Assessment of Apoptosis Pathway in Peripheral Blood of Autistic Patients

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Abstract

Autism spectrum disorder (ASD) includes a number of severe neurodevelopmental disorders known by defects in social interaction, impaired verbal and non-verbal interactions, and stereotypic activities and limited interests. Dysregulation of apoptotic pathways have been demonstrated in brain tissues of affected individuals. In the present study, we evaluated expression levels of apoptosis-related genes and miRNAs in peripheral blood of ASD patients compared with healthy subjects. Transcript levels of *BCL2*, *CASP8*, and *hsa-29c-3p* were significantly lower in total ASD patients compared with total normal children (P values = 0.003, 0.002, and 0.01 respectively). When sex of study participants was considered in the analysis, the difference in transcript levels of these genes was significant only in male subjects. Peripheral expression of *BCL2* and *hsa-29c-3p* had 100% sensitivity 92% specificity in ASD diagnosis. The diagnostic power of combination of transcript levels of these genes was estimated to be 78% based on the calculated AUC value. The present study provides evidences for dysregulation of apoptotic pathways in peripheral blood of ASD patients and suggests certain apoptosis-related genes as biomarkers in this regard.

Keywords Autism · Apoptosis · *BCL2* · *CASP* · *hsa-29c-3p*

Introduction

Autism spectrum disorder (ASD) comprises a group of profound neurodevelopmental disorders described by destruction of social interaction, impaired verbal and non-verbal interactions, and stereotypic activities and limited interests (Nazeer and Ghaziuddin 2012). Although the molecular underlying mechanisms of autism have not been clarified yet, postmortem and animal studies have demonstrated abnormal regulation of apoptotic pathways in autism (Wei et al. 2014). Apoptosis is regarded as a critical element of typical development of the nervous system. Dimensional, chronological, or quantitative dysregulation of this process would result in an anomalous quantity of neurons and deficient neural development (Wei et al. 2014). The mitochondrial, death receptor, and inflammatory pathways have been recognized as the main apoptotic pathways in the nervous system (Jarskog et al. 2005). Neuroimaging studies and assessment of postmortem brain have shown several anomalies in ASD patients including diminished Purkinje cell numbers in the cerebellar hemispheres and vermis (Bauman and Kemper 1985), absence of granular cells (Bauman and Kemper 2005), atypical widening of brain (Amaral et al. 2008), decreased corpus callosum size (Egaas et al. 1995), and enlarged amygdala size (Schumann et al. 2004) all of which can be at least partially attributed to dysregulation of apoptotic pathways. However, discoveries in the field of autism neuropathology are being limited by inaccessibility of brain tissue for analysis (Petinou and Minaidou 2017). Blood tissue is regarded as an alternative in this regard due to its availability especially during early phases of disease and relatively similar expression pattern with brain (Sullivan et al. 2006). In the current study, we selected three apoptosis-related mRNA coding genes (*BCL2*, *CASP2*, and *CASP8*) and four microRNAs (miRNAs) (*hsa-miR-17-5p*, *hsa-miR-20a-5p*, *hsa-miR-29a-3p*, and *hsa-29c-3p*) to assess their expression in the peripheral blood of ASD patients compared with

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normally developed children to find whether their expression pattern could be recruited as a diagnostic marker for ASD. Former studies have shown interaction between peripheral and central inflammation in the pathogenesis of ASD. Among the mentioned assessed molecules were those linked with the apoptosis (Cristiano et al. 2018). Moreover, as miRNAs can be transmitted from peripheral tissues into the bloodstream (Creemers et al. 2012), it is convincible to assume that alterations in miRNA levels in the peripheral blood mirror their concentrations in the central nervous system (Vaccaro et al. 2018). This speculation can be also applied to apoptosis-associated miRNAs. BCL2 is a principal up-stream protein in the intrinsic apoptotic pathway with anti-apoptotic effects (Cristiano et al. 2018). CASP2 cooperates with c-Myc to cytochrome *c* release into the cytosol leading to activation of the intrinsic apoptosis pathway (Vaccaro et al. 2018). CASP8 as another apoptotic initiator is a cysteine protease with a crucial function in the extrinsic apoptotic signaling pathway through death receptors (Creemers et al. 2012). *miR-20a* participates in the regulation of the extrinsic pathway through downregulation of Fas expression (Huang et al. 2012). Decreased expression of *miR-29a* is associated with overexpression of a chief anti-apoptotic BCL-2 family member (Desjobert et al. 2011). *hsa-miR-29a-3p* and *hsa-29c-3p* are possibly involved in extrinsic pathway (Venkatadri et al. 2016).

Material and Methods

Study Participants

The current study was conducted on peripheral blood samples obtained from 50 ASD patients (38 males and 12 females, age ± SD (range) = 6 ± 1.46(4–9)) and 50 healthy subjects (37 males and 13 females, age ± SD (range) = 6.04 ± 1.74(4–13)). Patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) (Association 2013). A team consisted of psychologists and psychiatrics confirmed the diagnosis of patients through structured developmental assessments such as Autism Diagnostic Observation Schedule and Autism Diagnostic Interview—Revised (Steiner et al. 2012). The results of developmental, speech, and motor testing were documented. Control subjects were chosen from normally developed children with no history of behavioral/psychiatric disorder or former psychiatric treatment in themselves or their first-degree relatives. The absence of behavioral/psychiatric disorder was verified by a psychologist. Informed consent forms were signed by the parents of study participants. The study protocol was approved by ethical committee of Hamadan University of Medical Sciences. This study was conducted in accordance with the Declaration of Helsinki.

Expression Study

Peripheral blood samples were collected from all study participants in EDTA tubes. All samples were gathered from study participants between 10 to 12 AM. Total blood cells were subjected to RNA extraction without enrichment of any kinds of blood cells. Hybrid-R™ blood RNA extraction Kit (GeneAll Biotechnology Co Ltd., South Korea) and the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit were used for isolation of total RNA and cDNA synthesis. Approximately, 3 μg RNA per 0.25 ml of blood was obtained from each sample and 250 ng of RNA samples were used for cDNA production. The relative transcript levels of genes were compared between study groups using the Applied Biosystems TaqMan® Universal PCR Master Mix in rotor gene 6000 Corbett Real-Time PCR System. About 50–100 ng of cDNA was used in each reaction. Reactions were performed in duplicate. The *HPRT1* gene was used as the reference gene. PCR program comprised a denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s, and a final extension step in 72 °C for 5 min. The nucleotide sequences of primers and probes used for amplification of apoptosis-related genes are shown in Table 1.

Approximately, 3 μg RNA per 0.25 ml of blood was obtained from each sample and 250 ng of miRNA samples were used for cDNA production. TaqMan® MicroRNA Reverse Transcription Kit (Invitrogen) was used to convert miRNAs to cDNA. About 50–100 ng of cDNA was used in each reaction. Relative expressions of miRNAs were compared between cases and controls using TaqMan® MicroRNA Assays kit (Applied Biosystems). *RNU6B* gene was used as normalizer. Reactions were performed in duplicate. The mean CT values of this housekeeping gene were 17.79 and 17.87 in cases and controls respectively.

Statistical Analysis

SPSS version 18 (Chicago, IL, USA) was used for statistical analysis. Relative expression of genes in ASD patients compared with controls was calculated using Efficiency^-ΔCT values. The significance of difference in mean values of gene expressions between patients and healthy subjects was assessed using independent *T* test. Spearman rank order correlation test was applied to assess the correlation between relative expression levels of genes. *P* values less than 0.05 were considered significant.

The receiver operating characteristic (ROC) curve was plotted to assess the diagnostic power of genes transcript levels for categorizing disease status. In order to evaluate gene expression probability cut-off, the Youden index (*j*) was applied to maximize the difference between sensitivity (true-positive rate) and 1—specificity (false-positive rate).

Table 1 The nucleotide sequences of primers and probes used for assessment of transcript levels of mRNA coding genes

Gene name	Primer and probe sequences	Primer and probe length	Product length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM-CATCTGGAGTCCTATTGACATCGC-TAMRA	24	
BCL2	F: GTGTGTGGAGAGCGTCAACC	20	78
	R: CGGTTCAGGTACTCAGTCATCC	22	
	FAM-CGCCCTGGTGGACAACATCGCC-TAMRA	23	
CASP2	F: GTGCAAGGAGATGTCTGAATACTG	24	149
	R: GAAGATCAAAGGCTCTATCACACC	24	
	FAM-TGCCGCCACCTCTACCTGTTCCCA-TAMRA	24	
CASP8	F: ACTAGAAAGGAGGAGATGGAAAGG	24	81
	R: CTGATAGAGCATGACCCTGTAGG	23	
	FAM-ACTTCAGACACCAGGCAGGGCTCA-TAMRA	24	

Sensitivity and specificity values were calculated based on the following equations:

$$\text{Sensitivity} = \text{true positive/true positive}$$
$$+ \text{ false negative; specificity}$$
$$= \text{true negative/true negative} + \text{false positive}$$

The diagnostic power of transcript level of each gene was scored based on the area under curve (AUC) values using the following system: 0.90–1 = excellent (A), 0.80–0.90 = good (B), 0.70–0.80 = fair (C), 0.60–0.70 = poor (D), and 0.50–0.60 = fail (F).

Results

Relative Expression of Genes in ASD Patients and Controls

The level of HPRT1 and RNU6B were similar between ASD and controls. Transcript levels of BCL2, CASP8, and hsa-29c-3p were significantly lower in total ASD patients compared with total normal children (P values = 0.003, 0.002, and 0.01 respectively). When sex of study participants was considered in the analysis, the difference in transcript levels of these genes was significant only in male subjects. Figure 1 shows relative expression of genes in ASD patients and controls. Fold change values and the corresponding P values are shown in Table 2.

Correlation Between Relative Expressions of Genes and Age of Study Participants

No significant correlation was found between relative expression of genes and age of ASD cases. However, expressions of hsa-miR-17-5p and hsa-miR-20a-5p were inversely correlated with age in controls. Table 3 shows R and P values of partial correlations, controlling for sex, between levels of gene expression and age of study participants.

Pairwise Correlation Between Expression Levels of Genes

BCL2 expression was significantly correlated with expression of both CASP8 and CASP2 genes in both cases and controls. CASP2 and CASP8 expressions were correlated only in healthy subjects. hsa-miR-29a-3p expression was correlated with expression levels of other miRNAs only in healthy children. Table 4 shows pairwise correlation between expression levels of genes. The most significant pairwise correlation was the correlation between hsa-miR-20a-5p and hsa-miR-17-5p (Fig. 2).

ROC Curve Analysis

ROC curves were depicted to demonstrate the diagnostic ability of transcript levels of each gene as a binary classifier system when its discrimination threshold is altered. The true positive rate (sensitivity) was plotted against the false positive rate (1—specificity) at different threshold sites. The AUC was defined as the likelihood that transcript levels of a certain gene could rank a randomly chosen sample from a patient higher than a randomly chosen control sample. As transcript levels of BCL2, CASP8, and has-29c-3p were significantly different between ASD patients and healthy subjects, we evaluated the diagnostic power of these genes in differentiating disease status (Fig. 3 and Table 4). BCL2 had 100% sensitivity, while hsa-29c-3p had 92% specificity in this regard. The diagnostic power of combination of transcript levels of these genes was estimated to be 78% based on the calculated AUC value (Table 5).
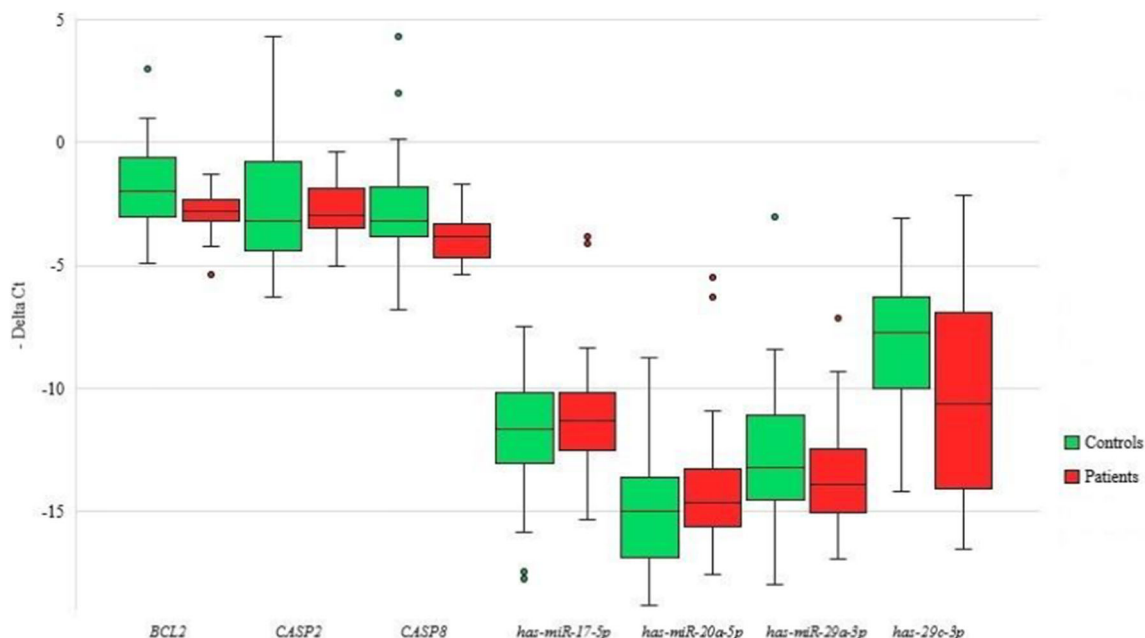
Fig. 1 Relative expression of genes in ASD patients and controls as described by -Delta CT values ($CT_{reference\ gene} - CT_{target\ gene}$)

Discussion

The role of apoptosis in the development of brain has been highlighted previously (Roth and D'Sa 2001). Several abnormal features of autistic brains can be attributed to dysregulation of apoptotic pathways. Recently, Dong et al. have shown the occurrence of endoplasmic reticulum (ER) stress and the consequent induction of apoptosis in distinct regions of autistic brains and suggest that cellular stress and apoptosis might participate in the pathogenesis of autism (Dong et al. 2018).

In the current case-control study, we demonstrated lower levels of *BCL2* transcripts in the peripheral blood of ASD patient compared with healthy subjects. The role of BCL2 in the neuropsychiatric diseases has been highlighted previously. Jarskog et al. have assessed expression of apoptosis-related genes in middle temporal gyrus of postmortem tissues and found elevated Bax/Bcl-2 ratio in the schizophrenia patients compared with controls and proposed that cortical cells are susceptible to apoptosis in chronic schizophrenia (Jarskog et al. 2004). Fatemi et al. have evaluated expression of Bcl-2

Table 2 Relative expression of genes in ASD patients and controls. Efficiency ^CT reference gene-efficiency ^CT target gene were compared between two study groups

		Patients vs. controls (50 vs. 50)	Male patients vs. male controls (38 vs. 37)	Female patients vs. female controls (12 vs. 13)
BCL2	Expression ratio	0.56	0.46	0.95
	P value	0.003	0.001	0.83
CASP2	Expression ratio	0.9	0.88	1.09
	P value	0.6	0.64	0.9
CASP8	Expression ratio	0.56	0.48	0.85
	P value	0.002	0.002	0.54
hsa-miR-17-5p	Expression ratio	1.58	1.22	2.39
	P value	0.17	0.57	0.25
hsa-miR-20a-5p	Expression ratio	1.84	1.33	3.54
	P value	0.1	0.5	0.1
hsa-miR-29a-3p	Expression ratio	0.7	0.7	0.81
	P value	0.28	0.38	0.73
hsa-29c-3p	Expression ratio	0.31	0.27	0.44
	P value	0.01	0.01	0.33

Table 3 Partial correlations, controlling for sex, between levels of gene expression and age of study participants

	Case		Control	
	R	P value	R	P value
BCL2	−0.03	0.87	0.02	0.88
CASP2	0.12	0.52	0.001	0.99
CASP8	0.01	0.93	−0.01	0.92
hsa-miR-17-5p	−0.22	0.24	−0.35	0.02
hsa-miR-20a-5p	−0.29	0.11	−0.34	0.02
hsa-miR-29a-3p	−0.22	0.24	−0.06	0.69
hsa-29c-3p	−0.12	0.51	−0.1	0.5

in cerebellar cortex of autistic patients and normal controls and found significant decreased level of this protein in autistic cerebellum which is suggestive of its participation in certain structural and behavioral defects seen in autism (Fatemi et al. 2001). Moreover, Bcl-2 levels have been decreased in autistic superior frontal cortex of autistic patients compared to control tissues (Araghi-Niknam and Fatemi 2003). These results have been confirmed by other independent studies in both brain tissues (Sheikh et al. 2010a, b) and lymphoblast cell lines (Malik et al. 2011). Totally, these observations are consistent with the reported neuroprotective role for Bcl-2 which is exerted through inhibition of apoptosis and increasing cell survival (Sasaki et al. 2006).

We also detected downregulation of CASP8 in peripheral blood of autistic patients compared with controls. Siniscalco et al. have previously assessed transcript levels of Caspases in peripheral blood mononuclear cells (PBMCs) of autistic patients and healthy subjects and demonstrated elevated mRNA levels for caspase-1, -2, -4, -5 in ASD children as compared to

healthy subjects. However, they demonstrated no significant difference in transcript levels of caspase-8 between cases and controls (Siniscalco et al. 2012). The inconsistency between our results and Siniscalco et al. might be due to source of expression analysis (total blood vs. PBMCs) or their small sample size (15 cases and 10 controls). Alternatively, the ethnic-based differences including both environmental and genetic factors might be involved in alteration of genes expressions.

Although our study shows dysregulation of genes involved in apoptosis process in ASD patients, the consequence of this dysregulation should be assessed by functional studies. Moreover, assessment of protein levels of these genes could be beneficial in this regard.

In addition, we demonstrated lower levels of hsa-miR-29c-3p in peripheral blood of autistic patients compared with controls. Kichukova et al. have assessed the relative expression levels of 42 miRNAs in the serum of ASD patients compared to normal controls and found higher expression of 11 miRNAs while lower expression of 29 miRNAs (including hsa-miRNA-29c-5p) in ASD patients compared with controls (Kichukova et al. 2017). hsa-miR-29c-3p has been identified as a pro-apoptotic miRNA which targets both pro- and anti-apoptotic genes in breast cancer MCF-7 cell line (Venkatadri et al. 2016). On the other hand, this miRNA is expressed in in the brain and decreases beta secretase 1 (BACE1) protein as demonstrated by both in vitro and animal studies (Zong et al. 2011). Although evidences support the role of BACE1 in neuronal synapses, such function is dependent on synaptic developmental stages as its deletion has not changed synaptic proteins in the previously developed neuronal synapses (Kamikubo et al. 2017). So although the observed downregulation of hsa-miR-29c-3p in our study might be accompanied by elevation of BACE1, the contribution of such elevation in

Table 4 Pairwise correlation between expression levels of genes (*correlation is significant at $P < 0.05$ level, **correlation is significant at $P < 0.01$ level)

		hsa-miR29c-3p	hsa-miR-29a-3p	hsa-miR-20a-5p	hsa-miR-17-5p	CASP8	CASP2
BCL2	Patients	0.05	0.01	0.000	0.003	0.16*	0.27*
	Controls	0.01	0.05	0.000	0.03	0.38**	0.2*
CASP2	Patients	0.02	0.003	0.02	0.003	0.06	
	Controls	0.004	0.009	0.04	0.02	0.18*	
CASP8	Patients	0.13*	0.23*	0.04	0.03		
	Controls	0.01	0.007*	0.02	0.008		
hsa-miR-17-5p	Patients	0.23*	0.01	0.66**			
	Controls	0.12*	0.29**	0.49**			
hsa-miR-20a-5p	Patients	0.17*	0.05				
	Controls	0.16*	0.12*				
hsa-miR-29a-3p	Patients	0.09					
	Controls	0.14*					

Fig. 2 The correlation between *hsa-miR-20a-5p* and *hsa-miR-17-5p* in patients (**a**) and in controls (**b**)
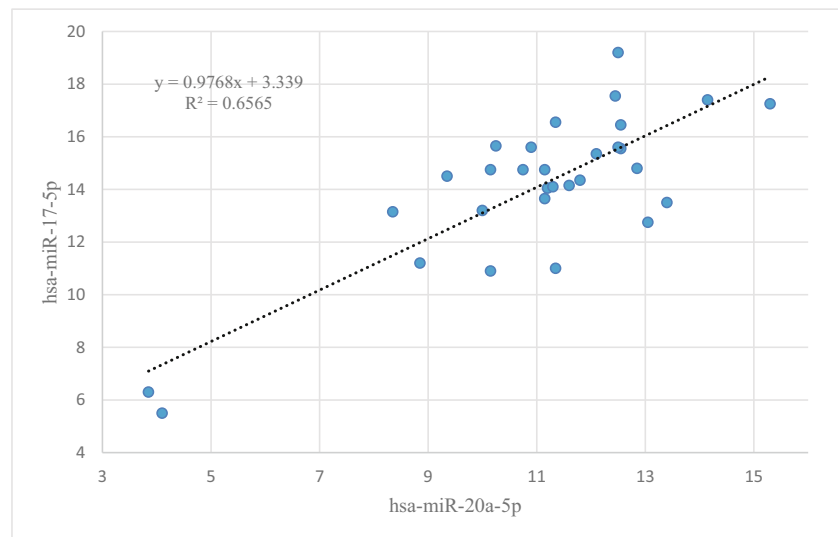
a
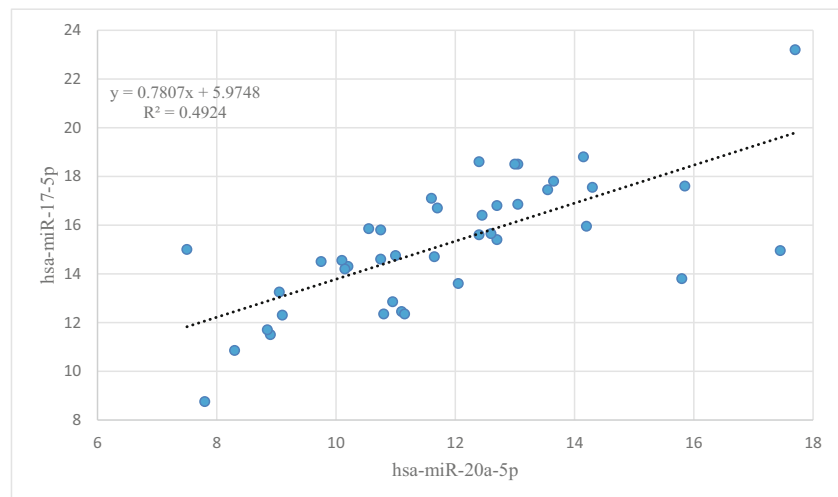
b

the remedy of autism is questionable as the development of neuronal synapses is expected to be accomplished at this diseased stage.

The lack of difference in expression of these genes in female subjects might be due to small number of subjects or sex-based difference in the underlying mechanism or phenotypes of ASD. Phenotypic differences have been noted between affected girls and boy. For instance, restricted interests and behaviors and stereotypes are less frequent in girls than boys. Such different patterns can be attributed to sexually dimorphic underlying causative mechanisms including those related to sex chromosomal gene dosage and sex hormone levels (Van Wijngaarden-Cremers et al. 2014).

We detected no significant correlation between relative expression of genes and age of ASD cases which means that their peripheral expression is independent of age. So the transcript levels of these genes might be regarded as age-independent markers of disease. The importance of such markers has been highlighted by a previous report which has suggested ineffectiveness of age as a character to illustrate neurodevelopment in ASD. Categorizing ASD patients by age might haze essential data about their developmental milestones and influence their clinical rehabilitations (Wu et al. 2018).

When evaluating the correlation between expression levels of these genes, we found absence of expected correlations in ASD patients in some cases. For instance, *CASP2* and *CASP8* expressions were correlated only in healthy subjects. *hsa-miR-29a-3p* expression was correlated with expression levels of other miRNAs only in

Fig. 3 The results of ROC curve analysis showing diagnostic power of transcript levels of *BCL2*, *CASP8* and *has-29c-3p* in ASD
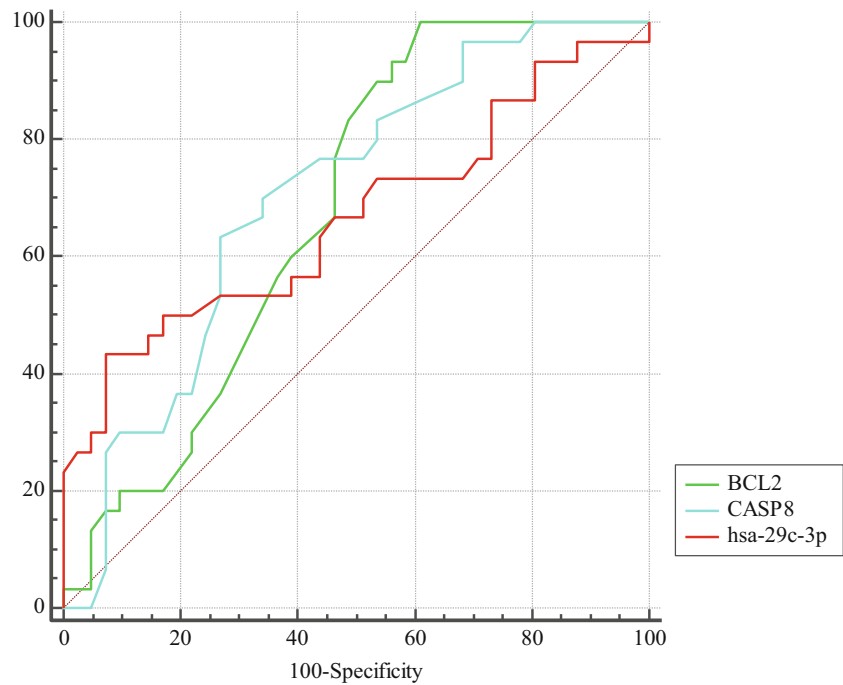
healthy children. Such observations might imply disturbance of regulation of apoptotic pathways in ASD which should be assessed in future studies. A recent study has demonstrated correlations between expression level of *hsa-miR-29a-3p* and environment pollution. Such finding might indicate a possible correlation between ASD, gene expression, and environment (Espin-Perez et al. 2018).

We also assessed diagnostic power of transcript levels of genes in ASD and found that *BCL2* had 100% sensitivity, while *hsa-29c-3p* had 92% specificity in this regard. The diagnostic power of combination of transcript levels of these genes was estimated to be 78% based on the calculated AUC value. Consequently, we proposed these genes as putative elements in a diagnostic panel for ASD. Based on the observed dysregulation of genes only in male subjects, such panel is only appropriate for male subjects at this step. However, lack of significant changes in female subgroup might be due to small number of subjects in this subgroup.

Consequently, assessment of their diagnostic power in larger samples of females is needed to explore their significance in this regard. Future studies are also needed to find more suitable genes to be incorporated in this panel to increase the diagnostic power.

Finally, although we reported dysregulation of apoptosis-related genes in ASD, there may be an environmental component/interaction at play. There are many types of exposures that can cause apoptosis, e.g., fluoride (Jothiramajayam et al. 2014), microwave (Zuo et al. 2014), mercury (Shenker et al. 2000), high glucose (Peng et al. 2013), diesel exhaust (Yun et al. 2009), etc. These environmental factors might affect gene activity as well.

Our study has some limitations. We state lack of assessment of other pro-/anti-apoptotic genes such as other members of CASP family as a limitation of our study. Moreover, the small sample size of the study is another shortcoming of our study which should be overcome in future studies.

Table 5 The results of ROC curve analysis (a: Youden index, b significance level *P* (area = 0.5), Estimation criterion: optimal cut-off point for gene expression; the cut-off point that resulted in the maximum sensitivity and specificity values. As sensitivity and specificity were similarly weighted, the best cut-off point was the point with the highest *J* value)

	Estimation criterion	AUC	*J*[a]	Sensitivity	Specificity	*P* value[b]
BCL2	> 1.2	0.67	0.39	100	39	0.004
CASP8	> 3.5	0.7	0.36	63.3	73.2	0.001
hsa-29c-3p	> 11.65	0.66	0.36	43.3	92.7	0.01
Combination of *BCL2, CASP8* and *hsa-29c-3p*	≤ 0.65	0.78	0.47	86.7	61	< 0.0001

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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