

ORIGINAL ARTICLE

Identification of novel targets for seasonal allergic rhinitis during and outside the pollen season by microarray analysis

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Abstract

Conclusion: *FOS*, *JUN*, and *DUSP1* could be used as candidate targets for the treatment of seasonal allergic rhinitis (SAR) during the pollen season, while *KLF4* and *CD163* could be used as candidate targets outside the pollen season. **Objectives:** The aim of this study is to screen novel genes related to SAR during and outside the pollen season, by using microarray analysis. **Methods:** The mRNA expression profile (GSE50101) of CD4⁺ T cells from SAR and healthy controls during and outside the pollen season was downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) were then screened with the cut-off criteria of fold change larger than 1.5 and *p*-value less than 0.05. **Results:** A total of 47 DEGs were identified. Ten DEGs were shared by SAR patient samples during and outside the pollen season, while four and 23 DEGs were specific to during and outside the pollen season, respectively. Five miRNAs were screened in this study. Among these miRNAs, miR-139, miR-101, miR-29A, and miR-181 could target *FOS*; miR-200 and miR-29A could target *KLF4*; miR-101 and miR-200 could target *DUSP1*; miR-139 and miR-181 could target *JUN* and *CD163*, respectively.

Keywords: Seasonal allergic rhinitis, differentially expressed genes, microRNA, network analysis

Introduction

Allergic rhinitis (AR) is a very common disorder that affects people of all ages, which is caused by exposure to an allergen to which a person is sensitized [1]. AR has been classified as seasonal or perennial, depending on sensitization to cyclic pollens or year-round allergens, among which seasonal allergic rhinitis (SAR) usually occurs in particular during pollen seasons [1]. Although AR is not a serious illness, it affects 10–20% of the global population and has a deleterious impact on quality-of-life [2]. Avoidance of allergens and irritants is an effective measure to prevent or reduce symptoms of AR [3]. However, it has proved difficult to implement in daily life.

Currently, gene therapy has been reported to be impressive potential for the treatment of allergic

airway diseases including SAR [4]. Therefore, it is critically needed to screen out candidate target molecules for the treatment of SAR. Nestor et al. [5] have generated genome-wide DNA methylation and gene expression profiles of CD4⁺ T cells to explore the difference between SAR patients and controls, both during and outside the pollen season. Accordingly, they have identified several changes in DNA methylation that might be associated with SAR. In addition, apolipoprotein A-IV was significantly increased in sublingual immunotherapy (SLIT)-treated SAR patients. However, the effective target for treatment of SAR is still unknown. Therefore, there is an urgent need to identify novel genes related to SAR and explore the underlying causative mechanisms.

In the present study, we re-analyzed gene expression profiles (GSE50101) of CD4⁺ T cells from SAR

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patients and healthy controls during and outside pollen season using bioinformatics methods. First, differentially expressed genes (DEGs) were screened in each group and *T*-test was used to test the gene sets in each group. Then, according to the DEGs, enriched microRNAs (miRNAs) were predicted and an interaction network was constructed respectively to identify upstream regulators and interaction pairs among DEGs. Finally, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed for DEGs in the regulatory network to identify key pathways involved by the DEGs. Our study aimed to identify candidate targets for SAR in different periods and develop new treatment methods.

Materials and methods

Microarray data and data pre-processing

The mRNA gene expression profiles (GSE50101) [5] were obtained from the GEO (Gene Expression Omnibus) database (<http://www.ncbi.nlm.nih.gov/geo/>) based on GPL20558 Illumina Human HT-12 V4.0 expression beadchip. The microarray data contained 19 samples of CD4⁺ T cells, respectively collected from nine SAR patients ($n_{\text{female}}:n_{\text{male}} = 4:5$) and 10 age- and sex-matched healthy controls ($n_{\text{female}}:n_{\text{male}} = 5:5$) during the pollen season, as well as 19 samples re-collected from those participants outside the pollen season. The patients with SAR were confirmed by a positive seasonal history and positive ImmunoCap Rapid tests, while healthy controls were defined by a negative seasonal history and ImmunoCap Rapid tests. Those patients with perennial symptoms or asthma were not enrolled in this study. Probes that mapped with the gene names labeled in annotation platform were performed through log2 transformation that could normalize the data from skewed normal distribution to the approximate normal distribution.

Screening of DEGs

Linear Models for Microarray Data (LIMMA) package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>) was used to normalize the data and identify the DEGs between SAR patients and healthy controls both during and outside the pollen season. Finally, the DEGs in each group were screened out with the cut-off criteria of fold change (FC) larger than 1.5 and *p*-value less than 0.05.

Comparison of the gene sets

The specific DEGs during and outside the pollen season and the shared DEGs were considered to three genes sets. Student's *t*-test was used to identify the difference of each gene set between SAR patients and healthy controls with the threshold of *p*-value < 0.05.

Prediction of PPI (protein-protein interaction)

Varieties of cell physiological activities and the reactions of cells to the external and internal environments were connected by the protein interaction network. Therefore, a further investigation of the PPI is necessary for recognizing and understanding the biological phenomena. The Search Tool for the Retrieval of Interacting Genes (STRING, <http://string.embl.de/>) database provides lots of experimental and predicted information for researchers and a combined score of 0.4 is the commonly used threshold to screen out PPIs. The DEGs were scanned by the STRING, and PPI pairs were selected with the default cut-off criterion of combined score larger than 0.4.

Prediction of miRNA and construction of miRNA regulatory network

MiRNAs play important gene-regulatory roles in plants and animals by paring to the mRNAs of protein-coding genes [6]. WebGestalt (<http://www.webgestalt.org/>) was used to search the miRNAs that were significantly related to the DEGs. A *p*-value of less than 0.05 was set as cut-off criterion. Then the regulatory network that contains miRNAs and the PPI pairs were constructed using Cytoscape software (<http://cytoscapeweb.cytoscape.org/>).

KEGG enrichment analysis of the genes in the network

Researches may be confused with the large number of genes derived from microarray data. KEGG pathway enrichment analysis can give biological interpretation of the large gene lists. Herein, the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) was used to identify the enriched KEGG pathways in DEGs with the commonly used criterion of *p* < 0.05.

Results

Screening of DEGs

In the study, 14 and 33 DEGs were respectively screened in SAR patients during and outside the pollen season when compared to healthy controls.

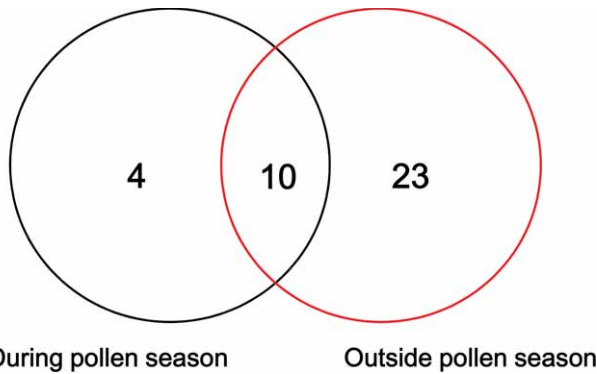


Figure 1. Venn diagram of the differentially expressed genes in seasonal allergic rhinitis patients during and outside the pollen season.

Among the total of 47 DEGs, 10 DEGs were shared by SAR patient samples during and outside the pollen season, while four and 23 DEGs were specific to during and outside the pollen season, respectively (Figure 1). Thus, there was a total of 37 DEGs during and outside the pollen season. We further performed a clustering analysis on the 37 DEGs to show the expression of those DEGs in each patient (Figure 2).

Analysis of the gene sets

The Student's *t*-test was performed for the DEGs in each group. The results showed that there was a significant difference between SAR patients and healthy controls in each gene set ($p < 0.05$) (Figure 3).

Prediction of PPI

In order to explore the interactions among DEGs, STRING was used to analyze the DEGs. The results showed that 44 PPI pairs were predicted in the study (Table I).

Screening and analysis of miRNAs

To obtain the relative miRNAs of the DEGs, WedGeatalt was used to identify the miRNA. With the cut-off criterion of *p*-value less than 0.05, five miRNAs were screened. The results showed that DEGs such as *FOS* and *JUN* were modulated by miR-139; *FOS* and *DUSP1* were modulated by miR-101; *KLF4* and *DUSP1* were modulated by miR-200; *FOS* and *KLF4* were modulated by miR-29A; *FOS* and *CD163* were modulated by miR-181 (Table II). In order to research

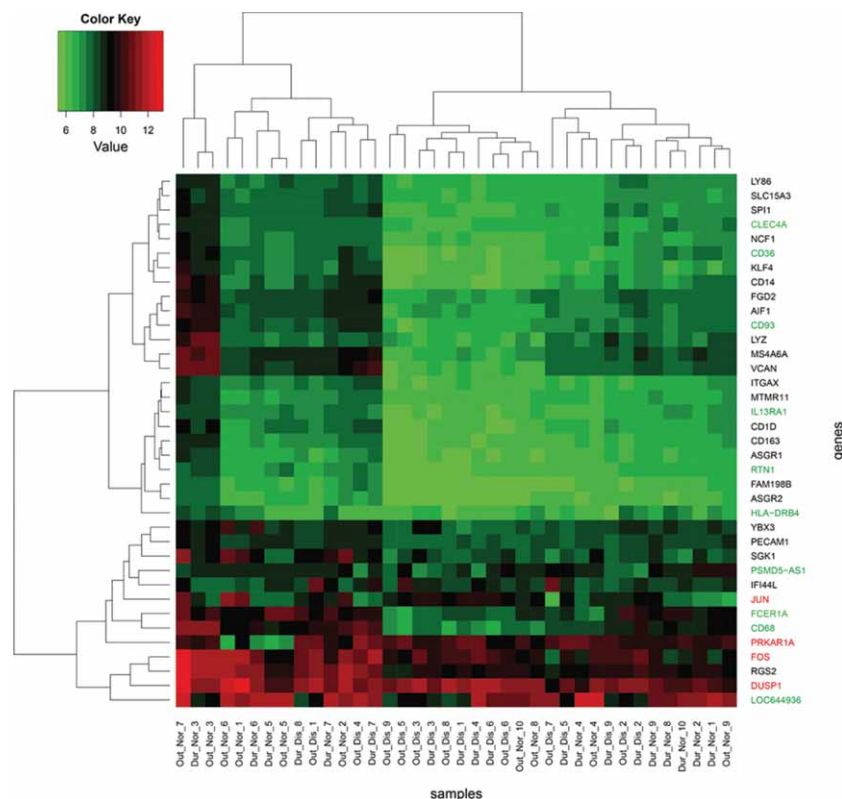


Figure 2. Heatmap of clustering of the 37 DEGs during and outside the pollen season. Genes in red, four specific DEGs during the pollen season; Genes in green, 10 common DEGs during and outside the pollen season; Genes in dark, 23 specific DEGs outside the pollen season. Out, outside the pollen season; Dur, during the pollen season; Nor, normal controls; Dis, disease samples; DEGs, differentially expressed genes.

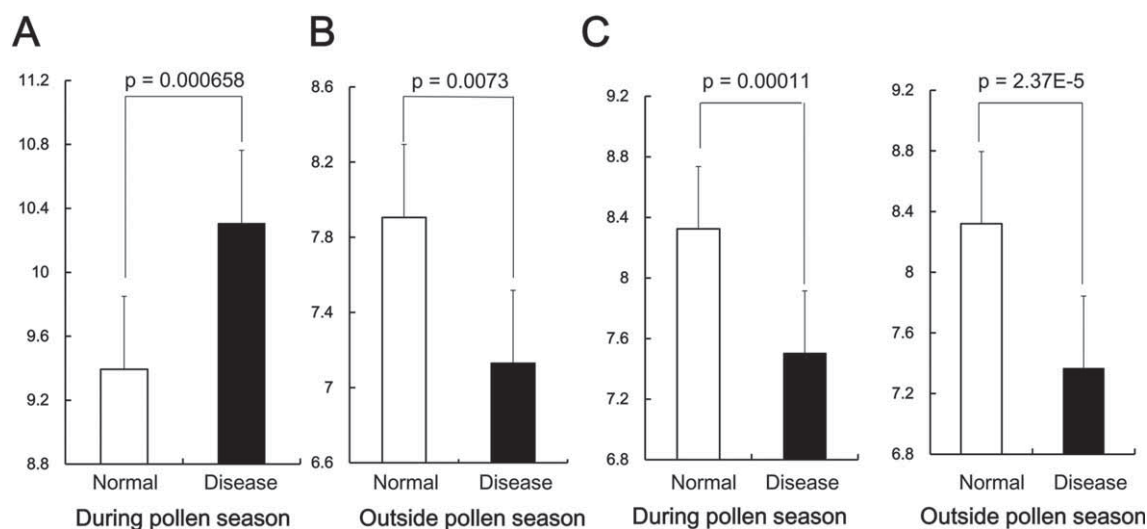


Figure 3. The comparison of the gene sets in each group. (A) Comparison of the specific DEGs during pollen season between seasonal allergic rhinitis (SAR) patients and healthy controls. (B) Comparison of the specific DEGs outside pollen season between SAR patients and healthy controls. (C) Comparison of the shared DEGs during (left) and outside pollen season (right) between SAR patients and healthy controls.

the regulatory functions of the miRNAs, miRNAs and the related DEGs was mapped into the miRNA regulatory network. The miRNA regulatory network contained 22 DEGs including three specific DEGs during the pollen season, 14 specific DEGs outside the pollen season, and five shared DEGs. In the regulatory network, *FOS* was modulated by miR-139, miR-101, miR-29A, and miR-181; *KLF4* was modulated by miR-200 and miR-29A; *DUSP1* was modulated by miR-101 and miR-200; *JUN* and *CD163* were modulated by miR-139 and miR-181, respectively (Figure 4).

KEGG pathway enrichment analysis

To explore the enriched pathways of the DEGs in the network, KEGG pathway enrichment analysis was performed. The results showed that the DEGs in the network were significantly enriched in two pathways. The DEGs such as *FOS*, *JUN*, and *DUSP1* were enriched in Toll-like receptor signaling pathway ($p = 0.013\ 042$); *FOS* and *JUN* were enriched in MAPK signaling pathway ($p = 0.015\ 844$) (Table III).

Table I. The predicted protein-protein interaction pairs of the differentially expressed genes.

Node 1	Node 2	Combined score	Node 1	Node 2	Combined score	Node 1	Node 2	Combined score
JUN	FOS	0.999	MS4A6A	CLEC4A	0.721	ITGAX	CD68	0.490
JUN	SPI1	0.977	LYZ	CD68	0.691	CD14	NCF1	0.470
FOS	DUSP1	0.955	DUSP1	RGS2	0.686	CD14	CLEC4A	0.468
LYZ	SPI1	0.929	CD14	SLC15A3	0.670	MS4A6A	CD68	0.463
CD14	LY86	0.927	CD36	CD68	0.647	CD14	CD93	0.460
CD14	VCAN	0.924	CD68	SPI1	0.640	MS4A6A	SLC15A3	0.455
JUN	DUSP1	0.912	CD14	CD68	0.619	NCF1	SLC15A3	0.455
ASGR2	ASGR1	0.911	CD163	CD36	0.617	ITGAX	SLC15A3	0.448
JUN	FCER1A	0.899	FOS	RGS2	0.614	SLC15A3	SPI1	0.446
FCER1A	FOS	0.899	CD68	SLC15A3	0.583	CD36	CD14	0.432
CD163	CD68	0.811	KLF4	FOS	0.547	MS4A6A	CD93	0.423
CD163	MS4A6A	0.804	NCF1	SPI1	0.533	ITGAX	CD93	0.408
CD163	CD14	0.761	CD163	CLEC4A	0.531	CD163	CD93	0.406
FOS	SPI1	0.755	MS4A6A	LY86	0.514	CD14	LYZ	0.400
MS4A6A	CD14	0.743	MS4A6A	CD36	0.498			

Table II. The enriched miRNAs of the differentially expressed genes.

miRNA	ID	Parameters	Targets
Has-ACTGTAG,MIR-139	DB-ID:727	O = 2; raw p -value = 0.0035; adjusted p -value = 0.0280	<i>FOS</i> , <i>JUN</i>
Has-GTACTGT,MIR-101	DB-ID:792	O = 2; raw p -value = 0.0132; adjusted p -value = 0.0291	<i>FOS</i> , <i>DUSP1</i>
Has-CAGTATT,MIR-200	DB-ID:865	O = 2; raw p -value = 0.0373; adjusted p -value = 0.0468	<i>KLF4</i> , <i>DUSP1</i>
Has-TGGTGCT,MIR-29A	DB-ID:674	O = 2; raw p -value = 0.0468; adjusted p -value = 0.0468	<i>FOS</i> , <i>KLF4</i>
Has-TGAATGT,MIR-181	DB-ID:841	O = 2; raw p -value = 0.0435; adjusted p -value = 0.0468	<i>FOS</i> , <i>CD163</i>

DB-ID, database identifier.

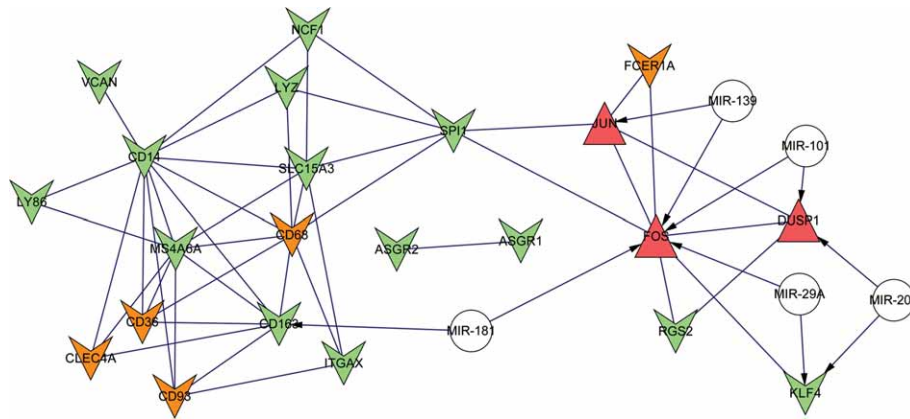


Figure 4. The microRNA (miRNA) regulatory network. The reverse and regular triangles indicate up- and down-regulated differentially expressed genes (DEGs), respectively. The red and green nodes indicate the specific DEGs during and outside pollen season, respectively. The orange nodes indicate the shared DEGs in two groups. The circular nodes indicate miRNAs.

Table III. The enriched KEGG pathways of differentially expressed genes in the regulatory network.

ID	Pathway	Count	p -value	Genes
hsa04010	Toll-like receptor signaling pathway	4	0.013 042	<i>FOS</i> , <i>JUN</i> , <i>DUSP1</i> , <i>CD14</i>
hsa04620	MAPK signaling pathway	3	0.015 844	<i>FOS</i> , <i>JUN</i> , <i>CD14</i>

Discussion

In this study, we re-analyzed the gene expression profiles of CD4⁺ T-cells in SAR to identify new targets for the therapy of SAR during and outside the pollen season. A total of 14 and 33 DEGs were obtained in SAR groups during and outside the pollen season, respectively. Five significantly enriched miRNAs (miR-139, miR-101, miR-200, miR-29A, miR-181) were then screened. Since miRNAs play key roles of post-transcriptional regression by targeting mRNAs, we performed interaction network analysis of these miRNAs and their target genes (such as *FOS*, *JUN*, *DUSP1*, *KLF4*, *CD163*). The KEGG enrichment analysis results showed that DEGs in

the network such as *FOS*, *JUN*, and *DUSP1* were significantly enriched in Toll-like receptor signaling pathway; *FOS* and *JUN* were significantly enriched in MAPK signaling pathway.

According to the regulatory network, miR-139, miR-101, miR-29A, and miR-181 could directly target *FOS*. As previously demonstrated, *FOS* (FBJ murine osteosarcoma viral oncogene homolog), also known as *c-FOS*, could interact with *JUN* (jun proto-oncogene, c-JUN) to form the transcription factor activating protein 1 (AP-1) that is crucial for cell adaptation to environmental changes [7]. *AP-1* has an integral function in certain immune cells such as monocytes/macrophages, lymphocytes, and mast cells [8]. Besides, epithelial *JUN* promotes keratinocyte proliferation and differentiation during development

by controlling the expression of epidermal growth factor receptor (EGFR) and heparin-binding EGF-like growth factor [9]. In this study, *JUN* was one of the down-regulated DEGs and modulated by miR-139. Further, T-cell immunoglobulin and mucin domain 3 (*TIM3*) has been also reported to inhibit *JUN* and *FOS* expression, leading to a reduction in *AP-1* activity, which plays a key role in negative CD4⁺ T cell regulation in human autoimmune disease [10]. Therefore, *FOS* and *JUN* might be closely related to immune reactions by interacting with *TIM3* in patients with SAR.

DUSP1 is a central regulator of innate immunity and affects the outcome of inflammatory challenges [11]. *FOS*, *JUN*, and *DUSP1* were significantly down-regulated in SAR during the pollen season, which implied *FOS*, *JUN*, and *DUSP1* might be involved in SAR during the pollen season. Further, *FOS*, *JUN*, and *DUSP1* were found to be enriched in a Toll-like receptor signaling pathway. Toll-like receptors are found to recognize specific pathogen-associated molecular patterns, triggering a host defense response, and activate signaling pathways that result in immune responses against microbial infections, including MAPK signaling pathway [12]. It has been shown that the expressions of Toll-like receptors 2, 3, and 4 were up-regulated in the nasal mucosa of SAR patients during the pollen season when compared to the SAR patients outside the pollen season [13]. In addition, *FOS* and *JUN* were also enriched in MAPK signaling pathway in the results. The p38 MAPK is involved in the regulation of the synthesis of inflammation mediators and is a potential target for anti-inflammatory therapeutics [14]. Although the role of *FOS*, *JUN*, and *DUSP1* in SAR remains unknown, they might be used as potential markers for SAR during natural allergen exposure.

KLF4 is an essential transcription factor for monocyte development which regulates host defenses, inflammation, and tissue homeostasis as part of the innate immune system [15]. *CD163* is found to directly induce intracellular signaling resulting in secretion of anti-inflammatory cytokines, and soluble CD163 might be a diagnostic parameter for monitoring macrophage activation in inflammatory conditions [16]. Our results showed that *KLF4* and *CD163* were significantly up-regulated in SAR patients outside the pollen season, which implied *KLF4* and *CD163* might be involved in SAR outside the pollen season.

Besides, miRNAs are considered to play a critical role in the development of cells of immune system and in the regulation of immune response [6]. In this study, five mRNAs were identified, including

miR-139, miR-101, miR-200, miR-29A, and miR-181. MiR-101 was shown to regulate the innate immune responses of macrophages through targeting MAPK phosphatase-1 (*MKP-1*) [17]. The MiR-200 family was considered to play an essential role in suppression of diseases by repressing epithelial-mesenchymal transition (EMT) related to immune response [18]. MiR-29 is related to innate and adaptive immune responses to intracellular bacterial infection and miR-29A could regulate the secretion of pro-inflammatory cytokine [19]. MiR-181 has been found to participate in the control of the antibody response [20]. The results showed that miR-101 could modulate *JUN* and *DUSP1*; miR-200 could modulate *KLF4* and *DUSP1*; miR-29A could modulate *FOS* and *KLF4*; miR-181 could modulate *FOS* and *CD163*. The network also showed that miR-139 might target *FOS* and *JUN* to influence SAR. Although the relationship between miR-139 and immune system or SAR is seldom reported, miR-139 was shown to regulate *FOS* in the regulatory network. Our results also suggested that these miRNAs might be involved in SAR by regulating the target genes.

In conclusion, we screened several DEGs (such as *FOS*, *JUN*, *DUSP1*, *KLF4*, *CD163*) in total CD4⁺ T-cells from SAR patients compared to healthy controls during and outside the pollen season by analyzing mRNA expression profile. The circulating total CD4⁺ T-cells were complex, consisting of several sub-sets which may vary between individuals. The complexity might make it difficult to identify subtle allergy-specific transcriptional signals using microarray data [5]. However, we still obtained some useful therapeutic targets using bioinformatics methods. Our study suggested that *FOS*, *JUN*, and *DUSP1* could be used as candidate targets for the treatment of SAR during the pollen season, while *KLF4* and *CD163* could be used as candidate targets outside the pollen season. Furthermore, miR-139, miR-101, miR-200, miR-29A, and miR-181 might also be related to human SAR. The results were obtained from bioinformatics analyses which could provide more information on molecular mechanisms underlying SAR. However, the present study can only be a preliminary study, and further experiments are recommended to validate the results.

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