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Assessment of miR-181b-5p, miR-23a-3p, BCL-2, and IL-6 in Peripheral Blood Mononuclear Cells of Autistic Patients; Likelihood of Reliable Biomarkers

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ABSTRACT

Autism is a neurodevelopmental disorder that is recognized by stereotypic and repetitive behaviors after 2 years of old. Dysregulation of the immune system, especially inflammation which is mostly regulated by IL-6, imposes a deficit in CNS development. Along with this crucial biomarker, researchers have proposed BCL-2, micro RNA-23a-3p (miR-23a-3p), miR-181b-5p as other probable biomarkers involved in inflammation and apoptosis. The aim of the study was to evaluate the alteration in the expression of these biomarkers in a group of autism spectrum disorder (ASD) children.

Peripheral blood mononuclear cells (PBMCs) were obtained from 37 autistic patients. After RNA extraction with precipitation method, the Syber green qReal-time Polymerase Chain Reaction (PCR) was performed in order to evaluate the possible alteration in the expression of IL-6, BCL-2, miR-181b-5p, and miR-23a-3p. The results were compared with healthy controls.

IL-6 was significantly upregulated in ASD patients (p=0.003). On the other hand, miR-23a was upregulated and BCL-2 downregulated in ASD patients but the changes were not significant. In initial evaluations, expression changes of miR-181b-5p were not statistically significant. However, when Patients were divided into two groups of upregulated and downregulated, re-evaluation showed that both up- (p=0.005) and down-regulation (p=0.004) (i.e. changes regardless of the direction) of miR-181b were significant in autistic children.

IL-6 and miR-181b-5p can have proper diagnostic values and are reliable biomarkers with high sensitivity and specificity. On the other hand, PBMC can be utilized for such studies and also evaluation of patients' condition instead of brain tissue as it is less accessible.

Keywords: Autistic disorder; BCL-2; Interluekin-6; MicroRNA; miR-181b; miR-23a

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder including autism, Asperger, and other related conditions.[1,2] This syndrome is determined by the inability to make social interactions with other individuals mainly due to stereotypic and repetitive behaviors.[3] It should be noted that not all the patients manifest the same symptoms and it can be ranged from mild to severe in different patients.[4, 5] The mean age for diagnosis is 4-5 years. On the other hand, the effectiveness of the treatments is directly related to the time of diagnosis.[6] Henceforth, there is a need for alternative reliable diagnostic methods such as reliable biomarkers for early diagnosis.

The immune system (IS) and the nervous system have a very close and complex relationship. Various IS functions affect different aspects of the nervous system including the development and function of the brain. If the IS malfunctions, the brain would be affected subsequently.[7] Inflammation as one of the main phenomena, controls the growth and development, as well as functions of the nervous system including memory and cognition through inflammatory cytokines such as IL-6.[8] The excessive peripheral IL-6, can get passed from the blood-brain barrier and affect normal brain functions; while its physiological level is essential for homeostasis, cognition, learning, and memory.[9]As previously reported in the literature, the elevation of IL-6 in the brain as well as circulation can lead to the development of autism and/or worsening the patient's condition.[10]On the other hand, its up-regulation has been reported along with a reduction in BCL-2 and an increase in apoptosis in some cases. Hence, apoptosis may have a major role in the pathophysiology of autism.[11,12] During these studies, it has been also stated that the lymphoblastoid cell line can be a proper tool in the assessment of changes in the nucleus level in autistic individuals. However, the brain tissue was not accessible in these studies, practically during the follow-ups.[11,13]

Micro RNAs (miRs) are small noncoding RNAs that act as regulators in the gene expression by silencing the target gene during the transcription.[14] Investigations suggest that miRs can be utilized as a new group of sensitive and non-invasive biomarkers in the diagnosis and monitoring of various complications including neural disorders.[15] Extracellular miRs can be detected in various body fluids[16] and may reflect changes in other tissue such as the brain as it is not an easily accessible organ.[17,18] In this effort, researchers have tried to determine the miR expression profile of ASD patients; especially the miR-23a-3p and miR-181b-5p which are mainly found in both circulation and brain tissue.[19-21] Target genes of these miRs are somehow assigned to be related to inflammation and apoptosis in the pathogenesis of autism.[22,23] miR-23a is upregulated in brain tissue as well as lymphoblastoid cell line (LCL) and peripheral blood mononuclear cells (PBMCs);[24] while miR-181b has shown downregulation in some studies in several tissues along with an increase in the inflammatory cytokine levels,[25,26] However, some other studies have detected an increase in the expression of miR-181b in brain and circulation.[27,28]

As it has been stated, inflammation and apoptosis are amongst the most important factors related to the etiopathogenesis of autism. Two hallmarks of these phenomena are IL-6 and BCL-2 which are both dysregulated in ASD patients. On the other hand, miR-23a and miR-181b have been documented to express differentially in ASD patients. Based on the previous findings, these four factors have the potential to be used as a convenient and reliable biomarkers in this regard. Henceforth, in the present study, the objective was to determine if these elements can be considered as proper biomarkers to be utilized in different aspects of the ASD including diagnosis and treatment by measuring their expression levels. In previous researches, each of these possible biomarkers was studied separately and the correlation between these elements was speculated and hypothesized and it was not directly evaluated. However, to verify these speculations it was needed to assess the mentioned biomarkers together. Therefore, in this effort, we tried to bring all the disperse previous findings together in one patient group in order to shed more light into this aspect of ASD and to prove that these expected changes are in fact related to each other and happen simultaneously in each ASD patient and can be utilized as one biomarker panel in ASD patients.

MATERIALS AND METHODS

Patients

Patients group was chosen from boys and girls admitted as ASD patients in autism centers in two cities of Iran including Tehran and Amol, which ranged from

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3 to 15 years old. The inclusion criteria consisted of patients who were diagnosed with ASD without any other neurodegenerative disorders, infectious disease or inflammation. Based on these criteria, patients were chosen according to their medical history record and examination by a physician at the time of specimen collection. Healthy controls were chosen from elementary schools in Amol city with the same criteria as patient groups except for being diagnosed with ASD.

All the parents were gathered in several meetings in order to be informed regarding the procedures and the objectives of this study and the informed consent were signed by them. Additionally, each patient was allocated a certain code at the beginning of the survey so that their identity and information would not be revealed.

EDTA blood was drawn from both groups and it was immediately sent to the laboratory for downstream procedures.

PBMC Isolation

After venipuncture, the 10ml drawn EDTA-blood tubes were transferred to the lab in 4°C cold chain. Subsequently, the PBMC isolation was performed based on the protocol introduced in Tayebi et. al study.[29] Briefly, diluted EDTA was added to Ficol, the respective layer containing the mononuclear cells was harvested and dissolved in QIAZOL (QIAGEN, Germany), and the isolated PBMCs were stored at -80ºC for the next step.

Extraction

Total RNA extraction was performed by precipitation method as previously described in Tayebi et. al study.[29] Briefly, the chloroform was added and the supernatant was separated and diluted after the centrifugation. Then, after overnight incubation, the samples were centrifuged and the pellet was washed by ethanol. In the end, the pellets were re-suspended in Diethyl Pyrocarbonate (DEPC) water and stored at -80°C.

In order to determine the quality of extraction, absorbance was assessed at 260 and 280 to see whether it was contaminated with proteins and/or DNA. Furthermore, the agarose gel electrophoresis was performed on the extracted RNA to make sure that it was extracted properly.

Real-time PCR

Prior to performing the qPCR, a poly-A tail was added by using Poly (A) Tailing Kit (ThermoFisher Scientific, USA) according to the manufacturer user manual. Next, the cDNA was synthesized with oligo dt primers with Hyperscript First Strant Synthesis kit (Geneall, Korea) according to the manufacturer user manual. The Real-time PCR reaction was performed in duplicate by RealQ Plus 2x Mastermix Green (Ampliqon, Denmark) according to the manufacturer user manual on the Rotorgene Q (Qiagen, Germany) Real-time PCR machine.PCR was initiated with 15minutes of denaturation at 95°C, followed by 40 cycles of 95°C for 30seconds, 60°C for 30seconds, and 72°C for 30seconds. The relative quantity of mRNA was obtained using the $2^{-\Delta\Delta Ct}$ method. The primers that were used in this study are shown in table-1. In order to normalize the data for target genes and miRs, GAPDH and miR-16 expression were utilized. In the end, a standard curve was drawn; using the diluted PCR products of each gene as the template and performing Real-time PCR on them. In this way, the quality of the results can be assessed and the confidence rate would increase significantly.

Data Analysis

The statistical analysis was performed; using the Statistical Package for the Social Sciences (SPSS)

Table 1. Primers sequences for Real-time PCR assay

Gene	Forward Primer 5′ → 3′	Reverse Primer 5′ → 3′	Product Size
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138
BCL-2	ATGTGTGTGGAGAGCGTCAA	ACAGTTCCACAAAGGCATCC	136
IL-6	CGAGCCCACCGGGAACGAAA	CAGCCCCAGGGAGAAGGCAACT	107
miR-16	CTAGCAGCACGTAAATAT	CGTCCAGTTTTTTTTTTTTTTTCGCC	46
miR-181b-5p	CAACATTCATTGCTGTCGG	CGTCCAGTTTTTTTTTTTTTTTACCC	47
miR-23a-3p	CATCACATTGCCAGGGAT	CGTCCAGTTTTTTTTTTTTTTTGGAA	45

Table 2. The demographic information of the studied groups. It demonstrates the number and percentage of patients and healthy controls in each studied group

Group	Age Mean (min-max)	Age %		Sex %		City			
		<5yrs	>5yrs	Male	Female	Amol		Tehran	
						M	F	M	F
Patient	7 (3-15)	29.7	70.3	70.3	29.7	11	6	15	5
Control	9 (4-12)	12.5	87.5	67.5	32.5	27	13	-	

Table 3.The details of the ROC analysis in all studied parameters. As it can be seen, along with the IL-6, miR-23a and Mir-181b subgroups are showing promising sensitivity and specificity for the mentioned Cut-off point based on the Ct value which patients over/below these designated cut-offs can be considered autistic

Target genes	Area Under Curve	Sensitivity %	Specificity %	Cut-off Ct
IL-6	0.681	70.27	67.50	<22.75
BCL-2	0.310	-	-	-
miR-181b	0.528	-	-	-
miR-23a	0.703	70.27	65.00	<28.90
miR-181bUP	0.810	80.00	77.50	<26.45
miR-181b DOWN	0.670	68.18	60.00	>27.48

Version 20.0 software (IBM Corp., Armonk, N.Y., USA). The graphs were drawn; using Graphpad Prism 6 (GRAPHPAD SOFTWARE, LLC, CA, USA). The p-value smaller than 0.05 was considered to be significant. In this study, various statistical tests such as Kolmogorov-Smirnov, independent t-test, Mann-Whitney U, Krus-Kalwalis, and ROC curve were performed to evaluate the results properly.

RESULTS

The demographic information of the patients is shown in table 2.

Based on their age, patients were categorized into two groups: patients aged <5 years old and those aged >5 years. The reason for choosing such a category was the fact that over 85% of brain development happens in the first five years, and after that, it can be considered as a new period in brain function and development. Regarding the pattern of the gene expression of BCL-2, IL-6, miR-16, miR-181b-5p, and miR-23a-3p, no significant statistical differences were found between these two groups for the above-mentioned variables (IL-6 p=0.54, BCL-2 p=0.54, miR-181b p=0.63, miR-23a p=0.78).

The results of gender-based assessments demonstrated that the expression of the target markers was not significantly different between genders (IL-6 p=0.42, BCL-2 p=0.93, miR-181b p=0.42, miR-23a p=0.11).

Further analysis was performed in order to determine the expression of these markers according to different cities the patients were living in. As depicted in Figure 1, the expression of the miR-181b was significantly different between Tehran and Amol cities and it was downregulated in Tehran comparing to Amol (p=0.02 Figure 1).

Patients in this study were separated into three groups (mild, moderate, severe) based on their symptoms and their scores in the diagnostic tests. The results demonstrated that the expression of IL-6 gradually increased with the severity of the disorder in ASD patients (p=0.04, Figure 2).

The final analysis between patient and control groups indicated that IL-6 expression was significantly increased in ASD patients (p=0.003, Figure 3). However, no similar results were obtained for other parameters.

BCL-2 was down-regulated in ASD patients as compared with healthy controls (FC=-1.74). However, the difference was not significant (p=0.1, figure 3). In addition, miR-23a was upregulated in ASD patients (FC=1.99) with no statistically significant difference compared to controls (p=0.1, Figure 3).

The miR-181b results were categorized into two groups, one for the upregulated patients compared to mean Ct in the control group and the other one for downregulated patients (Figure 4). The comparison between these two groups revealed that there was a significant difference in the expression of miR-181b in both groups. (p<0.01 Figure 4)

In order to evaluate the predictive value of the target parameters and to find a cut-off for each of them, ROC analysis was performed and graphs were drawn. ROC analysis was able to predict the specificity and sensitivity of the parameters based on the certain cut-off points (Figure 5 and Figure 6). Also, the details of ROC analysis for all parameters are shown in table 3.

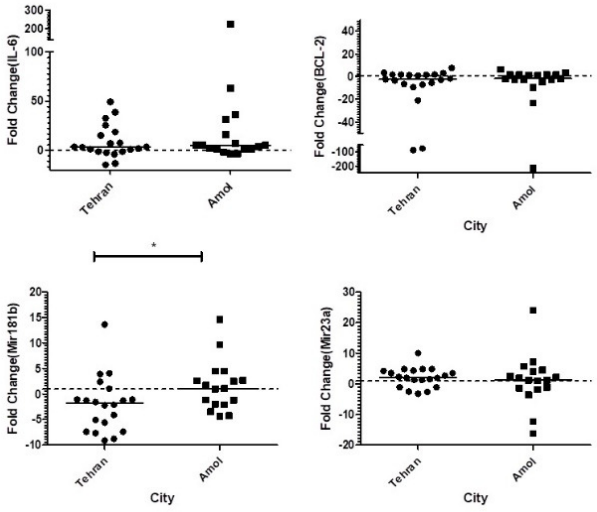

Figure 1. Comparison of target genes expression between Tehran (n=20) and Amol (n=17). The results of comparison among two groups are as follows: p (IL-6=0.53, BCL-2=0.66, miR-181b=0.02, miR-23a=0.40). The results show that miR-181b downregulates significantly in patients from Tehran comparing to Amol. *p<0.05

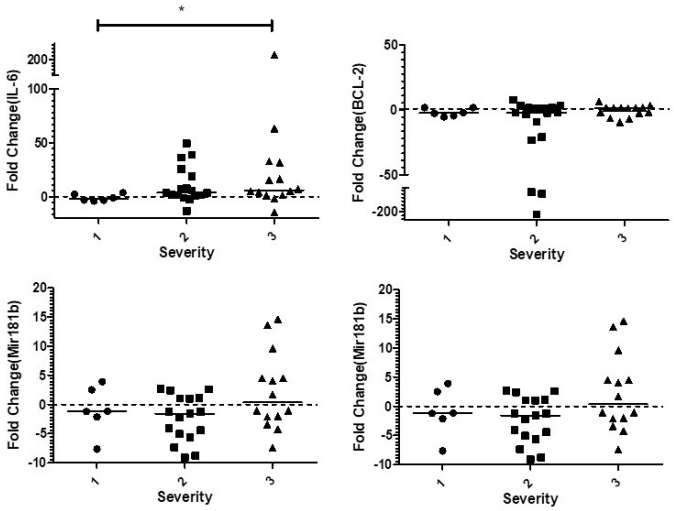

Figure 2. Comparison of target genes expression in patients with different severities (n=37). The results of comparison among three groups are as follows: p (IL-6= 0.04, BCL-2= 0.43, miR-181b= 0.10, miR-23a=0.32). The results demonstrate that the IL-6 expression significantly increases with the severity of the disorder. *p<0.05

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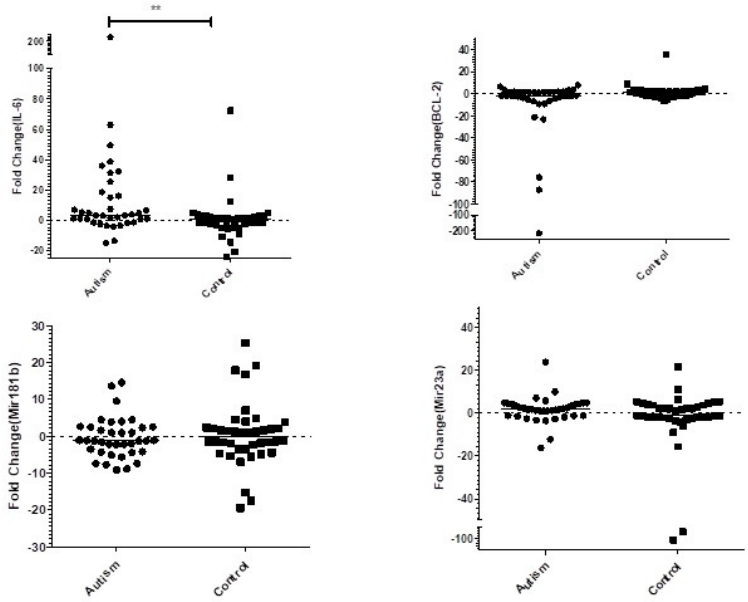

Figure 3. Comparison of target genes expression between patient (n=37) and control group (n=40). The results of comparison among patients and control groups were as follows: *p* (IL-6= 0.003, BCL-2= 0.1, miR-181b= 0.61, miR-23a=0.18). The data shows that IL-6 expression was increased significantly in the patient group. However, the upregulation of miR-23a and downregulation of BCL-2 was not statistically significant. **p<0.01.

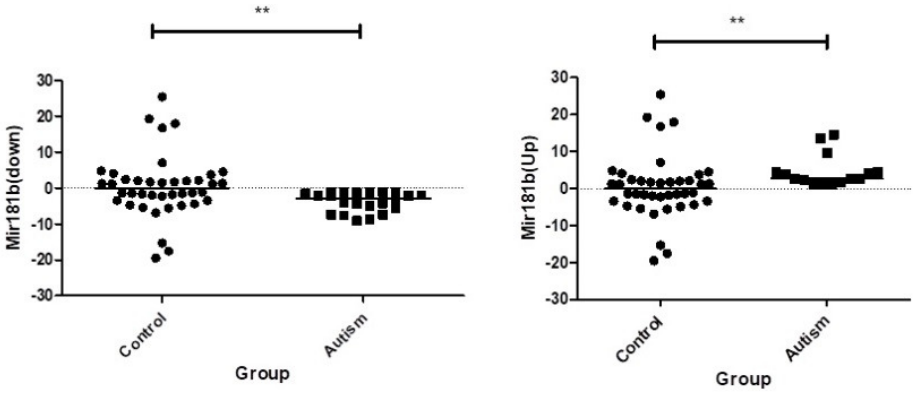

Figure 4. Comparison of miR-181b expression in two groups of up (n=16) and down (n=21)-regulation. *p*: upregulated=0.005 and downregulated=0.004. The evaluations show that if the changes in miR-181b assessed separately in up and downregulated groups, it would be significant. **p<0.01

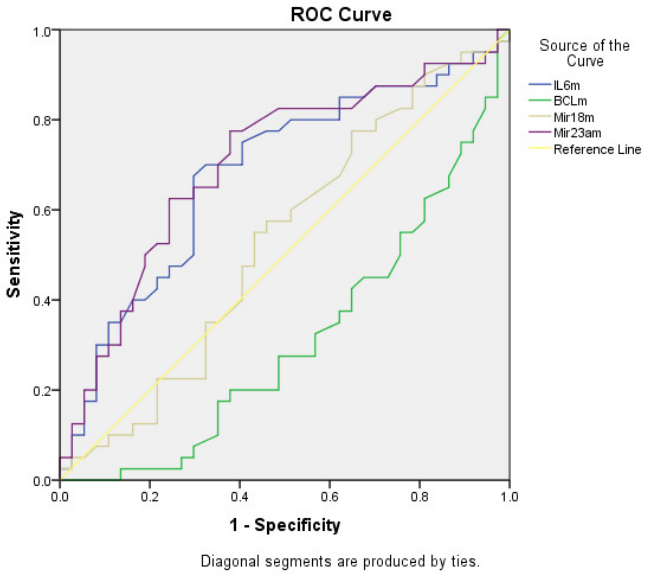

Figure 5. Compiled ROC curve for all target genes. The curves that are inclined to the upper left have higher predictive value. As it is demonstrated, IL-6 and miR-23a show a fairly strong predictive value

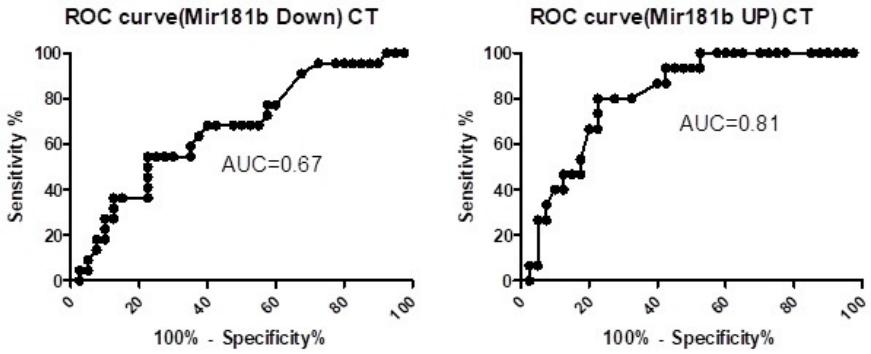

Figure 6. ROC analysis for miR-181b two separated groups (up and down-regulated patients). As it is stated, both graphs are shifted to the left and up which indicates that the changes in miR-181b (regardless of the direction of the change) have a quite strong predictive value in autistic patients

DISCUSSION

ASD is recognized with a deficit in social interactions and repetitive and stereotyped behaviors.[1-4] It is diagnosed mostly by the age of 2-3 years old with losing some or all ability to make interaction with others or speaking ability. Investigating the individual's behaviors and interaction are the gold standard in the diagnosis of autism. In spite of precise diagnosis, these methods cannot diagnose autism earlier than 2 years after birth. On the other hand, the effectiveness of the treatments is directly related to the time of diagnosis.[6] Hence, finding definitive early markers can greatly impact the quality of life in ASD patients.

Based on the previous studies, inflammation and apoptosis are amongst the pathways that are skewed in ASD and therefore it seems that it is more plausible to find reliable biomarkers related to these phenomena. IL-6,[10] BCL-2,[11,12] miR-181b-5p,[25,28] and miR-23a-3p[24] are the possible biomarkers and the evaluation of

which, could shed more light on this matter. The present study has tried to assess all the above-mentioned biomarkers in PBMCs of ASD patients in order to find a convenient and reliable biomarker in the diagnosis and monitoring of the autism disorder.

IL-6 assessments in PBMC of ASD patients indicated that its expression increased significantly compared to the control group. This finding is in line with previous studies regarding IL-6 level and its gene expression in brain and peripheral blood.[10] On the other hand, since the IL- 6 expression in ASD patients was steady in different age groups (<5 and >5 years old) it can be concluded that the IL-6 increase and inflammation are chronic phenomena which can be traced in different stages of life. Therefore, it is a proper candidate as a reliable early biomarker and monitoring tool in ASD patients.

Along with other studies,[30,31] the results showed that if the symptoms are more severe, the production of IL-6 would increases. This finding brings up the potency of IL-6 in the monitoring of the effectiveness of the treatments or in the evaluation of the patient's present condition. In this regard, ROC analysis suggested that a patient with a Ct below 22.75 is considered to be autistic with a sensitivity of 70.27% and specificity of 67.5%. Therefore, it is possible to determine a certain cut-off for IL-6, however, it needs more accurate and thorough investigations in order to reach a definitive point. Also as the results of IL-6 evaluation on PBMC were promising, it is deducible that PBMC is a proper alternative to brain tissue in this regard. This finding is somehow in line with other studies that introduced the evaluation of serum and LCL in ASD studies.[11,13] Also, results proposed that the changes in peripheral blood were not necessarily a passive reflection of changes in brain tissue, but PBMCs actively produce IL-6 in high levels in ASD context; and the inflammation might be generalized rather than limited to brain tissue.

Assessment of the BCL-2 indicated that its expression decreased in autistic patients although it was not statistically significant. However, it confirmed the findings in previous studies[11] which proposed that apoptosis is not limited to the brain but it is dispersed in other tissues including peripheral blood.[32] Also it merely suggests that apoptosis does not happen only in the fetal period but also continues to stay increased as a chronic phenomenon of different ages.

miR-23a evaluations were able to demonstrate its increase in autistic patients which was in line with previous studies in peripheral blood andbrain,[21] However, it failed to be conclusive due to statistical insignificance. The reason for that might be because of the limitation of the study design. On the other hand, since it does not change in different age groups, it could be considered as a potential biomarker; however, it needs more accurate evaluations. Also, in spite of being insignificant, the ROC analysis determined a fairly good predictive value for miR-23a which calls for more in-depth assessment in the context of neurodevelopmental disorders.

In the initial assessments of the miR-181b, there was no significant and meaningful relationship between the patient and control groups. According to previous studies, there should have been a significant difference in the expression of miR-181b between these two groups and either up- or down-regulation should be determined in autistic patients.[19,21,33] Based on the selected population and extrinsic factors, it can be deduced that it is difficult to perform conclusive comparative analysis for this specific miR, but it seems to be definitive that miR-181b expresses significantly different in all ASD cases. Therefore, secondary analyses were performed on patients' results regarding the miR-181b. In these analyses, patients were separated into two groups based on their miR-181b expression: up-regulation sub-groups and down-regulation sub-group, in order to find out whether the changes are significant if the direction of the changes is excluded from the assessment. Since the results showed that both up- and down-regulation were significant in autistic patients, it is suggested that miR-181b expression is significantly different from the control group regardless of the direction of the changes.

The reason for such findings might be the heterogeneity and bias in the patients' selection and even the control group (all of them being chosen from one city) and for that, the data are quite difficult to interpret. On the other hand, independent studies have demonstrated the effect and the role of lifestyle and ethnicity in the probability of developing and/or the manifestation of symptoms in ASD patients[34,35] which might be the reason for results in the present study.

Moreover, based on the findings in different ethnic groups in Tehran and Amol for miR-181b, it can be hypothesized that lifestyle and ethnicity might influence the manifestation, severity and/or development of ASD. However, due to the limitations, it is suggested to evaluate this marker in a larger and

more comprehensively selected group and utilize more accurate analytic techniques to achieve more reliable results. Also, the use of quantitative assessments rather than Ct based analysis is recommended for future studies as it can generate more consistent data.

In conclusion, the results of the present study proposed that IL-6 and miR-181b-5p are promising potential biomarkers in the diagnosis and monitoring of ASD patients throughout life, while BCL-2 and miR-23a-3p need more elaborate and further assessments to unravel their true potentials. Also, PBMCs are a practical and proper specimen in these types of studies and can be utilized in the early diagnosis and monitoring of ASD patients. It is suggested to analyze other inflammatory and apoptotic factors in future cohort studies in order to monitor newborns right after birth or even before, and track their changes in the hope of finding a new gold standard diagnostic criteria.

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