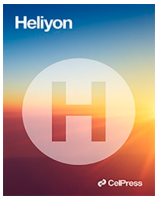
Heliyon 10 (2024) e32685

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

miR-24-3p and miR-484 are potential biomarkers for neurodegeneration in multiple sclerosis

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ARTICLE INFO

Keywords:
Multiple sclerosis
Biomarkers
microRNA
Relapsing-remitting MS
Secondary-progressive MS
Semaphorin 3A
Lipocalin 2

ABSTRACT

Multiple sclerosis (MS) is a complex, neurodegenerative chronic disorder. Circulating diagnostic biomarkers for MS have remained elusive, and those proposed so far have limited sensitivity and specificity to MS. Plasma-circulating microRNAs (miRNAs) have advantageous biochemical and physiological attributes that can be utilized in clinical testing and disease monitoring. MS miRNA expression microarray datasets analysis resulted in four candidate miRNAs that were assessed for their expression in a separate MS case-control study. Only miR-24-3p was downregulated in all MS patients compared to healthy controls. MiR-484 was significantly upregulated in relapsing-remitting MS (RRMS) patients compared to healthy controls. Mir-146-5p and miR-484 were significantly downregulated in secondary-progressive MS (SPMS) compared to RRMS. MiR-484 downregulation was associated with worsening disability and increased lipocalin-2 levels. Mir-342-3p and miR-24-3p downregulation were associated with increased semaphorin-3A levels in MS and RRMS patients. In conclusion, mir-24-3p downregulation is diagnostic of MS, and mir-484 upregulation and downregulation are potential biomarkers for RRMS and SPMS conversion, respectively. The differential expression of miR-146a-3p in MS subtypes suggests its potential as an SPMS transition biomarker. The association of downregulated mir-24-3p and mir-484 with increased neurodegeneration biomarkers suggests they play a role in MS pathogenesis and neurodegeneration.

1. Introduction

Multiple Sclerosis (MS) is a chronic autoimmune, neurodegenerative disorder characterized by autoimmune neuronal demyelination in the central nervous system (CNS). The most common MS clinical type, which comprises 85 % of all MS cases, is relapsing-remitting MS (RRMS). Some RRMS patients will progress to irreversible MS disability and convert to secondary progressive MS (SPMS) type in which a lower rate of inflammatory lesion activity is detected with constant axonal loss and limited repair. The least common MS clinical type is primary progressive MS (PPMS), where individuals show gradual worsening from onset without periods of remission. Despite ongoing refinement of the diagnostic criteria for MS, diagnosing MS is still hard to ascertain, and MS misdiagnoses are estimated to range between 30 and 67 % in different healthcare centers [1,2]. Delayed accurate diagnosis of MS would delay early clinical intervention, which is critical in managing MS and enhancing the quality of life of MS patients [3]. There is abundant research investigating MS biomarkers encompassing genetic risk factors, HLA haplotypes, cerebrospinal fluid (CSF) and serum markers,

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https://doi.org/10.1016/j.heliyon.2024.e32685
Received 20 April 2024; Received in revised form 5 June 2024; Accepted 6 June 2024
Available online 12 June 2024

immune cell expression profiles, and, to a lesser extent, microRNAs (miRNAs). So far, none of the reported MS biomarkers have established clinical applicability due to procedural or biomarker diagnostic performance limitations [4]. HLA haplotypes and genetic risk factors have a low incidence in confirmed MS patients. Though CSF biomarkers have an undisputed diagnostic application, the procedure is invasive, and repeat testing is limited. In addition, serum MS biomarkers are of limited specificity and sensitivity to MS. MiRNAs are yet to be thoroughly explored as potential biomarkers for MS, and reports on various miRNAs associated with MS are rarely replicated in different MS populations. Nevertheless, miRNAs offer valuable biomarker characteristics similar to protein biomarkers, including ease of sampling, superior stability to other biomarkers, dysregulation that can be detected in early disease, sensitive real-time monitoring, longitudinal assessment of disease and treatment dynamics, and high specificity to clinical phenotypes [5].

MiRNA's potential use in clinical diagnostics is valuable, specifically in neurodegenerative disorders [6,7]. Many studies have investigated miRNAs in MS using different sample types, including whole blood, immune cell subtypes, CSF, serum, and plasma samples [8]. Results from these studies generated a list of miRNAs differentially expressed in MS, which collectively point to a critical role for miRNAs in MS pathogenesis, diagnosis, prognosis, response to treatment monitoring, and potential therapeutics [9]. However, these studies' heterogeneity in terms of the type of sample used and analytical approach have generated inconsistent candidate miRNAs for clinical testing applications. In this study, we aim to sift through differentially expressed miRNAs from different MS sample types and identify candidate miRNAs with consistent differential expression across different MS sample types. We assessed the selected miRNAs' expression in an accessible sample type, namely plasma, to determine their potential use in streamlined, rapid clinical diagnostic testing for MS. Plasma samples can be easily retrieved and rapidly processed for miRNA biomarker analysis without cell isolation methods and cell culture. These technical features of plasma-derived biomarkers, in combination with miRNA's characteristic specificity and sensitivity to disease states, should facilitate a more accurate and rapid diagnosis testing for MS. The four selected miRNAs are mir-24-3p, −146a-3p, -342-3p and miR-484 [10–13].

2. Results

2.1. Analysis of miRNAs in MS

The expression of the four miRNAs and their association with MS clinical characteristics was investigated in MS. MS patients, and healthy control demographics and characteristics are shown in Table 1.

The miRNA fold expression in MS plasma was compared to healthy controls' fold expression (Table 2). No association was found

Table 1
MS and healthy control cohorts' demographics and clinical characteristics.

Criteria	Multiple sclerosis Patients (n = 76)	Healthy controls (n = 75)
Sex [n (%)]	50 (65.8)	51 (68)
Female	26 (34.2)	24 (32)
Male		
Age in years [Mean, (±SD[a])]	35.15 (±9.5)	27.52 (±6.6)
Disease duration in years [Mean, (±SD)]	8.77 (±6.11)	NA[b]
MS subtype [n (%)]	56 (73.7)	NA
RRMS	20 (26.3)	
SPMS		
EDSS[c] [Median (Range)]	2.5 (1–8)	NA
MSSS[d] [Mean, (±SD)]	4.48 (±2.88)	NA
Treatment [n (%)]	5 (6.6)	NA
None	13 (17.1)	
Interferon beta-1 formulations	22 (28.9)	
Fingolimod	32 (42.1)	
Natalizumab	4 (5.3)	
Other		
Neurodegeneration markers	16	NA
MRI load	32	68.8 ± 17.5
Mild	28	1.01 ± 0.57
Moderate	74.72 ± 21.6	25.6 ± 6.4
High	1.2 ± 0.55	297.5 ± 135.1
Sphingomyelin, ng/mL [Mean, (±SD)]	28.0 ± 4.4	3.9 ± 3.7
Neurofilament-L, ng/mL [Mean, (±SD)]	411.6 ± 163.7	
Lipocalin 2, ng/mL [Mean, (±SD)]	2.5 ± 2.3	
Semaphorin 3A, ng/mL [Mean, (±SD)]		
BDNF[e], ng/mL [Mean, (±SD)]		

[a] Standard Deviation.
[b] Not Applicable.
[c] Expanded Disability Status Scale.
[d] MS Severity Score.
[e] Brain Derived Neurotrophic Factor.

between any of the analyzed miRNAs and MS using the difference between means statistical analyses, except for a weak significant miR-24-3p reduced expression in MS. We hypothesized that other factors, such as age and sex, might contribute to this lack of association, and the influence of biological sex and age on miRNA expression was ascertained. Biological sex showed an impact on miR-342-3p expression, with males having higher fold expression when compared to females in the healthy control cohort ($\beta = 0.114$, 95% CI: 0.036–0.191, $p = 0.005$), whereas older age was associated with increased miR-484 fold expression in healthy controls ($\beta = 1.19$, 95%CI: 0.24–2.14, $p = 0.015$). Therefore, age is a confounding factor for miR-484, and biological sex is a confounding factor for miR-342-3p. All follow-up statistical analyses were adjusted for these confounding factors where appropriate. Linear regression analysis of the tested miRNA expression showed miR-24-3p fold expression is significantly different between the total MS cohort and healthy controls ($\beta = 0.06$, 95%CI: 0.002–0.116, $p = 0.043$). We next considered RRMS patients alone, and only miR-484 increased fold expression was significantly associated with RRMS compared to healthy controls ($\beta = -0.078$, 95%CI: −0.13 – (−0.03), $p = 0.002$). None of the expression of other miRNAs significantly differed between RRMS and healthy controls.

To determine if any of the assessed miRNAs have the potential to discriminate RRMS from SPMS, we performed intra-cohort comparisons based on MS types (Fig. 1). MiR-24-3p and miR-342-3 fold expression did not differ between RRMS and SPMS patients. Whereas miR-146-5p was significantly lower in SPMS than RRMS patients ($\beta = -0.11$, 95%CI: −0.184 – (−0.034), $p = 0.005$). MiR-484 expression was higher in RRMS compared to SPMS patients ($\beta = -0.075$, 95%CI: −0.122 – (−0.026), $p = 0.003$).

2.2. miRNA expression and MS clinical and biochemical markers

Increasing MS disease disability as assessed by EDSS associated with reduced miR-484 expression alone ($\beta = -0.4$, 95%CI: −0.654 – (−0.134), $p = 0.003$), and when adjusted for sex and age ($\beta = -0.3$, 95%CI: −0.545 – (−0.05), $p = 0.02$). No association was found for assessed miRNA fold expression and MSSS or MRI load or the type of treatment administered. Neurodegeneration biomarkers NFL and sphingomyelin levels were not associated with the expression of any of the assessed miRNAs. However, lipocalin 2 levels were higher in MS patients with low levels of miR-484 ($\beta = -12.24$, 95%CI: −23.6 – (−8.7), $p = 0.035$), whereas no association was found in healthy controls. Moreover, lipocalin 2 levels did not differ between total MS or RRMS patients and healthy controls. Semaphorin 3A levels were higher in MS patients with low miR-24-3p expression ($\beta = -41.6$, 95%CI: −74.7 – (−8.5), $p = 0.015$), and low miR-342-3p ($\beta = -31.8$, 95%CI: −63.7 – (−3.5), $p = 0.05$). Semaphorin 3A levels were significantly higher in MS and RRMS patients than in healthy controls ($p < 0.001$). No associations were found for BDNF level with any of the assessed miRNAs. Considering RRMS patients alone, the association of high semaphorin 3A levels with low miR-24-3p was retained ($p = 0.044$). Moreover, miR-342-3p low expression was associated with high levels of semaphorin 3A in RRMS patients ($\beta = -35.9$, 95%CI: −68.1 – (−3.8), $p = 0.028$).

Since miR-484 fold expression differed between RRMS and SPMS patients, the test performance metrics of miR-484 were analyzed separately for RRMS and SPMS groups. miR-484 accuracy in diagnosing RRMS was significant (ROC 0.657, 95%CI: 0.562–0.753, $p = 0.002$). Overall, the performance metrics for miR-484 were superior to the other tested miRNAs in the RRMS group ($\beta = 0.705$, 95%CI: 0.562–0.884, $p = 0.002$). MiR-484 had 46.4 % sensitivity, 74.3 % specificity, 58 % positive predictive value, 64.7 % negative predictive value, and classification accuracy of 62.3 %. In SPMS, none of the tested miRNAs had acceptable test performance metrics.

3. Discussion

Mir-24-3p was previously reported to be overexpressed in serum samples of RRMS patients and associated with increased disability [10]. However, this finding contradicts the reported functions of this miRNA. Our findings show miR-24-3p is significantly reduced in MS. Mir-24-3p increased expression has been shown to induce proliferation and tissue remodeling effects by regulating the proliferation/apoptotic pathways in different injured tissues [14–18]. Therefore, miR-24-3p's gradual downregulation in MS types suggests that the reparative repertoire of injured tissues is gradually exhausted and can no longer promote proliferation and regeneration. Moreover, the association of miR-24-3p with reduced semaphorin 3A in our study, which is a neuronal cell death promoter, further supports our finding [19]. A similar trend of reduced miR-24-3p has been reported in Parkinson's disease peripheral blood mononuclear cells (PBMCs), in which miR-24-3p downregulation was associated with advanced disease [20].

We have shown that miR-146a-5p is reduced in SPMS compared to RRMS. In reported MS studies, miR-146a expression was increased in PBMC of RRMS patients compared to healthy controls [21] and decreased in CSF of oligoclonal band positive RRMS patients compared to CSF of other neurological diseases [22]. However, in RRMS sera, miR-146a-5p was found to be downregulated and associated with increased disability [11]. Functional evidence for miR-146a-5p suggests its active involvement in regulating the immune response, with functions in immune cell differentiation, maturation, and death [23]. Evidence from rheumatoid arthritis (RA),

Table 2
Log2 transformed mean miRNAs fold expression ± Standard Error of the Mean (SEM) in multiple sclerosis patients (MS) compared to healthy controls (HC).

miRNA	Number expressed		Mean fold expression (±SEM)		*P*-value
	MS	HC	MS	HC	
miR-24-3p	76	74	−0.47 (±0.15)	−0.072 (±0.1)	0.049
miR-146a-5p	75	75	−0.465 (±0.19)	−0.072 (±0.15)	0.082
miR-342-3p	74	75	0.142 (±0.17)	−0.052 (±0.16)	0.11
miR-484	76	75	0.407 (±0.21)	−0.066 (±0.19)	0.092

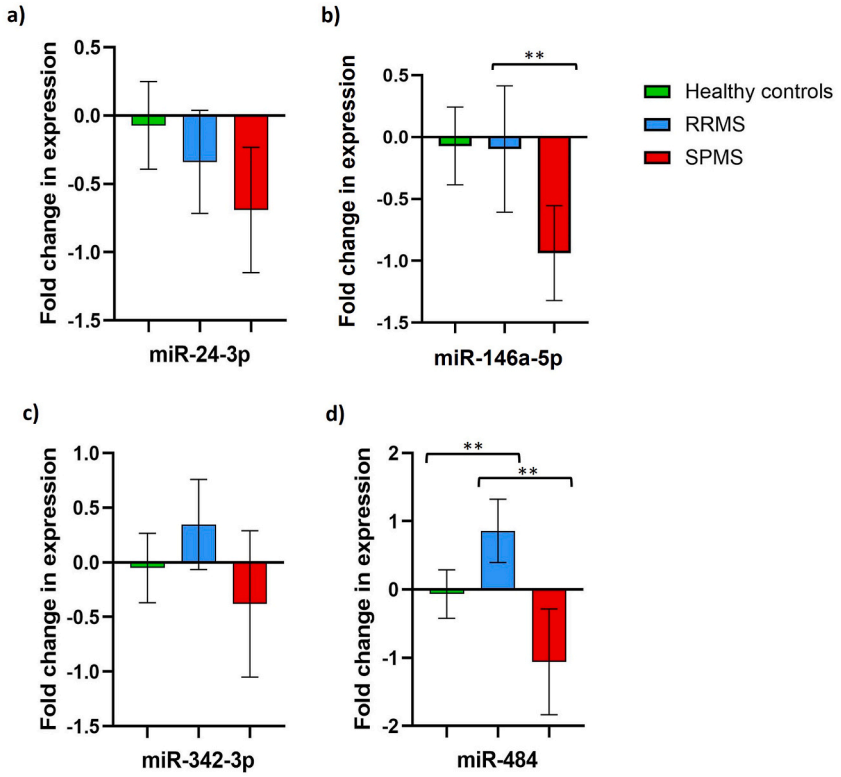

Fig. 1. Log2 transformed fold change in expression of assessed miRNAs in healthy controls, RRMS, and SPMS patients (a–d). Error bars are 95 % Confidence Interval of the mean. (**) denotes the degree of significance $p < 0.01$.

type-1 diabetes, and obesity suggests a conflicting inflammatory role for miR-146a-5p in these disorders, supporting a pro-inflammatory role in RA and obesity but an anti-inflammatory role in type-1 diabetes [24]. Our findings agree with its role as an anti-inflammatory miRNA since it was reduced in progressive MS disease in our study. However, given this miRNA's multiple functions in immune system regulation, its conflicting disease associations, and its indistinguishable expression in RRMS from healthy controls in our study, miR-146a-5p potential as a biomarker for MS is uncertain.

Based on our findings, mir-342-3p is a biological sex-influenced miRNA, and RRMS patients with elevated semaphorin 3A levels had reduced mir-342-3p expression. Mir-342-3p has been shown to have invasion and metastasis inhibitory effects in cancer [25,26] and is involved in ischemic injury repair [27,28]. Both functions highlight its role in regulating angiogenesis and vascular health, a feature known to be disrupted in MS [29]. In a small MS study, miR-342-3p was upregulated in RRMS and downregulated in SPMS when compared to RRMS, and the authors suggested miR-342-3p to be a biomarker for neuro-injury [12]. In addition, a recent study found miR-342-3p to be upregulated in CSF and serum exosomes of untreated RRMS patients when compared to healthy controls [30]. Moreover, we noted a trend for miR-342-3p upregulation in RRMS, but it did not reach statistical significance ($p = 0.12$). Genetic, environmental, and demographic factors dictate the microvasculature of the CNS. While miR-342-3p may play a mediatory role in controlling CNS microvasculature in MS, its reduced expression in progressive MS disease and association with high semaphorin 3A levels suggest that the damage in progressive MS disease may be beyond mediation.

We also found that miR-484 expression was increased in RRMS, decreased in SPMS, and at baseline in healthy controls. This suggests miR-484 may be a candidate biomarker for RRMS diagnosis and conversion to SPMS. We also found it to be lowest in patients with high disability and high lipocalin 2 levels, a neuroinflammatory protein [31]. MiR-484 has been investigated in many pathological conditions and has been suggested to be a marker for health and disease [32]. There are numerous reports that miR-484 mediates multiple pathways, such as endoplasmic reticulum and oxidative stress, modulating mitochondrial shape and functions, inflammation, cell proliferation, and apoptosis. Its physiological roles have been shown to include mitigating endothelial injury, glucolipid metabolism, mitochondrial fusion, and neurological cognition and synaptic transmission pathways. In the CNS, miR-484 is a biomarker for neuronal plasticity and cognitive decline, especially in older adults [33]. These functions corroborate its elevated expression in RRMS during remission and its decrease in SPMS patients with progressive disease. Mir-484 assessment in MS has been reported with findings supporting ours [13,34]. These findings suggest miR-484 may be a candidate for MS diagnosis and prognosis in an MS miRNA biomarker panel. Further investigation into miR-484's role in MS disease and its therapeutic potential in curbing MS progression should be pursued.

A significant limitation of our study is the sample size, specifically of SPMS patients. However, the majority of SPMS patients refused participation due to their increased MS disability and inability to withstand additional blood withdrawal other than physician-

ordered tests. In addition, the assessment of these miRNAs should be conducted in different neurological diseases that mimic MS to establish their specificity to MS. In conclusion, among the miRNAs investigated, miR-24-3p is reduced in MS, miR-146a-5p is a potential SPMS conversion biomarker, and miR-484 had the most significant RRMS diagnostic potential, albeit with moderate test performance metrics. Moreover, we have provided evidence associating the expression of these miRNAs with established neurodegeneration and remyelination markers. Future studies should focus on how these miRNAs may be used for the clinical diagnosis and potential therapeutic intervention in MS.

4. Materials and methods

4.1. MS and healthy control samples

All study protocols were approved by Kuwait's Health Sciences Centre's Joint Committee for The Protection of Human Subjects (VDR/JC/883) and by the Dasman Diabetes Institute ethical review committee (RA/018/2013) both of which adhere to the declaration of Helsinki's Ethical Principles for Medical Research Involving Human Subjects guidelines. All information related to study protocols was clearly explained to all participants before obtaining informed written consent. MS patients' inclusion criteria included a detailed clinical history, an MS disease duration of $\geq$2 years, a clear MS clinical course, and consent to provide a blood sample. Patients who have an expanded disability status scale (EDSS) score of 0 and disease duration of $\leq$1 year were excluded, and patients who are in relapse and receiving intravenous medications at the time of sample collection were also excluded. Healthy controls' exclusion criteria were having a positive family history of MS and having a diagnosis of other autoimmune or neurodegenerative disorders. All fasting blood samples were collected in EDTA-coated vacutainers, and blood fractionation was performed to isolate plasma fractions that were stored in duplicates at $-80\ ^{\circ}C$ until use. A total of 247 MS samples and 172 healthy control samples were collected, of which 76 MS patients (56 RRMS, 20 SPMS) and 75 healthy controls were selected of approximate age and sex-matching. A prospective power analysis showed that our sample size had a 95 % power to detect an average difference between means of 0.98 with a significance level (alpha) of 0.05 (two-tailed) based on StatMate version 2 (Graphpad Inc., USA). Plasma neurodegeneration and remyelination biomarkers results for these samples were retrieved from the laboratory database. Neurodegeneration biomarkers included sphingomyelin, neurofilament light chain (NFL), lipocalin 2, semaphorin 3A, and brain-derived neurotrophic factor (BDNF).

4.2. Selection of candidate miRNAs

Publicly available datasets of MS miRNA expression microarray profiling studies predating December 2018 were extracted from GEO Datasets (www.ncbi.nlm.nih.gov/) and ArrayExpress (www.ebi.ac.uk/arrayexpress/) using the keyword "Multiple sclerosis.". Resultant studies were scrutinized for completeness of study and dataset descriptions. Study inclusion criteria were as follows: having a clinically confirmed MS subtype, the sample assessed is sourced from blood (cells, plasma, serum), and the type of platform used. Only case-control studies were included in our analysis. Studies using biological material from MS animal models and cell culture studies were excluded, as were studies focusing on analyzing specific therapeutic interventions. Six datasets passed our inclusion criteria and data quality checks (Supplementary table). A differential expression analysis was performed on the datasets using linear models for microarray (LIMMA) data software package [14]. Multiple and general comparisons were performed to identify MS risk miRNAs. Resultant miRNAs of the highest significant p-values from dataset comparisons with the largest sample size were prioritized for reproducibility analysis in plasma miRNA extracts. These miRNAs were hsa-miR-342-3p, hsa-miR-484, hsa-miR-24, and hsa-miR-146a. MiR-24-5p and miR-146a-3p specific strands were selected based on functional evidence from the literature on their association with neurodegeneration or inflammation.

4.3. Plasma miRNA isolation and cDNA synthesis

Plasma circulating miRNAs were extracted from 200 μL of plasma using miRNeasy plasma/serum kit (Qiagen, CA, USA) according to standard manufacturer protocol. A Cel-miR-39-3p spike-in control (Qiagen, CA, USA) was added at a concentration of 4×109 copies/μL to all samples after Trizol lysis to ensure that miRNA extraction and retrieval efficiency are comparable. Extracted miRNA samples were assessed by spectrophotometry for purity, integrity, and concentration. Extracted miRNA samples were converted to cDNA in a multi-step method using TaqMan™ Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific, MA, USA) that involves poly-A tailing, adaptor ligation, universal reverse transcription (RT), and a 14-cycle amplification using 5 μL of RT product to allow detection of low transcript miRNA within the cycling conditions (miR-Amp). All incubations were performed in thermal cycler GeneAmp polymerase chain reaction (PCR) system 9700 (Applied Biosystems, CA, USA).

4.4. MiRNA expression analysis

Relative quantitation by Real-Time PCR (RT-PCR) was performed using 5 μL of 1:10 diluted miR-Amp cDNA products, Taqman's human advanced miRNA assays (ThermoFisher Scientific, MA, USA), and TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, MA, USA) according to manufacturer's protocol. MiR-*Cel*-39-3p levels were assessed in all samples at two stages: following cDNA synthesis to ensure comparable extraction and cDNA synthesis efficiencies across all samples and after the 14-cycle amplification. TaqMan advanced miR-*Cel*-39-3p assay was used for these assessments (Assay ID: 478293_mir, ThermoFisher Scientific, MA, USA). The set acceptable standard deviation (SD) value between samples' cycle threshold (Ct) for miR-*Cel*-39-3p was $\pm$0.6, and a Ct $\leq$ 31 for

cDNA synthesis and a Ct $\leq$ 28 for miR-Amp efficiency assessments. Any sample that did not meet this criterion was re-amplified and reassessed. TaqMan human miR-191-5p assay (Assay ID: 477952_mir) was used as an endogenous control and was found to be consistently detectable at comparable Cts across all samples (Average Ct 23.8 SD $\pm$ 1.2). Four Taqman advanced miRNA assays for mir-24-3p, −146a-3p, -342-3p, and miR-484 (Assay ID: 477992_mir, 478714_mir, 478043_mir, and 478308_mir respectively) were used for expression analysis in all samples. All reactions were run in triplicates on an ABI7500 Fast Real-time PCR system (Applied Biosystems, CA, USA), and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to determine miRNA fold expression.

4.5. Statistical analysis

Resultant miRNA raw fold expression values were graphed into scatter plots, and outliers were identified and excluded from the analysis. Fold expression values were log2 transformed to determine fold expression change and evaluated for normality using the Shapiro-Wilks test in both cohorts. Non-parametric tests included the Mann-Whitney *U* test, and normally distributed data were analyzed using an unpaired Student t-test. Analysis of miRNA fold change association with study variables was performed using linear regression, and binary logistic regression was used to determine test performance metrics. Adjustment for confounding factors was applied where appropriate. An area under the curve analysis (ROC) was performed to assess test accuracy in predicting disease status. All statistical analyses were performed using the statistical package for the social sciences (SPSS) software (IBM, NY, USA). A prospective power analysis was conducted using StatMate software (GraphPad Software Inc., CA, USA).

Funding

This work was supported by Kuwait University Research Sector grant number MG01/19.

Data availability

All data supporting the conclusions have been provided in the manuscript.

CRediT authorship contribution statement

Rabeah Al-Temaimi: Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Raed Alroughani:** Resources, Data curation.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

We thank Mohammad Dashti for the bioinformatic analysis of datasets.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32685.

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