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ORIGINAL ARTICLE

Expression analysis of inhibitory B7 family members in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a global health problem due to its complexity, which frequently makes the development of treatment methods extremely difficult. Therefore, new methodologies are necessary to investigate the pathophysiology of AD and to treat AD. The interaction of immune modulation and neurodegeneration has added new dimensions in current knowledge of AD etiology and offers an attractive opportunity for the discovery of novel biomarkers and therapies. Using quantitative polymerase chain reaction, we compared the expression levels of inhibitory B7 family members (*B7-1*, *B7-2*, *B7-H1*, *B7-DC*, *B7-H3*, *B7-H4*, *B7-H5*, *B7-H7*, and *ILDR2*), as immune regulators, in the peripheral blood of late-onset AD (LOAD) patients (n = 50) and healthy individuals (n = 50). The levels of *B7-2*, *B7-H4*, *ILDR2*, and *B7-DC* expression were significantly higher in-patient blood samples than in control blood samples. Furthermore, we discovered a substantial positive correlation between all gene expression levels. In addition, the current study indicated that *ILDR2*, *B7-H4*, *B7-2*, and *B7-DC* might serve as diagnostic biomarkers to identify LOAD patients from healthy persons. The present work provides additional evidence for the significance of inhibitory B7 family members to the etiology of LOAD.

Keywords Alzheimer's disease · B7 family · Biomarker · Expression analysis · Immune checkpoint

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a kind of dementia leading to memory, behavioral, and thinking problems (Kang et al. 2020). The Alzheimer's Association has stated that AD causes 60–80% of people with dementia. Today, there are 50 million people in the world suffering from AD as well as other kinds of dementia. By the 65-year age, AD's occurrence doubles every five years (Fan et al. 2019). Usually, symptoms are

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revealed gradually and deteriorate with time. AD is genetically a heterogeneous polygenic disorder. It was classified into two kinds of late-onset AD (LOAD) and early-onset AD (EOAD) in terms of the age of initiation. LOAD is the most common dementia, which is known commonly as sporadic AD. As a complicated disease, AD results from susceptible genes and environmental variables (Rezazadeh et al. 2019). A huge deal of effort has been made in the treatment of AD. However, only modest effects have been revealed by presently accepted interventions for AD in the modification of clinical symptoms rather than the progression of the disease. Thus, new approaches are required to assess the pathogenesis and treatment of AD. The progressive loss of cognitive functions and memory are the characteristics of AD (Hardy and Selkoe 2002; Weiner and Frenkel 2006; Ballard et al. 2011). The amyloid-β (Aβ) plaques, hallmark protein aggregates, and neurofibrillary tangles are responsible for cognitive decline. They can quicken neuronal cell death and affect synaptic function adversely, thus leading to neuron loss (Eisele and Duyckaerts 2016; Ballard et al. 2011; Hardy and Selkoe 2002; Weiner and Frenkel 2006). Immune system dysregulation is a cardinal property of AD. Moreover, substantial data suggests that peripheral and central immune responses undergo pathological alterations throughout time (Kinney et al. 2018; Huang et al. 2022; Bettcher et al. 2021). Traditionally, the brain is regarded as immune privileged, although the central nervous system (CNS) is immunocompetent actually, according to compelling data. Furthermore, neuroimmune communication is vital in CNS function, homeostasis, and brain repair, particularly in pathological circumstances such as AD (Liu et al. 2021). It has been evidenced that AD should be regarded as a systemic disease involving dynamic responses in the central and peripheral immune compartments (Bettcher et al. 2021). Immune checkpoint regulators are some molecules expressed on antigen-presenting cells (APCs) or T cells. They can present co-inhibitory and co-stimulatory signals during the activation of T cells. Such a balance between negative and positive signals is vital for increasing antigen-specific immune responses, although the risk for autoimmunity is limited. An extensive contribution of NCRs to CNS pathologies is suggested by previous studies (Baruch et al. 2016; Borggrewe et al. 2018; Joller et al. 2012; Yshii et al. 2017; Cuzzubbo et al. 2017).

B7 family members of the immune regulators containing co-inhibitory and co-stimulatory molecules have a vital role in the regulation of immune responses. The FDA approval of several drugs has emphasized the importance of the B7 family in treating cancers and autoimmune diseases by targeting the receptors or ligands of the B7 family members *CTLA-4* and *PD-L1*/*PD-1* (Liu et al. 2021; Ahangar et al. 2021). Presently, 11 members exist in the growing B7 family including *B7-1* (*CD80*), *B7-2* (*CD86*), *B7-H1* (*PD-L1*, *CD274*), *B7-DC* (*PDCD1LG2*, *PD-L2*, *CD273*), *B7-H2* (*B7RP1*, *ICOS-L*, *CD275*), *B7-H3* (*CD276*), *B7-H4* (*B7x*, *B7S1*, *Vtcn1*), *B7-H5* (*VISTA*, *Gi24*, *DD1*, *Dies1*, *SISP1*), *B7-H6* (*NCR3LG1*), *B7-H7* (*HHLA2*), and Ig-like domain-containing receptor 2 (*ILDR2*) (Bolandi et al. 2021). *B7-DC*, *B7-H1*, *B7-H5*, *B7-H4*, and *ILDR2* possess a co-inhibitory function, *B7-H6* and *B7-H2* act within a co-stimulatory function, while *B7-1*, *B7-H3*, *B7-2*, and *B7-H7* possess both co-stimulatory and co-inhibitory functions (Bolandi et al. 2021). It has been highly evidenced that inhibitory B7 family members can regulate immune responses substantially and maintain immune tolerance (Ahangar et al. 2021; Bolandi et al. 2021). Nevertheless, their expression pattern in LOAD patients' peripheral blood has not been thoroughly studied.

In the present work, expression levels of inhibitory B7 family members (*B7-1*, *B7-2*, *B7-H1*, *B7-DC*, *B7-H3*, *B7-H4*, *B7-H5*, *B7-H7*, and *ILDR2*) were compared in the peripheral blood of healthy controls and LOAD patients to assess their role in the LOAD etiology as well as their potential function as the biomarker of the disease.

Methods

Participants and peripheral blood samples

A hundred people were included in this case-control study as 50 LOAD patients and 50 healthy controls based on age and gender. The current study was approved by Tabriz University of Medical Sciences' clinical research ethics committee (Ethical code: IR.TBZMED.REC.1401.539). The subjects were selected from the Department of Neurology of Tabriz University of Medical Sciences' Imam Reza Hospital. The individuals were identified by a neurology specialist utilizing the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-V) criteria (Association 2013). The inclusion criteria were the age of 65 years and higher and the lack of other psychiatric/neurologic diagnoses except for LOAD. Among people aged 65 years and higher and people without LOAD in the same department, the control group was selected. The exclusion criteria were diabetes, chronic or active infectious diseases, cancer, thyroid disorders, liver, and renal failure, receiving anti-inflammatory drugs, inflammatory diseases, severe ischemic heart disease, metabolic disease, cerebrovascular accident, alcohol abuse, and receiving corticosteroids in the last eight weeks of assessment. Informed written consent was achieved from all subjects or their legal representatives before enrollment in the research. Ultimately, peripheral

blood was sampled (5 ml) from each person in tubes treated with EDTA.

Expression assays

To isolate total RNA from the whole blood, the Hybrid-R™ Blood RNA purification kit was used based on the instructions of the manufacturer (GeneALL, Seoul, South Korea), and DNase I was used for removing contamination of DNA. Using NanoDrop, the quality and quantity of the extracted RNA were assessed (Thermo Scientific, Wilmington, DE). CDNA was synthesized using the cDNA synthesis Kit (GeneALL, Seoul, South Korea) based on the manufacturer's guidelines. For further examination, the cDNA was kept at -20 °C. Table 1 shows the primer sequences employed in quantitative polymerase chain reaction (qPCR) reactions. The internal control was ubiquitin C (*UBC*) for normalizing the mRNA levels. The RealQ Plus2x Master Mix (Ampliqon, Odense, Denmark) and Step OnePlus™ Real-Time PCR were employed for the qPCR. The qPCR reactions were all conducted in duplicate. The mean of ΔCT was approximated for the samples, and the $2^{-\Delta\Delta Ct}$ technique was used to calculate the relative gene expression finely.

Statistical analysis

To perform Statistical analysis, GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) was used. The normal/gaussian distribution of the values was obtained using the Shapiro–Wilk test. The differences in gene expression levels between the two groups were examined using a t-test. *P* values less than 0.05 was significant. By calculating Pearson's coefficient, the correlation between the variables was assessed. The receiver operator characteristics (ROC) analysis defined the genes' diagnostic value. Then, the plot of the true positive rate was made versus the false positive rate at different thresholds.

Results

General demographic data

Based on the exclusion and inclusion criteria, 50 healthy controls (men/women %: 30.6/69.4) and 50 LOAD patients (men/women %: 31.4/68.6) were included in the study with the age of (mean ± standard deviation (SD)) and 76.36 ± 6.26 (mean ± SD) of 74.3 ± 6.22, respectively.

3.2. Expression assays

The relative expression levels of inhibitory B7 family members' in LOAD patients and controls are displayed in Fig. 1. The expression of *B7-2*, *B7-H4*, and *ILDR2* was significantly higher in the patient's blood samples compared to the control blood samples (fold change = 3.575, *P* value < 0.0001, fold change = 3.415, *P* value < 0.0001, fold change = 3.482, *P* value < 0.0001, respectively). An expression difference was also found between the male and female subgroups (Table 2). Moreover, the *B7-DC* expression was significantly higher in the blood samples of LOAD patients than in the controls (fold change = 1.480, *P* value = 0.0241). No expression difference was found between the gender subgroups. No difference was also found in the expression of *B7-1*, *B7-H1*, *B7-H3*, *B7-H5*, and *B7-H7* in the blood samples of the case and control groups (Table 2).

Correlation analysis

In the present study, the association between expression levels of all gene pairs was calculated using the Pearson correlation test (Fig. 2). A considerably positive correlation was found between all genes' expression levels. Furthermore, there was a correlation between *B7-H3* expressions and the age of the patients.

ROC curve analysis

The ROC analysis was conducted to obtain the specificity and sensitivity of the expression of *ILDR2*, *B7-H4*, *B7-2*, and *B7-DC* as biomarkers. Significant results were presented for all four genes (Fig. 3; Table 3). Considering the values of AUC (area under the curve), all four genes can be utilized as diagnostic biomarkers in LOAD, specifically *ILDR2* (sensitivity = 84% and specificity = 74%), *B7-H4* (sensitivity = 80% and specificity = 74%), as well as *B7-2* (sensitivity = 82% and specificity = 70%). Besides, ROC analysis was carried out to combine *B7-2*, *B7-H4*, and *ILDR2* expression levels. AUC was found to be 0.90 to discriminate between LOAD patients and controls (Fig. 4; Table 3).

Discussion

According to various independent investigations in animal models, it is revealed that AD pathology could be effectively reduced by decreasing systemic immune suppression through the depletion of peripheral regulatory T cells (Baruch et al. 2015). These findings are in line with an independent observation revealing the important role

Table 1 Sequences of primers used in qPCR reactions

Gene name	Gene reference ID	Primer sequences	Product length (bp)
B7-1	NM_005191.4	Forward: CTCTTGGTGCTGGCTGGTCTTT	136
		Reverse: GCCAGTAGATGCGAGTTTGTGC	
B7-2	NM_001206925.2 NM_001206924.2 NM_176892.2 NM_006889.5 NM_175862.5	Forward: TGCTCATCTATACACGGTTACC Reverse: TGCATAACACCATCATACTCGA	92
B7-H1	XM_047423262.1 NM_001314029.2 NM_014143.4	Forward: TGCCGACTACAAGCGAATTACTG Reverse: CTGCTTGTCCAGATGACTTCGG	150
B7-DC	XM_005251600.4 NM_025239.4	Forward: CTCGTTCCACATACCTCAAGTCC Reverse: CTGGAACCTTTAGGATGTGAGTG	149
B7-H3	XM_017022638.2 XM_047433148.1 XM_011522095.3 XM_005254700.5 XM_047433147.1 NM_025240.3 NM_001329629.2 NM_001329628.2 NM_001024736.2	Forward: CTGGCTTTCGTGTGCTGGAGAA Reverse: GCTGTCAGAGTGTTTCAGAGGC	126
B7-H4	XM_017002335.3 NM_001253850.2 NM_001253849.2 NM_024626.4 XM_011542143.2	Forward: TCTGGGCATCCCAAGTTGAC Reverse: TCCGCCTTTTGATCTCCGATT	207
B7-H5	NM_022153.2	Forward: AGATGCACCATCCAACTGTGTGG Reverse: AGGCAGAGGATTCCTACGATGC	108
B7-H7	XM_011512367.4 XM_005247080.4 XM_047447369.1 XM_024453326.2 XM_011512364.3 XM_011512363.3 XM_047447368.1 XM_011512362.3 NM_001282556.2 NM_001282558.2 NM_001282559.2 NM_001282557.2 NM_007072.4 NM_001370244.1	Forward: TGAAGCAAACATGGACAGGG Reverse: CAGATAGTAAGCCAGGACAGAG	176
ILDR2	NM_001410891.1 NM_001410892.1 XM_017001257.2 XM_047419950.1 XM_017001256.2 XM_047419940.1 XM_017001255.2 XM_017001254.2 XM_017001253.2 XM_017001252.2 NM_199351.3	Forward: AGCCAGAAGGATGAGAGGCAGA Reverse: GTCAGGATTGCTCTCCAAGTCC	145
UBC	NM_021009.7	Forward: CAGCCGGGATTTGGGTCG Reverse: CACGAAGATCTGCATTGTCAAGT	72

of the adaptive immune system in AD progression in animal models. More importantly, anti-inflammatory cells and immunoregulatory T cells are required in the brain as anti-inflammatory cytokines sources to reduce the inflammatory response. Thus, well-controlled boosting was proposed as a novel method instead of suppression of systemic immunity for modifying the disease pathology by indirectly targeting any disease hallmarks of the brain (Schwartz et al. 2019).

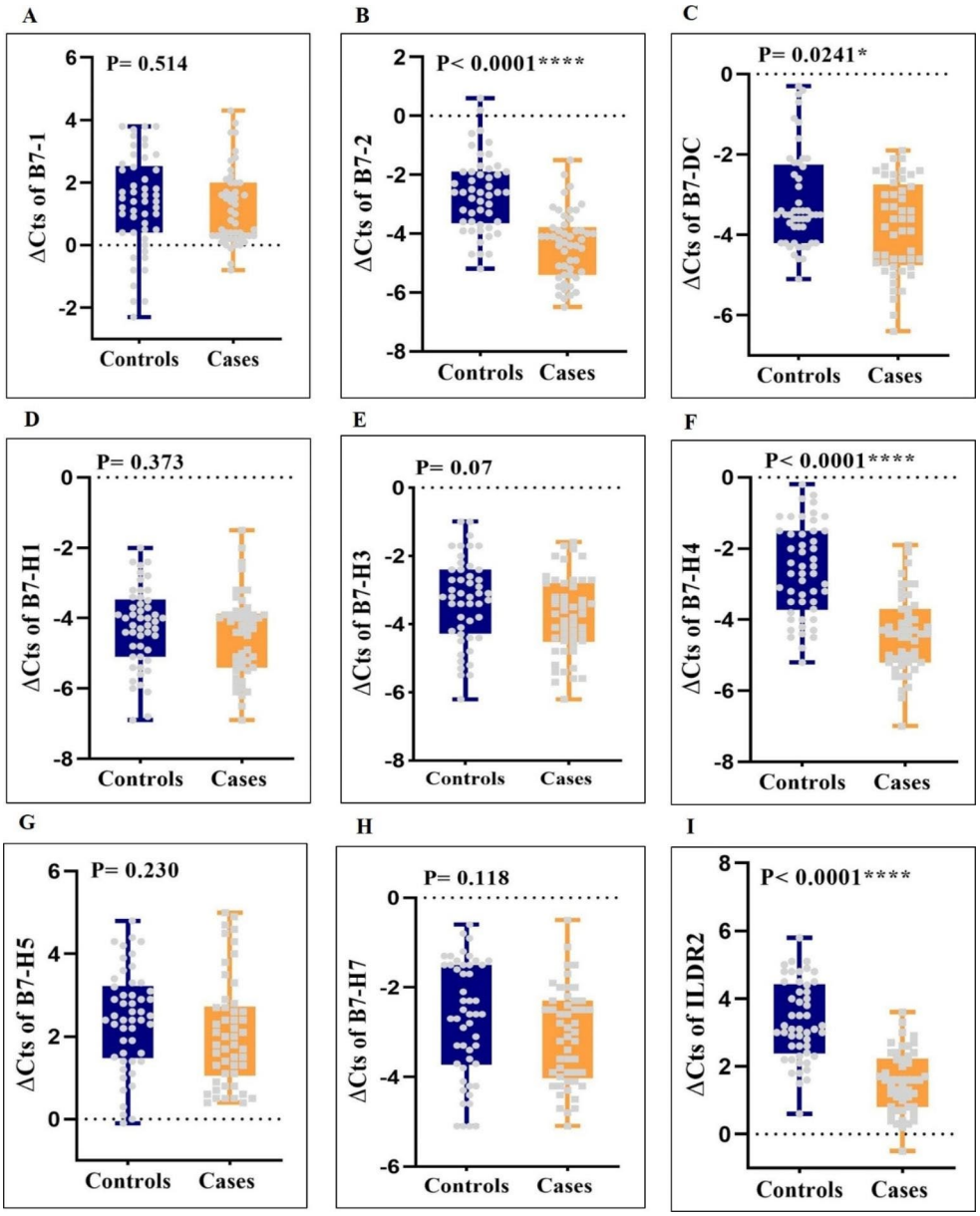

Fig. 1 The relative expression of **(A)***B7-1*, **(B)***B7-2*, **(C)***B7-DC*, **(D)***B7-H1*, **(E)***B7-H3*, **(F)***B7-H4*, **(G)***B7-H5*, **(H)***B7-H7*, and **(I)***ILDR2* in patients and controls. Gene expression levels of each sample were normalized relative to *UBC* expression. The t-test and the formula $2^{-\Delta\Delta Ct}$ were used to determine the transcripts' relative expression

Meanwhile, emerging human data suggests that neuroimmune interactions and cellular adaptive immunity play an important role in AD pathophysiology (Bettcher et al. 2021). According to several studies, there is a dysregulation of peripheral T cell immunity in AD (Bettcher et al. 2021). In line with this idea, we assessed the expression levels of inhibitory B7 family members (*B7-1*, *B7-2*, *B7-H1*, *B7-DC*, *B7-H3*, *B7-H4*, *B7-H5*, *B7-H7*, and *ILDR2*) in the peripheral blood of healthy controls and LOAD patients in this study.

A higher expression of *B7-2*, *B7-H4*, *ILDR2*, and *B7-DC* was found in LOAD patients. However, *B7-1*, *B7-H1*, *B7-H3*, *B7-H5*, and *B7-H7* were not significantly expressed. Two recognized B7 family proteins, B7-1 and B7-2, could attach to Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and CD28 and act as their ligands (Zhu and Li 2018). Inhibitory signals are delivered to activate T-lymphocytes by interacting with CTLA-4 with these ligands. Moreover, immune responses against malignant tumors and the production of cytokines are reduced. B7-H1 also assigned as Programmed cell death-ligand 1 (PD-L1), or CD274, while B7-DC assigned as programmed cell death-ligand 2 (PD-L2), or CD273 are two ligands of programmed cell death 1 (PD-1, CD279) (Sharpe et al. 2007; Riella et al. 2011). PD-L1/PD-L2 interacts with PD-1, thus increasing the tolerance of T-cells, inducing inhibitory effects on T-cell activation/proliferation, and preventing cytolysis of T cell (Freeman et al. 2000b). *B7-H3* shares almost identical amino acid sequences of 20–27% with other B7 family members. According to studies, B7-H3 has a negative regulatory role on T lymphocytes in murine and humans (48, 49) predominantly.

Table 2 Relative expression of genes in total, females, and males patients and healthy controls

Gene name	Parameters	ΔCt means		Up/Down	Fold Change	*P*-Value
		Cases	Controls			
B7-1	Total	1.160	1.342	-	-	0.514
	Female	1.326	1.150	-	-	0.648
	male	0.8895	1.630	-	-	0.0591
B7-2	Total	-4.402	-2.564	Up	3.575	< 0.0001****
	Female	-4.297	-2.383	Up	3.767	< 0.0001****
	male	-4.574	-2.835	Up	3.337	< 0.0001****
B7-H1	Total	-4.460	-4.254	-	-	0.373
	Female	-4.326	-4.09	-	-	0.428
	male	-4.679	-4.5	-	-	0.627
B7-DC	Total	-3.774	-3.208	Up	1.480	0.0241*
	Female	-3.719	-3.293	-	-	0.176
	male	-3.863	-3.080	-	-	0.065
B7-H3	Total	-3.780	-3.316	-	-	0.070
	Female	-3.687	-3.350	-	-	0.298
	male	-3.932	-3.265	-	-	0.123
B7-H4	Total	-4.412	-2.640	Up	3.415	< 0.0001****
	Female	-4.345	-2.530	Up	3.519	< 0.0001****
	male	-4.521	-2.805	Up	3.285	< 0.0001****
B7-H5	Total	2.102	2.408	-	-	0.230
	Female	2.161	2.417	-	-	0.409
	male	2.005	2.395	-	-	0.388
B7-H7	Total	-3.106	-2.730	-	-	0.118
	Female	-3.103	-2.793	-	-	0.325
	male	-3.111	-2.635	-	-	0.217
ILDR2	Total	1.530	3.330	Up	3.482	< 0.0001****
	Female	1.565	3.480	Up	3.77	< 0.0001****
	male	1.474	3.105	Up	3.097	< 0.0001****

*Significant *P* value < 0.05, ****significant *P* value < 0.0001

B7-H4 is also known as *B7S1* and *B7x*. It belongs to the B7 family's immune checkpoints. *VTCN1* is the encoding gene associated with B7-H4 (Prasad et al. 2003; Sica et al. 2003). B7-H4, as an inhibitory immunoregulatory molecule, does not bind to the Inducible T Cell Costimulator (ICOS), CTLA4/CD28, and PD-1 (Sica et al. 2003). No specific receptor has been recognized so far for binding to B7-H4. Though, B and T lymphocyte attenuator (BTLA) was proposed as a B7-H4 receptor binding indirectly (Watanabe et al. 2003, Sedy et al., 2005). *VSIR* is the encoding gene of B7-H5 (Liu et al. 2011). It was demonstrated that B7-H5 functions as a co-inhibitory ligand on APCs. However, it functions as a co-inhibitory receptor by expressing CD4 + T lymphocytes (Le Mercier et al. 2014; Nowak et al. 2017; Flies et al. 2014). *HHLA2*, the human endogenous retro virus-H long terminal repeat associating-2, is also called *B7y* and *B7-H7* (Janakiram et al. 2015). *B7-H7* was found as a B7 ligand family member. It imposes a co-inhibitory impact on the T lymphocytes' responses. Presently, ILDR2 is recognized as a B7-like ligand (Hecht et al. 2018). *C1orf32* is the encoding gene of ILDR2, which is situated on Chr1q23–25 in humans (Dokmanovic-Chouinard et al. 2008). It was revealed that recombinant ILDR2-Fc fusion protein results in cell division and inhibition of the activating the early TCR signaling without incrementing apoptosis of T cells. The CD4 and CD8 T lymphocyte activation is suppressed by the agonistic activity of ILDR2-Fc on inhibitory receptors through monoclonal antibodies against CD28 and CD3 in humans and mice (Hecht et al. 2018). As we know, except for *PD-L1*, *CD80*, and *CD86*, other genes investigated in our work were reported in LOAD patients for the first time. The activity of memory T cells is restrained by inhibitory immune checkpoints, mostly versus self-compounds, to inhibit autoimmune diseases. PD-1 is one of these checkpoints, which is expressed by various activated effector memory immune cells such as CD4 + T cells (Gotsman et al. 2007). Memory T-cell responses such as cytokine production, and proliferation are suppressed by the interaction between PD-L1 and PD-1 (Gotsman et al. 2007; Carter et al. 2002). Potentially, the blockage of PD-L1/PD-1 pathway increases the activation of T cells (Freeman et al. 2000a; Fife et al. 2009; Karwacz et al. 2011). Furthermore, it has been comprehended that targeting systemic PD-1/PD-L1 can activate such protective/reparative immune responses

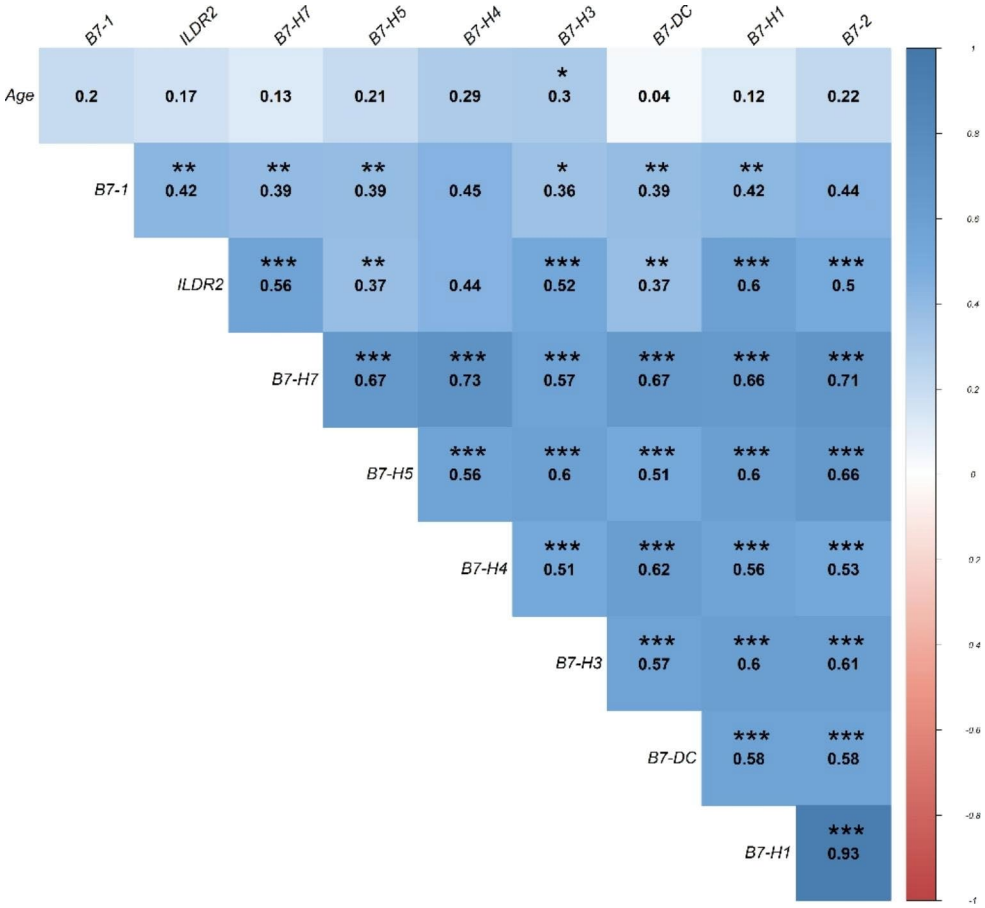

Fig. 2 Correlation between variables in the cases group using a pairwise approach. The correlation coefficients plus the significance level as stars are displayed. *Significant *P* value < 0.05, **significant *P* value < 0.01, ****significant *P* value < 0.0001

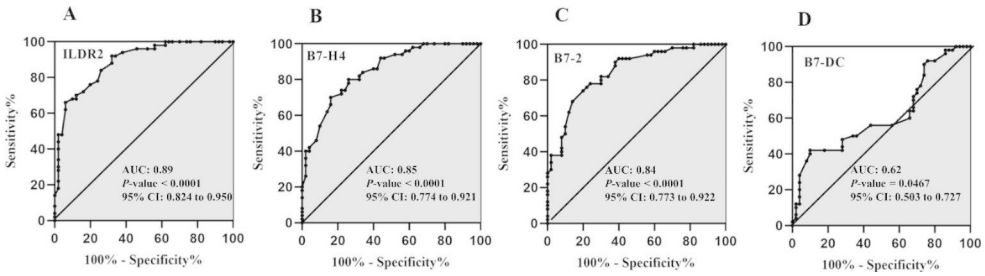

Fig. 3 Receiver operator characteristics (ROC) curve analysis of (**A**) *ILDR2*, (**B**) *B7-H4*, (**C**) *B7-2*, and (**D**) *B7-DC*

Table 3 The results of receiver operator characteristics (ROC) curve analysis of genes in the separation of cases and controls

Gene Name	AUC	Sensitivity%	Specificity%	95% CI	*P*-Value
ILDR2	0.89	84	74	0.824 to 0.950	< 0.0001
B7-H4	0.85	80	74	0.774 to 0.921	< 0.0001
B7-2	0.84	82	70	0.773 to 0.922	< 0.0001
B7-DC	0.62	56	56	0.503 to 0.727	0.0467
Model	0.90	88	74	0.839 to 0.957	< 0.0001

(Schwartz et al. 2019). Moreover, Busse et al. indicated that alterations in the expression of *CD86* and *CD80* in peripheral blood are resulting from immunosenescence, not AD pathology (Busse et al. 2015). However, we found significant expression changes in CD86. Such contradictory result can be justified by different methodology, the small number of examined patients, and the patients treated with rivastigmine in Busse's study.

Besides, a considerable positive association was found between expression levels of all genes. In the meantime, similar regulatory functions may be indicated by the pairwise associations between gene expressions in LOAD patients within immune paths vital for AD pathogenesis.

According to meta-analyses of observational and epidemiological studies, immune markers are dysregulated in AD

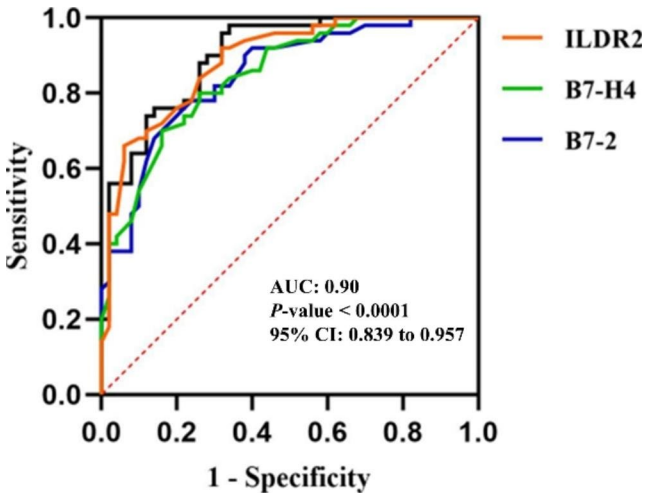

Fig. 4 Receiver operator characteristics (ROC) curve for the combination of expression levels of *B7-2*, *B7-H4*, and *ILDR2*

(Swardfager et al. 2010; Lai et al. 2017) resulting in a higher risk of developing dementia (Darweesh et al. 2018; Koyama et al. 2013). Researchers have shown that alterations in peripheral immune markers may predict cognitive changes and/or symptomatic signs of disease in late life, based on early clinical studies of immune dysregulation. According to these studies, baseline C-reactive protein (Schmidt et al. 2002; Engelhart et al. 2004) and IL-6 (Engelhart et al. 2004) and tumor necrosis factor (TNF) and IL-1β (Tan et al. 2007) levels predict future AD development. According to clinical studies, a biomarker must have high specificity (> 80%) and sensitivity to be useful in classifying and diagnosing diseases (Schneider and Prvulovic 2013). Based on our findings, *ILDR2*, *B7-H4*, *B7-2*, and their combination with *B7-DC* may serve as potential biomarkers for distinguishing LOAD from healthy individuals. In the future, further investigations should be conducted with a larger sample size and the effectiveness of the classifiers should be validated.

A complex pathogenesis for AD is emerging as a result of the interplay between immune regulation and neurodegeneration. In order to develop innovative immunotherapy approaches and identify new immune-related biomarkers of diagnostic interest, it is imperative to further understand peripheral-central immune dysregulation in AD and how these factors contribute to disease pathogenesis. In this study, we demonstrated that there is possibly a relation between increasing the peripheral blood expression of *ILDR2*, *B7-H4*, *B7-2*, and *B7-DC* and an incremented LOAD risk. Furthermore, a diagnostic biomarker may be provided by the combination of expression levels of *B7-2*, *B7-H4*, and *ILDR2* for LOAD. There is still a need for more study, as we can already draw preliminary conclusions.

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Authors' contributions PT, MRA and JG and MSB performed the experiment. ZSF analyzed the data. MR and HS wrote the draft and revised it. MT and AS designed and supervised the study. All the authors contribute equally and read the submission.

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Data Availability The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Tabriz University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication Not applicable.

Competing interests The authors declare they have nothing to report.

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