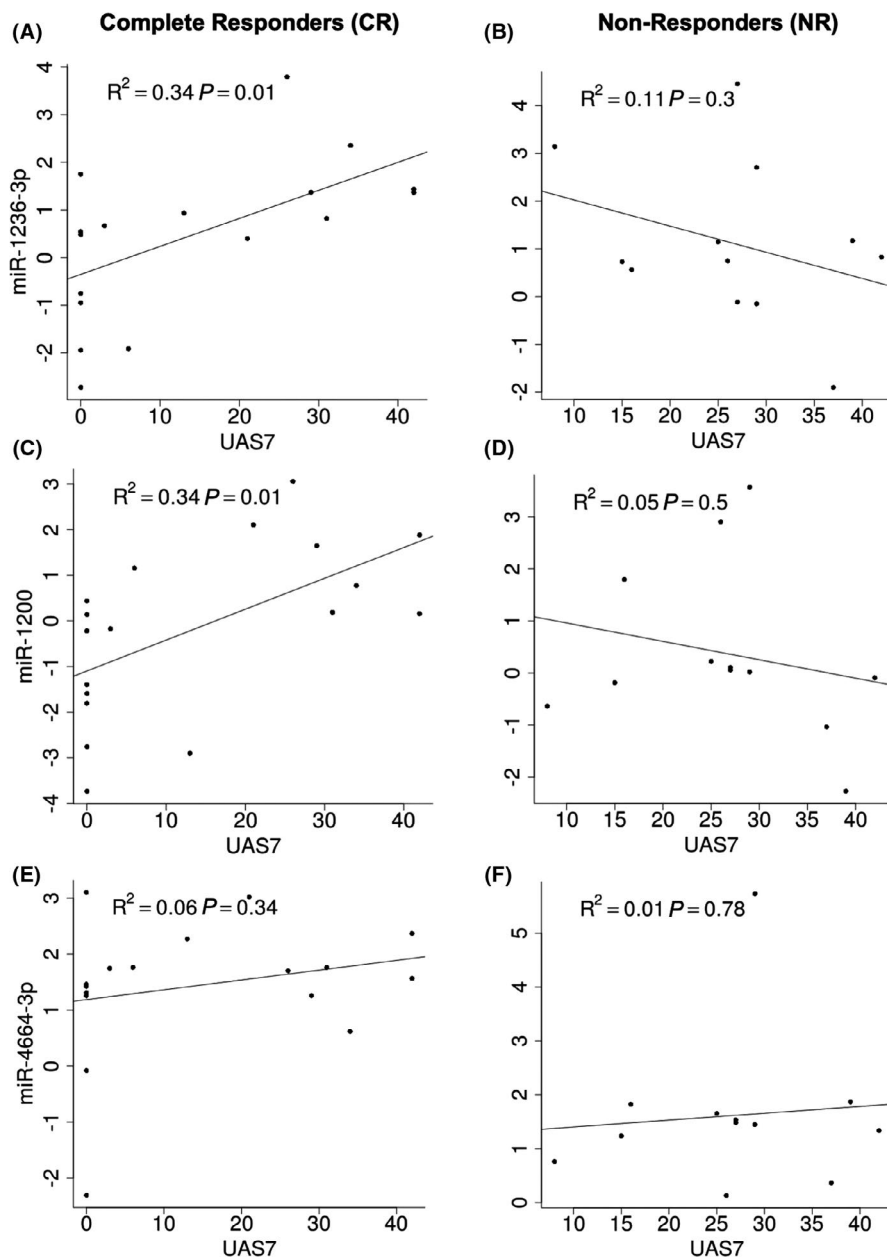


FIGURE 1 Basophil microRNAs associated with clinical response to omalizumab in CSU patients at baseline. (A) Heatmap of differentially expressed basophil microRNAs on Day 0. Expression values (CPM) were converted to z-scores. (C-D) Upper panels show geometric mean expression levels ($\pm$ SD) between CR and NR at baseline for the three differentially expressed basophil microRNAs. Lower panels show UAS7 scores ($\pm$ SE in grey) over 12-week treatment period of subjects with high (red) versus low (blue) expression. There were 7 subjects with high expression of both miR-1236-3p and miR-4664-3p, and 6 subjects with high expression of miR-1200. Statistical significance was determined LRT after fitting a linear mixed model with a random intercept for each subject and fixed effects defined by an interaction between a 2 degrees of freedom spline on the observational week and the expression level (high or low)

FIGURE 2 Correlation between basophil microRNAs and UAS7 scores amongst omalizumab-treated CSU patients based on clinical response. (A, B) miR-1236-3p level was positively associated with UAS7 score amongst CR, but not amongst NR. (C, D) miR-1200 was positively associated with UAS7 score amongst CR, but not amongst NR. (D, E). No association between miR-4664-3p and UAS7 score was seen in either CR or NR



for each microRNA), and their response (UAS7 scores over 12 weeks) was contrasted by LRT. Individuals with high expression of these microRNAs had better clinical response than those with low expression: miR-1236-3p (LRT = 34.7, $p = .003$), miR-1200 (LRT = 75.2, $p < .001$) and miR-4664-3p (LRT = 34.7, $p = .003$); all preceding p -values are Bonferroni corrected (Figure 1B-D, lower panels). We wondered if levels of these microRNAs would change in association with clinical response to omalizumab. By week 12, CR had a statistically non-significant decrease in expression levels of miR-1236-3p (LogFC, -2.03; FDR = 0.50), miR-1200 (LogFC, -2.62; FDR = 0.19) and increase in miR-4664-3p (LogFC, 1.42; FDR = .85). We next explored the correlation between differentially expressed basophil microRNAs at baseline and disease activity (Figure 2). Amongst CR, expression levels of both miR-1236-3p (Figure 2A) and miR-1200 (Figure 2C) correlated positively with the weekly UAS7 scores ($R^2 = 0.34$, $p = 0.01$ and $R^2 = 0.34$, $p = .01$,

respectively). Both microRNAs showed no significant association with UAS7 scores amongst NR: miR-1236-3p (Figure 2B, $R^2 = 0.11$, $p = .3$) and miR-1200 (Figure 2D, $R^2 = 0.05$, $p = .5$). There was no significant association between miR-4664-3p levels and UAS7 scores amongst CR (Figure 2E, $R^2 = 0.06$, $p = .34$) and NR (Figure 2F, $R^2 = 0.01$, $p = .78$).

Our pilot results highlight a set of differentially expressed basophil microRNAs that may serve as potential biomarkers predictive of response to anti-IgE therapy in CSU and help personalize care for these patients. Commercial microRNA panels have been developed to identify the origin or classification of cancers.⁹ The basophil microRNA expression profiles identified in our study (ie miR-4664-3p, miR-1200, and miR-1236-3p) could serve as a diagnostic panel to guide physicians in selecting CSU patients for omalizumab intervention. This could be a cost-effective approach considering the cost associated with omalizumab treatment and need for multiple doses

before determination of response. Since microRNA expression levels correlate with clinical response, corroboration of this basophil microRNA signature in a second cohort of CSU patients is needed for their validation as biomarkers.

KEYWORDS

Basophils, chronic spontaneous urticarial, microRNA, omalizumab, response biomarker

CONFLICTS OF INTEREST

T.A.-S. has patents MicroRNAs as Predictors of Response to Anti-IgE Therapies in Chronic Spontaneous Urticaria and Methods of Treatment using Omalizumab and Ligelizumab pending. J.W.M. reports grant from Novartis Pharmaceuticals Corporation, during the conduct of the study; In addition, J.W.M. has patents MicroRNAs as Predictors of Response to Anti-IgE Therapies in Chronic Spontaneous Urticaria and Methods of Treatment using Omalizumab and Ligelizumab pending. T.K.B. reports grant from Novartis Pharmaceuticals Corporation, during the conduct of the study; In addition, T.K.B. has patents MicroRNAs as Predictors of Response to Anti-IgE Therapies in Chronic Spontaneous Urticaria and Methods of Treatment using Omalizumab and Ligelizumab pending. M.C.A. reports personal fees from Regeneron, outside the submitted work. A.G.A. has nothing to disclose. D.H.P. reports grant from Novartis Pharmaceuticals Corporation, during the conduct of the study; grants from Genentech, DBV Technologies, Aimmune, Regeneron, and NIH/NIAID (Immune Tolerance Network), outside the submitted work. S.A.T. reports grant from Novartis Pharmaceuticals Corporation, during the conduct of the study; grants from Genentech, DBV Technologies, Aimmune, Regeneron, NIH/NIAID (Immune Tolerance Network), and personal fees from Aimmune, outside the submitted work. W.R.H. reports grant from Novartis Pharmaceuticals Corporation, during the conduct of the study; In addition, W.R.H. has patents MicroRNAs as Predictors of Response to Anti-IgE Therapies in Chronic Spontaneous Urticaria and Methods of Treatment using Omalizumab and Ligelizumab pending.

AUTHOR CONTRIBUTIONS

W.R.H. wrote the investigator-initiated grant proposal and obtained funding for the study. S.A.T., D.H.P. and W.R.H. were responsible for the overall conduct of the clinical study plan with assistance by M.C.A. and A.G.A. T.K.B. directed isolation of basophils and RNA-seq analysis. J.W.M., T.A.-S. and T.K.B. performed statistical analysis and preparation of figures. T.A.-S. and W.R.H. wrote the manuscript. All authors reviewed the manuscript and provided comments.

ETHICAL STATEMENT

The study protocol complied with all relevant ethical regulations in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Western Institutional Review Board. Prior to inclusion in the study, all participants provided written informed consent.

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