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A Study of Plasma miR-23a Expression in Vitiligo Patients

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Abstract

Background: **Vitiligo is a chronic autoimmune skin depigmentation disorder with multifactorial causation, involving genetic susceptibility, immunological events, and environmental triggers. The exact molecular mechanisms of vitiligo development and progression are poorly understood. Recent studies reported microRNAs as promising biomarkers for disease detection and molecular targets for future treatment.** ***Aim:*** **Evaluating the expression level of circulating miR-23a in vitiligo patients and its association with the clinical features of vitiligo.** ***Subjects and Methods*****: This is a case-control study comprising 50 vitiligo patients and 44 healthy controls. Plasma miR-23a expression levels were estimated by quantitative real-time PCR. Bioinformatic analysis for the miR-23a gene was performed.** ***Results:*** **Vitiligo patients displayed significantly lower circulating miR-23a expression levels compared to healthy controls. There was a significant negative correlation between miR-23a fold change and Vitiligo Area Severity Index (*p*= 0.003). Plasma miR-23a levels discriminated between vitiligo patients and controls with 60 % specificity and 64% sensitivity at the optimal cut-off value of 0.23 and likelihood ratio 1.61 (AUC=0.67).** ***Conclusion*****: miR-23a along with its putative target genes could play a role in vitiligo pathogenesis.**

Keywords: MicroRNA, Autoimmune, Vitiligo, Skin

Introduction

Vitiligo, the most common skin depigmentation disorder, is a chronic autoimmune skin disorder characterized by depigmented skin patches resulting from the selective destruction of melanocytes and is associated with psychosocial stigmatization[1,2]. Vitiligo is a multifactorial disease involving several mechanisms which lead to melanocyte loss; these include genetic predisposition and environmental factors, as well as immune, metabolic, and epigenetic alterations[1,2]. The two major forms of vitiligo, non-segmental vitiligo (NSV) and segmental vitiligo[3], are believed to have overlapping pathogenesis[1]. Progress in understanding the molecular and pathological basis of vitiligo offers the hope of advancing vitiligo treatment. MicroRNAs (miRNAs) are a group of short (19-24 nucleotide-long) noncoding RNAs (ncRNAs) that are involved in the regulation of gene expression at both the post-transcriptional and translation level. miRNAs are thus involved in both biological and disease processes[4]. Recently, miRNAs have been shown to be important players in the pathogenesis of autoimmune diseases[5] including those involving the skin[6]. MiR-23a is

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one of the mRNAs most extensively researched in cancer. It mediates tumor cell proliferation, survival, and migration, and controls the cancer microenvironment through modulating immune function and angiogenesis[7]. MiR-R23a has immune functions and has been shown to regulate B-cell development *in vivo*[8]. Moreover, a role for miR-23a has been identified in autoimmune diseases including psoriasis Vogt-Koyanagi-Harada syndrome, psoriasis, and rheumatoid arthritis[9-12]. Due to a lack of published data investigating miR-23a in vitiligo patients, this study was conducted to investigate the level of circulating miR-23a in the plasma of vitiligo patients, and the possible association with clinical features of vitiligo.

Subjects and Methods

Study population

This case-control study comprised 50 vitiligo patients and 44 age- and sex-matched healthy volunteers as a control group. Vitiligo patients were recruited from the Dermatology Clinic of the Suez Canal University Hospital (Ismailia, Egypt). A diagnosis of vitiligo was based on history, clinical examination, and using Woods's lamp. Patients with other hypopigmentation disorders were excluded. Clinical findings and assessment of the Vitiligo disease activity score (VIDA) [13], Vitiligo European Task Force (VETF) score [14], and Vitiligo Area Severity Index (VASI) score [15] were determined by the attending Dermatologist. The healthy volunteers were recruited from the Suez Canal University Hospital Blood Bank (Ismailia, Egypt). Individuals with chronic skin diseases or a family history of vitiligo were excluded. Written informed consent was obtained from all participants. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Medical Research Ethics Committee of the Suez Canal University Faculty of Medicine (Ismailia, Egypt).

MicroRNA-34a expression analysis

Blood Sample collection and RNA extraction: Three ml of fresh venous blood were collected in vacutainer tubes containing EDTA anticoagulant. Total RNA, including the miRNA, was isolated from plasma obtained after centrifugation of fresh blood samples using the Qiagen miRNeasy Mini kit (Cat No. 217004, QIAGEN, Hilden, Germany) following the protocol supplied by the manufacturer. Total RNA quantity and quality were measured by Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, USA).

Quantification of miR-23a expression: A two-step approach was implemented. The first step was reverse transcription to convert RNA to complementary DNA (cDNA). Reverse transcription was performed using TaqMan® MicroRNA Reverse Transcription Kit (Cat No. 4366596, Applied Biosystems, Foster City, USA), following the protocol supplied by the manufacturer, and was carried out in an Applied Biosystems™ Veriti™ HID 96-Well Thermal Cycler, 0.2mL (Applied Biosystems, Thermo Fischer, Waltham, USA). The second step involved quantitative real-time PCR amplification of cDNA was performed in the StepOne™ Real-Time PCR System (Cat No.4376357, Applied Biosystems, Foster City, USA) using TaqMan MicroRNA™ assay (20×) (Cat No.4427975, assay ID 000399, Applied Biosystems, Foster City, USA) containing a mixture of forward and reverse primers and a TaqMan probe (FAM TM dye) to amplify and detect the cDNA of miR-23a target. The expression levels of miR-23a in plasma were normalized by *GAPDH*. Each sample was run in duplicate and a nontemplate control was included in

all runs. The PCR cycle involved an initial cycle at 95 °C for 10 min, followed by 40 cycles of denaturation and annealing/extension at 92 °C (15 s) and 60 °C (1 min), respectively. All PCR reactions were carried out in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines[16].

Bioinformatic analysis

1-Selection of miRNA-23a

Predicted and experimentally validated miRNAs that significantly target melanogenesis and apoptosis KEGG pathway were identified by DIANA-mirPath v3.0 web server via Reverse Search module and TarBase v7.0 pipeline [17]. Hsa-miR23a was selected as it was previously examined as an apoptosis regulator in oxidative stress in age-related macular degeneration of the retina, a theory similar to that taking place in vitiligo.

2- Genomic characterization of miR23a

Chromosomal localization, genomic sequence and structure analysis, subcellular localization, and folding pattern were retrieved from different online tools; including Ensembl (http://www.ensembl.org/), GeneCards for human gene database (http://www.genecards.org/), National Center for Biotechnology Information (NCBI) (ncbi.nlm.nih.gov/), compartments subcellular localization (https://compartments.jensenlab.org/Search), KineFold (http://kinefold.curie.fr/).

3- Gene ontology and functional enrichment analysis

Gene ontology and pathway enrichment analysis of microRNA-23a was performed by Diana-miRPath v3.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php) using its experimentally validated gene targets.

Statistical analysis

Data were managed using R software (version 3.3.2, Vienna, Austria), GraphPad prism (version 7.0, Boston, USA), and SPSS software (version 23.0, Chicago, USA). Descriptive data were represented as mean ± standard deviation or median and quartiles for quantitative variables and percentages for qualitative variables. Chi-square, Fisher's exact, student's t, one-way ANOVA, Mann-Whitney U, and Kruskal-Wallis tests were used where appropriate. Correlation analysis was carried out using Spearman's rank test. The area under the curve (AUC) of ROC was plotted to evaluate the diagnostic and prognostic value of the studied miRNA. A *p-value* of <0.05 was considered statistically significant.

Results

Baseline characteristics of the study population

A total of 94 subjects were enrolled in the study: 50 vitiligo patients and 44 healthy controls. The mean age was 31.7 ± 18.3 years in patients and 36.59 ± 12.2 years in the control group (p= 0.131). Most of the study participants were females (Table 1). A positive family history of vitiligo and autoimmune disease was observed in 34% and 8%, respectively, of vitiligo patients. A few vitiligo patients had a co-existing autoimmune disease (8% had rheumatoid arthritis (RA) and 2% had alopecia). Moreover, type 2 diabetes mellitus was present in 12% of vitiligo patients (Table 1).

Clinicopathological features of vitiligo patients

As depicted in Figure 1, the most common type of vitiligo represented in patients was NSV (86%). The remainder of the patients displayed SV. Of patients with NSV, 68% had the generalized type, while 12% had acrofacial distribution and only 6% had the universal type. We used three different scoring systems to assess disease extent

and severity in vitiligo patients (Figure 2). According to the VDA score, most of the patients were stage +4 (40%) and +3 (40%). According to the VETF staging score, a little more than half of the cases were in stage 1, and 44% were in stage 2. Only 4% of patients were in stage 3. The VETF spreading score showed that 84% of vitiligo patients had actively progressing disease, and the disease was stable in only 16% of patients. Regarding VASI, 100% depigmentation, 90% depigmentation, 75% depigmentation, 50% depigmentation, 25% depigmentation, 10% depigmentation corresponded to 18%, 32%, 4%, 4%, 18%, and 18% of patients, respectively.

Table 1: Demographic data of study participants

	Controls (n=44) n (%)	**Vitiligo** (n=50) n (%)	***P* value**
Age			
≤ 40 years	30 (68.2)	34 (68.0)	1.000
> 40 years	14 (31.8)	16 (32.0)	
Sex			
Female	40 (90.9)	38 (76.0)	0.097
Male	4 (9.1)	12 (24.0)	
Positive family history			
Vitiligo	0 (0.0)	17 (34.0)	**<0.001**
Autoimmune disease	0 (0.0)	4 (8.0)	0.120
Autoimmune disease			
Thyroid disease	0 (0.0)	0 (0.0)	NA
Alopecia	0 (0.0)	1 (2.0)	1.00
Rheumatoid arthritis	0 (0.0)	4 (8.0)	0.120
Clinical illness			
Hypertension	0 (0.0)	4 (8.0)	0.
Coronary artery disease	0 (0.0)	1 (2.0)	1.00
T2DM	0 (0.0)	6 (12.0)	**0.028**

T2DM: type 2 diabetes mellitus. P value <0.05 was considered significant. using Chi-square and Fisher's exact tests

MiR-23a gene expression analysis

The expression levels of the miRNA miR-23a were measured in the plasma of both vitiligo patients and matched controls. Interestingly, miR-23a expression was significantly downregulated in vitiligo patients, being 0.57-fold lower than relative expression levels detected in controls (Figure 3A). Moreover, plasma miR-23a levels discriminated between vitiligo patients and controls with 62 % specificity and 76% sensitivity (AUC=0.67, P value = 0.004) (Figure 3B).

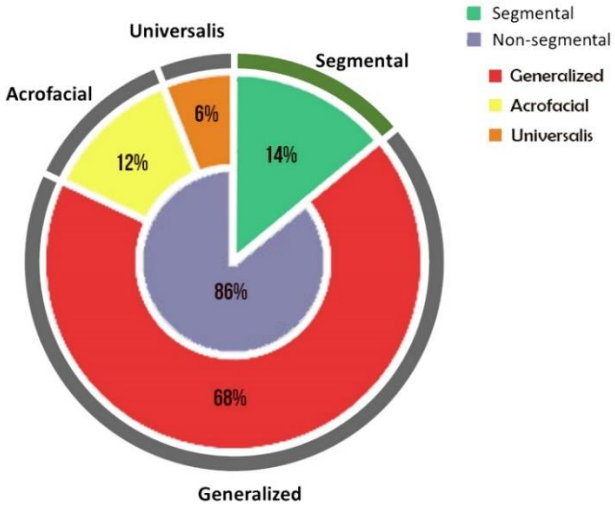

Figure 1. Types of vitiligo in study subjects
Donut plot representing the percentage of vitiligo patients for each vitiligo type

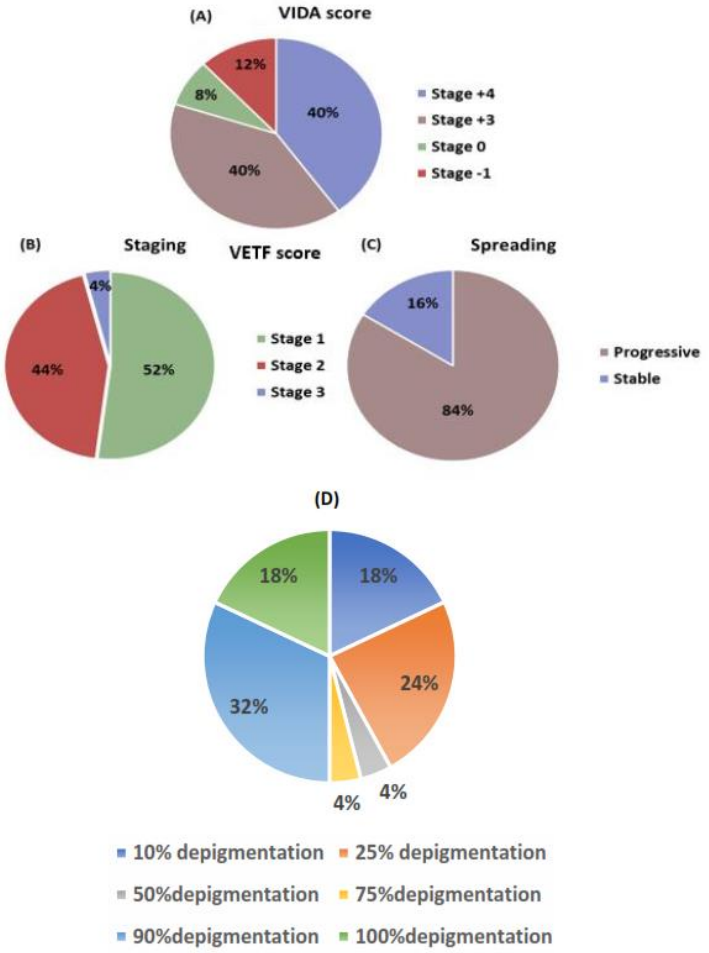

Figure 2: Assessment of disease extent and severity in vitiligo patients
Pie charts showing the distribution of vitiligo patients according to (A) Vitiligo disease activity (VIDA) score, (B) Vitiligo European Task Force (VETF) staging score, (C) VETF spreading score, and (D) Vitiligo Area Severity Index (VASI).

Bioinformatic analysis of miR-23a

1- Genomic location of miR-23a

MiR-23a (Has-miR-23a) is an ncRNA gene encoded by the *MIR23A* gene on chromosome 19p13.12 (Genomic coordination at 19: 13,836,587–13,836,659) on the negative strand (according to human genome assembly GRCh38). It consists of 1 exon, spanning 73 bases and encoding for 1 alternative splice (Figure 5A). Considering the subcellular localization, miR-23a predominantly existed within extracellular exosomes which are extruded into the circulation (Figure 5B). The architecture of the noncoding miR-23a is displayed in Figure 5C.

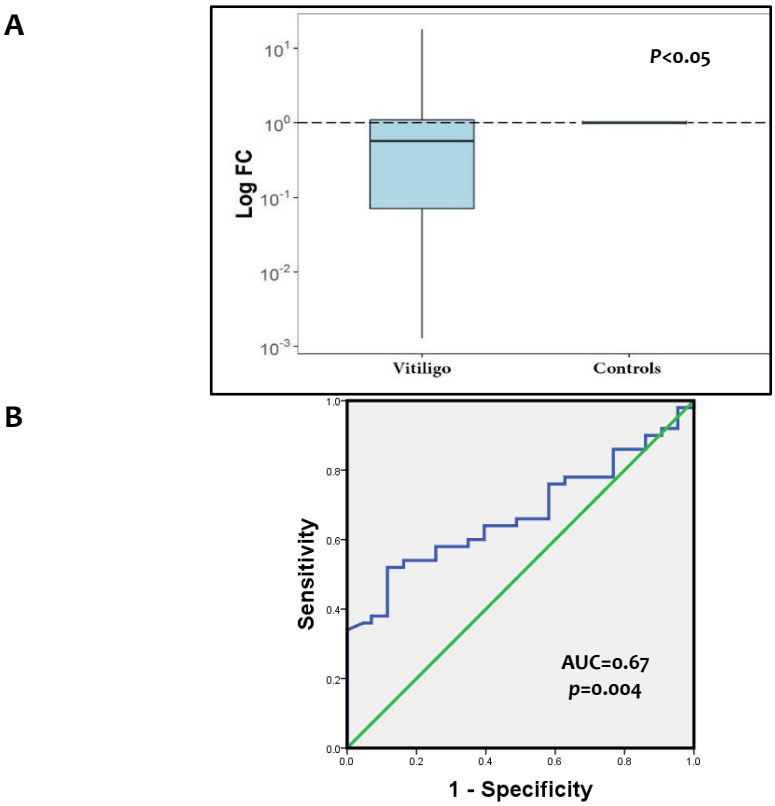

Figure 3: MiR-23a expression profile

A: Expression in vitiligo patients and healthy controls. Values represented as medians. The box defines the upper and lower quartiles (25% and 75%, respectively). Expression levels were normalized to *GAPI* Fold change was calculated using the delta-delta CT method ($2^{-\Delta}$ compared to controls. Mann-Whitney U test was used for comparison. **B:** ceiver operating characteristics (ROC) curve analysis for discriminating v ligo patients from healthy controls. AUC: area under curve; FC: fold chang

2- Gene Ontology for miR-23a

Gene ontology (GO) revealed that the miR-23a molecular function involved "posttranscriptional gene silencing via mRNA binding". For biological processes, miR-23a is involved in "gene silencing"; vascular function: "cellular response to vascular endothelial growth factor stimulus", "positive regulation of blood vessel endothelial cell proliferation involved in sprouting angiogenesis", "positive regulation of cell migration involved in sprouting angiogenesis", "regulation of sprouting angiogenesis", "negative regulation of vascular

permeability".

3- Enrichment pathway analysis of MIR-23a gene targets

Gene enrichment analysis revealed that miR-23a regulates "cell cycle" and "P53 signaling" pathways. Moreover, the relationship of the miR-23a with its target genes related to vitiligo pathways (apoptosis and melanogenesis) was uncovered and these were then filtered to select only strongly validated targets: 10 apoptosis genes and 8 melanogenesis genes (Figure 6).

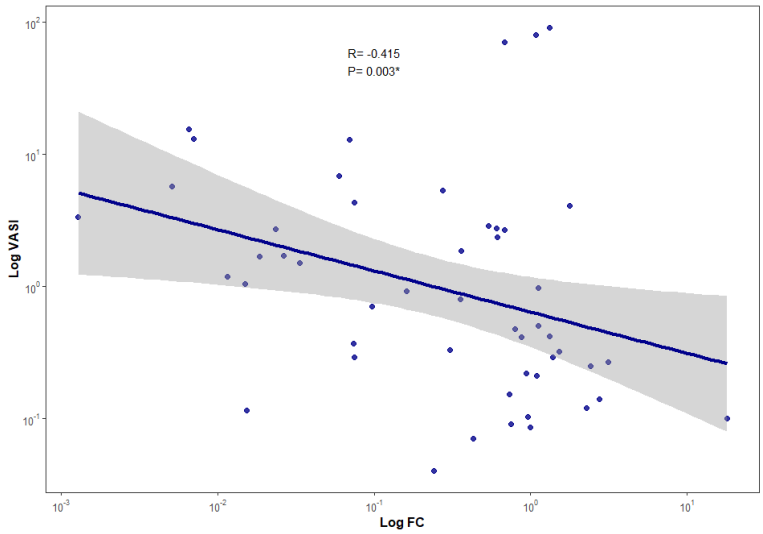

Figure 4: Correlation between log fold change (FC) of miR-23a expression and the Vitiligo Area Severity Index (VASI) in vitiligo patients

Pearson correlation analysis. *: statistically significant. R: correlation coefficient.

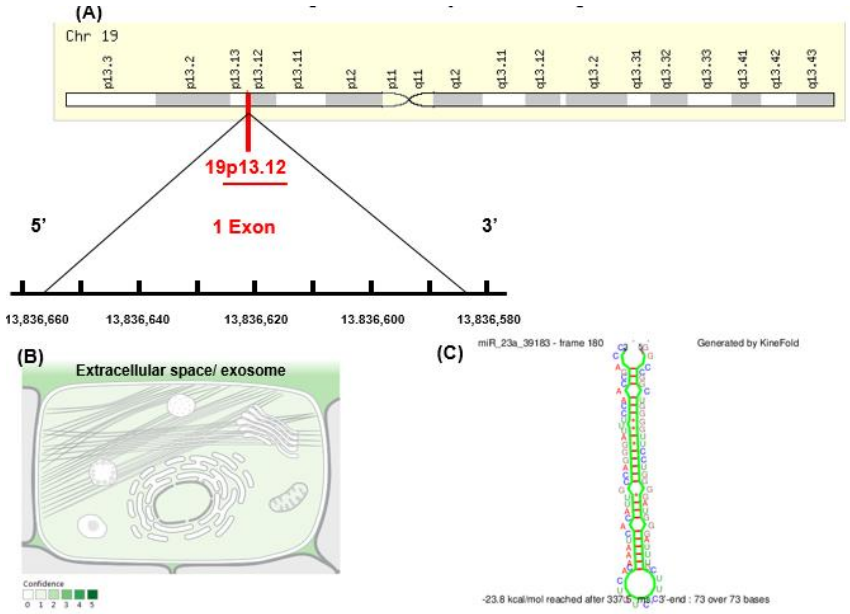

Figure 5: Structural and functional analysis of the miR-23a (*MIR23A*) gene

A: Chromosomal localization of the *MIR23A* gene. **B:** Subcellular localization of miR-23a. **C:** Folding pattern of miR-23a. *[Source: Ensembl.org, genecards.org, NCBI, COMPARTMENT database, and MFold]*

Discussion

Melanocyte loss is the central pathogenic feature of vitiligo[18], the cause of which is still far from deciphered. The currently available therapies for vitiligo are quite defective. Vitiligo patients require extended treatment plans that may last months to years and may still result in unsatisfactory outcomes. This lack of treatment success highlights the importance of searching for the root of the problem and discovering molecular therapies which target the precise defect. Moreover, the diagnosis of vitiligo is possible only after the exclusion of a wide range of depigmentation disorders[19], so finding suitable biomarkers for vitiligo diagnosis and prognosis would be of great value. miRNAs are a novel class of ncRNAs that play an important role in controlling gene expression. miRNAs are now known to regulate major cellular functions and deregulated miRNA expression plays important roles in human diseases[4]. miRNAs have been shown to be well preserved in the peripheral circulation, and found in stable forms in the serum and plasma, which suggests that they could serve as promising biomarkers for different diseases[20].

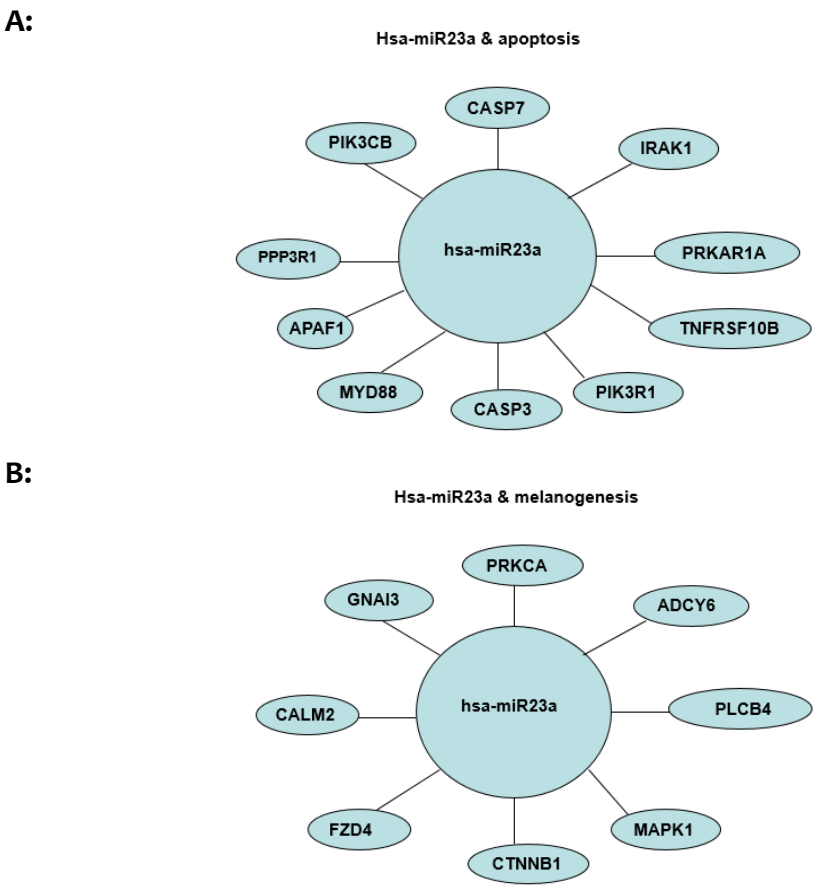

Figure 6: miR-23a (has-miR-23a) target gene network analysis in vitiligo
A: Apoptosis-related genes. **B:** Melanogenesis-related genes
Diana-miRPath v3.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php)

Based on the bioinformatic data we generated, we selected miR-23a as a plausible player in vitiligo. To the best of our knowledge, this is the first study to examine miR-23a expression in vitiligo. In the current study, we examined the circulating

expression level of miR-23a in vitiligo patients and compared it to the expression levels in healthy matched controls. We further stratified vitiligo patients according to demographic and clinical data, and according to disease extent and severity, to examine whether any of these factors were affected by the miR-23a expression levels. Since data on miR-23a in the literature is lacking, we also performed a detailed bioinformatic analysis to uncover the physiological roles and molecular pathways, and targets of miR-23a, and how it might be involved in the pathogenesis of vitiligo. The mean age of vitiligo patients was 31.7 ± 18.3 years. Most of the study participants were females but gender did not influence the characteristics of vitiligo. Less than a third of the patients had a positive family history of vitiligo and/or other autoimmune disease and less than 10% had a co-existing autoimmune disease. Type 2 diabetes mellitus was present in 12% of vitiligo patients. NSV was present in 86% of patients. We stratified vitiligo patients according to several scoring systems for vitiligo distribution and stage. According to the VIDA score, about 40% of patients were in stages 3 and 4. According to the VETF spreading score, most of our patients had progressive disease and only 16 % were stable, while none had a regressive course. According to the VASI score, 40% of patients exhibited 90-100% depigmentation. Numerous miRNAs have been implicated in vitiligo[21], but not miR-23a. Our study shows that plasma miR-23a expression was significantly downregulated in vitiligo patients (p<0.001). Moreover, the downregulation of miR-23a expression was associated with a higher VASI score (p= 0.003). ROC curve analysis demonstrated that plasma miR-23a could be used as a biomarker for vitiligo (AUC=0.67), with 76 % sensitivity and 62% specificity. Limited but comparable results have been found in other autoimmune diseases where miR-23a was downregulated in synovial tissue of psoriatic arthritis patients and was inversely correlated with disease activity and synovitis[10]. In addition, miR-23a downregulation was found to promote proliferation, invasion, and migration and inhibit apoptosis in rheumatoid arthritis fibroblast-like synoviocytes[12]. Thus, our data suggest that miR-23a may be involved in the pathophysiology of vitiligo. Bioinformatic analysis of miR-23a in this study has demonstrated that the GO pathways and gene enrichment analysis targets have been shown to be involved in vitiligo pathogenesis[21-24], particularly apoptosis and melanogenesis. This study is of limited sample size, and of single ethnicity, so selection bias cannot be excluded. Further *in vivo* and *in vitro* research is necessary to examine the molecular relationships uncovered by bioinformatic analysis.

Conclusion

Circulating miRNA-23a is downregulated in vitiligo and is negatively correlated with VASI score, highlighting its potential role in vitiligo pathogenesis.

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