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# Changing microRNA Expression during Three-Month Wasp Venom Immunotherapy

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## ABSTRACT

MicroRNAs are small non-coding molecules playing a significant regulatory role in several allergic diseases. However their role in tolerance induction remains unclear. The aim of this study was to determine the expression of selected microRNAs during the first three months of wasp venom immunotherapy (VIT). 5 adult patients with a history of severe systemic reactions after stinging by wasps and confirmed sensitization were included. Venous blood samples were collected before VIT, 24 hours after completing its initial phase and after 3 months of the maintenance therapy. A control group was comprised of 5 healthy individuals with no history of allergy. In the blood samples expression of 96 microRNAs was determined with the use of microfluidic cards. In a statistical analysis the expression was compared between the study groups as well as between the pre- and post-VIT samples. Significant differences were found between the patients with wasp venom allergy and the healthy controls in the expression of miR-601 and miR-1201 upregulated in allergic patients at every time point ( $p = 0.04$ ;  $p = 0.015$ , respectively). During VIT profile of microRNA was changing with lower expression of 6 microRNAs (including miR-182, miR-342, miR-375) and higher of 11 microRNAs (including let-7d, miR-34b, miR-143). To conclude, VIT has led to some changes in the expression of microRNA associated with Th2-type inflammation and tolerance induction.

## KEYWORDS

Hymenoptera allergy; miRNAs; venom immunotherapy; wasp venom allergy; let-7d; miR-34b; miR-143; miR-182; miR-342; miR-375

## Introduction

Allergen-specific immunotherapy is a recommended causative method of treatment of allergic asthma, rhinitis, atopic dermatitis, and insect venom allergy (Bonifazi et al., 2005; Dhami et al., 2017; GINA, 2018). It restores normal immunity against allergens by modulating both cellular and humoral responses. Very early effects of the immunotherapy result from mast cells and basophils desensitization. Intermediate effects are caused by several changes in allergen-specific T cells and late effects are related to decreased IgE synthesis and an altered function of mast cells, basophils, and eosinophils (Akdis and Akdis, 2007). It is generally believed that the key phenomenon in the tolerance induction is Th2 cells suppression by Treg cells secreting IL-10 and TGF $\beta$ . IL-10 has also

a suppressive effect on the effector cells (mast cells, basophils) and changes the type of humoral response from IgE to IgG<sub>4</sub>-dominated phenotype (Jutel and Akdis, 2011).

A process of tolerance induction in the immunotherapy has been investigated on a genetic level and expression of several genes involved in intracellular signalling and cell adhesion has been found to be changed (Niedoszytko et al., 2010). These processes are probably influenced by microRNAs (miRNAs) that are short, non-coding molecules regulating genes expression by degradation of mRNA or inhibition of translation. They are now considered to form one of the most important epigenetic mechanisms regulating cells proliferation, activation, and maturation (Bartel, 2009; Lagos-Quintana et al., 2001; Lu and Rothenberg, 2013). In recent years, the profiles of microRNAs expression in several allergic diseases have been demonstrated (Garbacki et al., 2011; Lu et al., 2012; Panganiban et al., 2012; Shaoqing et al., 2011). The crucial processes influenced by miRNAs are: eosinophils development, T cells differentiation and activation, development and activation of mast cells. However, their involvement in the process of tolerance induction is not understood and warrants a further investigation.

The aim of this study was to determine the profile of 96 miRNAs expression in patients undergoing specific immunotherapy with a wasp venom.

## Methods

The study group comprised five adult patients, including three women and two men, aged 23–60 (mean age: 45.8 years) with a history of a severe anaphylactic reaction after stinging by a wasp (grade III or IV in Mueller's classification) (Mueller, 1966). In all patients, sensitization to the wasp venom had been confirmed by both skin tests and sIgE measurement in the sera. Exclusion criteria were: pregnancy, mast cells disorders, acute or chronic infection, cancer diagnosed in previous 5 years, concomitant autoimmune disease, unstable cardiac insufficiency, kidney failure, hepatic cirrhosis, and poor compliance.

A control group was comprised of five healthy adult subjects (two women, three men) aged 27–50 years (mean age: 38 years). Exclusion criterion was any clinically significant disease including allergy or asthma.

Informed consent was taken from every participant before an inclusion into the study.

## Protocol of the study

Patients with the history of sting-related anaphylaxis were screened according to the aforementioned inclusion and exclusion criteria. The wasp venom immunotherapy (VIT) was performed in the Department of Allergology, Medical University of Gdańsk. The initial phase was conducted according to ultra-rush protocol. A cumulative dose of 101.1 µg of the wasp venom was administered over 210 min (0.1; 1; 10; 20; 30; 40 µg Venomenhal Wespe, HAL Allergy, the Netherlands). Consecutively, a maintenance phase was initiated during which the patients were admitted to the clinic every 4–5 weeks and the dose of 100 µg of wasp venom was administered. During the treatment clinical information concerning unwanted effects (anaphylaxis, large local reactions during VIT) and field stings were recorded.

Venous blood was collected before VIT, 24 h after completing its initial phase and after 3 months of the maintenance phase. In the healthy controls, blood sampling was performed once. Samples were stored at the temperature of  $-80^{\circ}\text{C}$  till the processing.

MiRNAs expression in participants' whole blood was determined in the Laboratory of Forensic Genetics, Medical University of Gdańsk. Total RNA containing miRNA was isolated from blood with Tempus<sup>TM</sup> Spin RNA Isolation Reagent Kit (*Applied Biosystems, USA*). The RNA concentration was assessed spectrophotometrically using NanoDrop<sup>®</sup>. Reverse transcription was carried out with 450 ng of total RNA with TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit (*Applied Biosystems, USA*). The obtained cDNA was quantified by qRT-PCR in duplicates with the use of miRNAs specific primer pairs, fluorescent TaqMan probes and polymerase with 5' nuclease activity in customized microfluidic cards (Custom TaqMan<sup>®</sup> Array Microfluidic Cards, *Applied Biosystems, USA*) in HT 7900 cyclor (*Applied Biosystems, USA*), with reaction conditions in accordance with manufacturer's recommendations. Totally the presence of 96 miRNAs was assessed. They had been selected on the basis of the pilot study published previously (Specjalski et al., 2016). The results were expressed as  $C_T$  (threshold cycle) – number of a cycle with fluorescence exceeding detection level, as well as RQ (relative quantification) – an amount of transcript in relation to a calibration sample. When comparing expression of miRNAs between the groups the 2-fold difference was regarded as significant.

In a statistical analysis, expression of selected miRNA was compared between the patients with wasp venom allergy and the control group, in the next step – among pre-VIT, post-initial phase, and post-maintenance phase samples. Due to the lack of normal distribution, Mann Whitney U test was used for comparing the study groups. In order to assess the variability of miRNAs expression over time, Wilcoxon rank test was applied. In all statistical tests,  $p < .05$  was considered to be statistically significant.

The protocol of the study has been approved by Independent Bioethics Committee of Medical University of Gdańsk (no. NKBBN/133–287/2016).

## Results

In all patients, the wasp VIT was conducted according to the protocol, with no significant adverse events and no field stings during a 3-month observation.

Out of 96 miRNAs determined, two were downregulated in group with insects' venom allergy compared to healthy controls: miR-601 ( $1.7809 \pm 1.5433$  vs.  $6.41 \pm 3.85$ ;  $p = .04$ ) and miR-1201 ( $1.6385 \pm 2.1329$  vs.  $4.133 \pm 0.93$ ;  $p = .015$ ). The differences remained significant despite immunotherapy.

During VIT, expressions of several miRNAs were changing significantly (Table 1). After the initial phase expression of six miRNA increased and two decreased in at least four patients. After 3 months of VIT, the profile of microRNA was changed with lower expression of 6 microRNA (including miR-182, miR-342, miR-375) and higher of 11 microRNAs (including let-7d, miR-34b, miR-143). The role of detected miRNAs in allergic diseases is presented in Table 2.

## Discussion

MiRNAs regulate functioning of the immune system by targeting multiple pathways. As a result some of them enhance inflammation and allergic reactions while the other

**Table 1.** MiRNAs with a significant (i.e. at least 2-fold) change after initial phase of wasp venom immunotherapy or 3 months of maintenance phase of immunotherapy. The results are presented as mean RQ (relative quantification)  $\pm$  standard deviation. The differences found in four patients are in bold; the differences found in five patients are additionally marked with asterisk.

|                                  |            | Mean RQ $\pm$ SD<br>before VIT | Mean RQ $\pm$ SD after<br>initial phase | Mean RQ $\pm$ SD after<br>3 months of VIT | <i>p</i> |
|----------------------------------|------------|--------------------------------|-----------------------------------------|-------------------------------------------|----------|
| Increase during<br>immunotherapy | miR-1201   | 1.6395 $\pm$ 2.1329            | <b>3.71 <math>\pm</math> 5.5368</b>     | <b>2.9638 *</b> $\pm$ 1.688               | 0.25     |
|                                  | miR-1303   | 3.398 $\pm$ 2.3305             | <b>9.1194 <math>\pm</math> 11.289</b>   | <b>23.5115*</b> $\pm$ 14.661              | 0.013    |
|                                  | let-7d     | 0.2433 $\pm$ 0.278             | 0.4135 $\pm$ 0.392                      | <b>0.6343</b> $\pm$ 0.673                 | 0.24     |
|                                  | miR-34     | 0.0236 $\pm$ 0.0121            | <b>0.676 <math>\pm</math> 0.095</b>     | <b>0.0484</b> $\pm$ 0.0322                | 0.04     |
|                                  | miR-143    | 1.8564 $\pm$ 3.340             | <b>3.7764 <math>\pm</math> 6.584</b>    | <b>3.4816</b> $\pm$ 4.3812                | 0.47     |
|                                  | miR-204    | 0.0065 $\pm$ 0.009             | <b>1.3329 <math>\pm</math> 2.325</b>    | <b>2.7946 <math>\pm</math> 3.204</b>      | 0.06     |
|                                  | miR-299-5p | 0.1732 $\pm$ 0.3161            | 0.0804 $\pm$ 0.1430                     | <b>0.6462</b> $\pm$ 1.523                 | 0.34     |
|                                  | miR-379    | 0.5223 $\pm$ 1.348             | 0.7930 $\pm$ 1.2931                     | <b>1.2482</b> $\pm$ 3.2087                | 0.34     |
|                                  | miR-485-3p | 0.6458 $\pm$ 0.6216            | 0.7941 $\pm$ 0.4171                     | <b>0.7233</b> $\pm$ 0.5901                | 0.76     |
|                                  | miR-516-3p | 0.8095 $\pm$ 0.7142            | 0.9841 $\pm$ 0.6956                     | <b>3.3108</b> $\pm$ 4.838                 | 0.2      |
|                                  | miR-601    | 1.7809 $\pm$ 1.5433            | <b>5.7167 <math>\pm</math> 6.4067</b>   | <b>2.4808</b> $\pm$ 2.313                 | 0.33     |
|                                  | miR-182    | 15.7313 $\pm$ 10.776           | 9.503 $\pm$ 6.289                       | <b>8.2269</b> $\pm$ 5.23                  | 0.08     |
| Decrease during<br>immunotherapy | miR-342-3p | 3.0959 $\pm$ 2.8348            | <b>1.6496</b> $\pm$ 0.9866              | <b>2.1275</b> $\pm$ 1.405                 | 0.34     |
|                                  | miR-375    | 0.9559 $\pm$ 1.544             | 2.3086 $\pm$ 4.437                      | <b>0.0446</b> $\pm$ 0.073                 | 0.17     |
|                                  | miR-489    | 0.0373 $\pm$ 0.414             | <b>0.018 <math>\pm</math> 0.015</b>     | <b>0.02538</b> $\pm$ 0.203                | 0.58     |
|                                  | miR-591    | 5.5183 $\pm$ 2.587             | 4.318 $\pm$ 4.708                       | <b>3.8885</b> $\pm$ 8.206                 | 0.77     |
|                                  | miR-643    | 1.0663 $\pm$ 1.826             | 1.0516 $\pm$ 1.747                      | <b>0.8361</b> $\pm$ 1.034                 | 0.8      |

**Table 2.** Possible role of selected miRNAs in the pathomechanism of allergy and tolerance induction.

| miRNA    | Possible role in allergic diseases                                                                                                                                                                                                    |
|----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| let-7d   | Decreases expression of IL-13, 6 and TLR4 (Kumar et al., 2011)<br>Negatively correlates with lung function in asthma (Panganiban et al., 2012)                                                                                        |
| miR-34b  | Down-regulated in asthma (Levanen et al., 2013). Repressed by IL-13 (Solberg et al., 2012).<br>Upregulated by IL-4 (Bao et al., 2018)                                                                                                 |
| miR-143  | Increases expression of FOXP3. Stimulates proliferation of CD25+ CD4+ lymphocytes. Downregulates IL-13 (Yu et al., 2013). Downregulated in allergic rhinitis (Shaoqing et al., 2011). Upregulated in asthma (Suojalehto et al., 2014) |
| miR-182  | Differentiation of Th17 cells (Kastle et al., 2017). Regulates proliferation of T cells and Treg cells function (Bothur et al., 2015; Stittrich et al., 2010)                                                                         |
| miR-204  | Regulates bronchial smooth muscles cells proliferation (Courboulin et al., 2011)                                                                                                                                                      |
| miR-299  | Downregulated in asthma (Panganiban et al., 2016)                                                                                                                                                                                     |
| miR-342  | Regulates Treg function. Targets NF $\kappa$ B (Hezova et al., 2010)                                                                                                                                                                  |
| miR-375  | Upregulated in bronchial epithelial cells in pollutants-induced exacerbations of asthma (Bleck et al., 2013). Blocks expression of TLR7 in asthmatic patients (Rupani et al., 2016).                                                  |
| miR-379  | Induced by IL-13. Regulated cell surface receptor linked signal transduction (Ooi et al., 2012)                                                                                                                                       |
| miR-485  | Upregulated in asthma (Liu et al., 2012)                                                                                                                                                                                              |
| miR-489  | Upregulated in mice model of allergic rhinitis (Rutledge et al., 2015). Correlates with IFN $\gamma$ in asthmatics (Suojalehto, 2014)                                                                                                 |
| miR-628  | Dowregulated in rhinosinusitis (Ma et al., 2015). Upregulated in eosinophilic esophagitis (Lu et al., 2012). Controls TLR signaling (Jun et al., 2015)                                                                                |
| miR-643  | Regulates expression of IL-17 (Mai et al., 2012)                                                                                                                                                                                      |
| miR-1180 | Activates NF $\kappa$ B (Zhu et al., 2018)                                                                                                                                                                                            |
| miR-1303 | Regulates gene ADAM33 whose altered function is related to bronchial hyperreactivity (Vishweswaraiah et al., 2014)                                                                                                                    |

facilitate resolution of inflammation and have tolerogenic properties. In the allergic diseases, the crucial processes influenced by miRNAs are: eosinophils development, T cells differentiation and activation, cytokines production, development and activation of effector cells (mast cells, basophils). For example, miR-155, upregulated in atopic dermatitis, enhances inflammation, and activates T cells, what is associated with increased

secretion of IL-4, IL-5, IL-13, and IL-17a (Zhang et al., 2017; Zhou et al., 2016). MiR-21 and miR-19a promote differentiation of T cells towards Th2 (Li et al., 2007; Long et al., 2017; Lu et al., 2011; Sawant et al., 2013). MiR-513-5p, miR-22-3p, and miR-625-5p decrease secretion of Th1 cytokines including IL-12 and IFN $\gamma$  (Dong et al., 2016). MiR-210 inhibits tolerogenic function of Treg cells (Long et al., 2017). On the contrary, let-7 family inhibits secretion of IL-13 and miR-146a negatively regulates IL-5 (Polikepahad et al., 2010; Yang et al., 2017). In the recent years, profiles of miRNAs expression in several allergic diseases have been demonstrated and some miRNAs have been proposed as the biomarkers characterized by satisfactory sensitivity and specificity (He et al., 2017; Jia et al., 2018). However, whether the changed expression of miRNAs could be reversed by the curative measures is unclear (Solberg et al., 2012).

In the recent years, some studies tried to investigate the profile of miRNAs during the allergen immunotherapy. In the study of Luo et al. (2016), 24 children with allergic rhinitis and house dust mites allergy were treated either with sublingual (SLIT) or subcutaneous immunotherapy (SCIT). After 3 months of treatment in both groups, an increased expression of miR-146a correlated positively with Foxp3 mRNA level and negatively with disease severity. Increased IL-10 and decreased of IL-5 levels were also found with no differences between SLIT and SCIT. However, changing expression of miRNAs was not confronted with a clinical response to the immunotherapy. Hou et al. (2015) assessed that in patients with allergy to Japanese cedar pollen undergoing SLIT. Out of 15 patients, 6 underwent sublingual immunotherapy with the pollens and 9 received placebo. During a pollinating season seven subjects in the placebo group and none administered SLIT developed symptoms of rhinitis, respectively. Sera miRNAs levels were determined in pre- and post-seasonal samples. The only miRNAs that changed significantly in both, the SLIT and the placebo group, was miR-223. In the placebo group, development of pollinosis was significantly associated with downregulation of let-7b. Thus, the available data do not indicate that any miRNAs can be used as a reliable biomarker in the monitoring of the allergen immunotherapy.

In the preliminary study published previously, we have determined 740 miRNAs in whole blood of patients with insects venom allergy in order to select those expressed reproducibly (Specjalski et al., 2016). Out of 740 miRNAs, 440 were detected in the study group. Expression of 67 miRNAs was found to differ at two time points – before and 24 after initial phase of VIT. These together with 29 miRNAs with reproducible expression, and described role in the development of allergic diseases, were chosen for this study.

The major limitation of the preliminary study was the comparison of miRNAs expression before VIT and after its initial phase only. However, tolerance induction is considered a long process and the efficacy of immunotherapy correlates with the cumulative dose of allergen administered. As a consequence, it seemed crucial to assess miRNAs expression during long-term maintenance phase of VIT as well.

In the current study, significant differences between the patients with insects venom allergy and the healthy controls were found in expression of miR-601 and miR-1201. So far the role of these molecules in the pathomechanism of allergic diseases has not been mentioned.

Overall 17 out of 96 miRNAs changed their expression significantly during VIT, including 8 miRNAs changing their expression as early as 24 hours after completing the

initial phase of VIT. The most significant findings included: upregulation of let-7d (anti-inflammatory and tolerogenic), miR-143 (increasing FOXP3 expression and repressing IL-13), miR-34b (downregulated by IL-13 in allergic diseases), and downregulation of miR-342 (regulating Treg function and NF $\kappa$ B expression), miR-375 (regulating IL-13 secretion), miR-182 (regulating Th17 and Treg cells function). Thus, we found some changes in miRNAs expression that could explain protective properties of VIT observed even on early stages of the therapy. The current study has confirmed some findings of the preliminary study, particularly an increase in expression of let-7d, miR-143, miR-204, miR-1201, miR-1303, miR-379, and miR-601 during VIT. However, for the remaining miRNAs the tendencies have not been reproduced. It is difficult to assess whether it was due to the small number of participants or some of the changes were random.

Changing profile of miRNAs expression at the early stages of immunotherapy can be explained by some previously published studies. Mamessier et al. (2006) have shown that some of the effects may be observed just after 3, 5 hour ultra-rush. They found decreased levels of IL-4 compared to results from before VIT, with an increase in a number of IL-10 + T cells and higher proportion of CD4<sup>+</sup> CD25<sup>high</sup> regulatory cells. The significant difference was also found in IL-10 concentration in the study of Bussmann et al. (2010) after the first day of bee VIT conducted according to rush protocol. The most important phenomenon leading to tolerance of an allergen is Th2-cells suppression by Treg cells secreting IL-10 and TGF $\beta$ . We revealed change in the expression of miR-143, miR-182, and miR-342 controlling Treg function that could be related to this effect. MiR-143 was found upregulated after 3 months of treatment while miR-182 and miR-342 already after completing the initial phase of VIT.

The induction of allergens' tolerance by the immunotherapy has been applied in a clinical practise for decades and its efficacy has been well proven. However, due to the complex immunologic and epigenetic processes that stand behind it, assessment of efficacy of immunotherapy has always been challenging. In relation to the immunotherapy with common airborne allergens such as pollens or mites symptom scores are applied to assess reaction to everyday exposure. In insects venom allergy exposure is usually rare, and considering history of life-threatening anaphylactic reactions, stings should be avoided (no patients were stung during the observation period). Provocation tests are also dangerous and performing them only for scientific reasons could be considered unethical. The lack of correlation between changing profile of miRNAs and clinical response is one of the major limitation of this study. It makes it difficult to assess which tendencies in miRNAs expression are truly related to protective properties of VIT.

Another limitation of this study was small population included and large number of parameters studied. That might result in an overestimation of the detected differences. Finally, the expression of miRNAs was assessed no later than 3 months of the maintenance phase of VIT. It is generally believed that the tolerance induction is much longer process involving different mechanisms in the subsequent phases.

## Conclusion

This study has led to better understanding of epigenetic mechanisms of the wasp VIT by demonstrating some changes in the miRNAs expression during the treatment. It has been shown that VIT is associated with an upregulation of tolerogenic let-7d and

a downregulation of miR-375 associated with secretion of Th2 cytokines, miR-182, and miR-342 regulating T cells function. Despite this, the results of this study may be a starting point for some future investigations on epigenetic regulation of tolerance induction and the relations between this regulation and efficacy of immunotherapy.

## Disclosure of interest

The authors report no conflicts of interests.

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