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The investigation of apoptosis-related genes in periodontitis

Arezou Sayad[1], Fatemeh Hashemian[1], Leila Gholami[2], Masoud Jamali[3], Elham Badrlou[1,4], Saba Sadeghpour[1,5], Naghme Nazer[6], Sheyda Khalilian[1] and Soudeh Ghafouri-Fard[7*]

Abstract

Objectives This study aims at determination of the roles of five apoptosis-related genes, namely *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* in the pathoetiology of periodontitis *via* measurement of their expressions in both peripheral blood and tissues of affected persons.

Results *CASP2* was over-expressed in gingiva of patients compared with healthy subjects (RME = 24.56, $P < 0.001$), and in both affected females and males (RME = 30.53, $P = 0.03$ and RME = 20.59, $P = 0.01$, respectively). *BCL2* was higher in affected tissues compared with controls (RME = 32.28, $P < 0.001$) and in affected tissues of males versus controls (RME = 69.03, $P < 0.001$). Finally, *HULC* had lower level in the blood of patients (RME = 0.21, $P = 0.01$) and in the blood of female patients compared with normal females (RME = 0.15, $P = 0.01$). Other comparisons yielded no significant results. *BCL2* and *CASP2* had the highest diagnostic values for separation of diseased gingival tissues from normal ones. *HULC* has the best values in the distinction of blood samples of affected persons from control persons. Combination of transcript levels of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* changed AUC to 0.84 and 0.72 in tissues and blood samples, respectively. To conclude, these genes might be regarded as putative contributors in the pathophysiology of periodontitis.

Keywords Periodontitis, B-Cell CLL/Lymphoma 2, Caspase2, Caspase 8, HULC, Pvt1

Background

Periodontitis is a chronic disease described by inflammation of the adjoining tissue of the teeth resulting in attachment loss and destruction of bone structures in a progressive manner [1]. Bacterial infections, particularly commensal microorganisms have important roles in the initiation of this disorder. However, the severity of disease is influenced by the interaction between immune system and microorganisms [2]. In fact, elevation of plasma cell, naive B cell and neutrophil populations has been reported in the affected tissue samples [3].

There is evidence that some bacteria exist around teeth and implants even when there is no sign of inflammation. Moreover, some bacterial species have been detected more commonly in supra-gingival peri-implant

*Correspondence:
Soudeh Ghafouri-Fard
s.ghafourifard@sbmu.ac.ir
[1]Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Periodontics, Dental Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
[3]Sarem Hospital, Tehran, Iran
[4]Pediatric Cell Therapy Research Center, Tehran University of Medical Sciences, Tehran, Iran
[5]Hematopoietic Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6]Department of Electrical Engineering, Sharif University of Technology, Tehran, Iran
[7]Dental Research Center, Research Institute for Dental Sciences, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran

biofilm [4]. Inflammatory responses contribute in bone loss through disturbing the balance between activities of osteoblasts and osteoclasts [5]. A number of other biomolecules and signaling pathways are also involved in pathoetiology of periodontitis [6]. Measurement of expression of apoptosis-related genes has led to identification of elevated levels of TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL R4 in gingival tissues of affected persons. Moreover, these tissues had higher levels of cleaved caspase (CASP)-3, X-Linked inhibitor of apoptosis (xIAP) and surviving compared with control tissues. Based on these results, authors have speculated that apoptosis may be suppressed in the periodontitis through elevation of TRAIL decoy receptors and inhibitors of cleaved caspase-3 [7]. Another study has shown remarkable increase or increasing trend in levels of DNA fragmentation and apoptotic proteins in affected tissues of these patients parallel with disease progression [8]. Moreover, several transcription factors, non-coding RNAs and mRNAs have been reported to be dysregulated in periodontitis, among them are a number of apoptosis-related ones [9].

Since apoptotic pathways have roles in the pathophysiology of this disorder, we aimed to identify expression levels of five genes in these pathways, namely *CASP2*, *CASP8*, *B-Cell CLL/Lymphoma 2* (*BCL2*), *hepatocellular carcinoma up-regulated long non-coding RNA* (*HULC*) and *Plasmacytoma Variant Translocation 1* (*PVT1*) in gingival tissue and circulation of patients with periodontitis. The BCL2 family of proteins have essential roles in determination of cell commitment to apoptosis [10]. *PVT1* is a long non-coding RNA (lncRNA) that has anti-apoptotic effects through enhancing expression of BCL2 [11]. *HULC* is another lncRNA that inhibits cell apoptosis, since its over-expression of this lncRNA increases expression of BCL2 and caspase-3 while decreasing levels of cleaved-caspase-3 [12]. The protein encoded by *CASP2* is located in the mitochondria and induces apoptosis directly from this organelle [13]. Finally, the protein encoded by *CASP8* propagates the apoptotic signals either through cleavage of downstream caspases or through cleavage of the BH3 Bcl2-interacting protein [14]. The role of the mentioned apoptotic genes in the periodontitis is unclear.

Objectives

Thus, this study is designed to document the involvement of five apoptosis-related genes in the pathoetiology of periodontitis by quantifying their expression levels in both peripheral blood and tissue samples obtained from individuals with the condition. The rationale is that aberrant expression patterns of these genes may indicate their potential contribution to the disease process.

Methods

Participants

Gingival specimens were collected from persons affected with chronic periodontitis (stages II-IV), in a similar way to our former study [15]. Blood and tissue samples were obtained at the first time of their visit to the clinic. The inclusion criteria were: presence of a minimum of two periodontal pockets in each sextant after non-surgical treatment, probing depth of 5 mm or more, bleeding on probing, and a minimum of 3 mm of attachment loss were included as cases. Tissues were obtained from cases met the inclusion criteria during periodontal flap surgery. Exclusion criteria were history of smoking, inflammatory disorder, cancer, diabetes or intake of antibiotics or anti-inflammatory medicines. All cases were evaluated in the periodontal clinic of Hamadan University of Medical Sciences (Ethical code: IR.SBMU.DRC.REC.1400.011, date of approval: April of 2019). Control tissues were collected during dental crown lengthening procedure from persons with no manifestations of periodontitis and from bleeding on probing free sites.

Expression assay

Blood samples were collected in EDTA tubes. Tissue samples were collected in RNase-free tubes. They were snap-frozen in liquid nitrogen and transferred to the Genetic Lab where they were stored in -70 °C until they were used for RNA extraction. They were stored and prepared in strict RNase-free conditions. GeneAll® Hybrid-R™ Blood RNA kit (South Korea, Cat. No. 315 – 150) was used for extraction of total RNA from 0.2 ml whole blood sample. In addition, GeneAll® RiboEx™ kit (South Korea, Cat. No. 301-001 / 301-002) was used for extraction of RNA from approximately 50 mg tissue samples. Quantities and quality of retrieved RNA were valued by spectrophotometer and gel electrophoresis. A260/A280 ratio of RNA was within the 1.8–2.0 range. Extracted RNA samples were stored in -70 °C or instantly used for cDNA synthesis. Repeated freeze-thaw cycles were avoided. cDNA was made from these specimens by using the cDNA production kit (Smobio Company, Hsinchu City, Taiwan, Cat. No. RP1300). Expression amounts of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes were quantified in tissues and blood samples using the commercial RT-PCR kit provided by GeneDireX Company (Taoyuan city, Taiwan, Cat. No. SQ201-0125). Each reaction included 10 µL of 2X SYBR Green Master Mix, 0.8 µL of each of forward and reverse primers (10 µM), 2 µL (~10 ng) of cDNA template and up to 20 µL nuclease-free water. qRT-PCR was done in LightCycler® 96. Primer sequences are shown in Table 1. Cycling protocol included 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Then, melt curve analysis was performed (65–95 °C, +0.5 °C/5 sec). Each run included

Table 1 Nucleotide sequence of primers

Genes name	Sequences
HPRT1 (internal control)	F: 5′-AGCCTAAGATGAGAGTTC-3′ R: 5′-CACAGAACTAGAACATTGATA-3′
BCL2	F: 5′-GTGTGTGGAGAGCGTCAACC-3′ R: 5′-CGGTTCAGGTACTCAGTCATCC-3′
CASP2	F: 5′-GTGCAAGGAGATGTCTGAATACTG-3′ R: 5′-GAAGATCAAAGGCTCTATCACACC-3′
CASP8	F:5′-ACTAGAAAGGAGGAGATGGAAAGG-3′ R: 5′-CTGATAGAGCATGACCCTGTAGG-3′
HULC	F: 5′-ACGTGAGGATACAGCAAGGC-3′ R: 5′-AGAGTTCCTGCATGGTCTGG-3′
PVT1	F: 5′-CCCATTACGATTTCATCTC-3′ R: 5′-GTTCGTACTCATCTTATTCAA-3′

a no-template control and a positive control sample. The recall rate of PCR runs was more than 90%. Cq (Quantification Cycle) and efficiency values were obtained. *HPRT1* was used as internal control and data showed its consistent expression among samples. All reactions were performed in duplicate. Primers were designed using the Primer3 and BLAST tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) in a way that the amplicon covers at least one exon-intron junction.

Post-PCR analyses included the following steps: Normalization of target gene Cq to reference gene and calculation of fold change using the efficiency adjusted Cq values. Melt curve analysis was conducted to confirm specificity of amplifications in a way that single peaks were considered as the presence of specific products; while multiple peaks implied primer-dimers or contamination. Tm (melting temperature) was compared to expected product.

Confounders were controlled at different levels: Pre-PCR: using high-quality RNA and consistent RT; PCR Run: using optimized primers, master mixes, and technical replicates; Post-PCR: selection of proper baseline/threshold and stable reference gene.

Statistical method

Statistical parameters were analyzed using R language. Expression of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes was calculated using Cq and PCR efficiency. These values were first log-transformed. Mean value of gene expression was compared between two groups using t-test. Correlations between expression of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes were measured using the Spearman correlation coefficient. Diagnostic power of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes was assessed *via* depicting ROC curves and estimation of AUC.

Results

General information

Totally, 26 patients with periodontitis and 28 controls were registered in the study. Patient group consisted of 16 female subjects and 10 male subjects (Mean age ± SD: 37.5 ± 2.4). Control group included 15 females and 8 males (Mean age ± SD: 38.1 ± 2.9).

Expression assays

Relative expressions of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes in gingiva and blood of patients and controls are demonstrated in Figs. 1 and 2, respectively.

CASP2 was more expressed in gingiva of patients versus control samples (RME = 24.56, $P < 0.001$), and in both female and male patients compared with matched control samples (RME = 30.53, $P = 0.03$ and RME = 20.59, $P = 0.01$, respectively). *BCL2* was more expressed in patients' tissues compared with controls (RME = 32.28, $P < 0.001$) and in patients' tissues of males compared with controls (RME = 69.03, $P < 0.001$). Finally, *HULC* was less expressed in the blood of patients versus controls (RME = 0.21, $P = 0.01$) and in the blood of female patients compared with normal females (RME = 0.15, $P = 0.01$). Other comparisons yielded no significant results (Table 2).

Correlations between expression levels of mentioned genes were assessed in each group of samples using correlation coefficients. Expressions of *CASP2*, *CASP8* and *BCL2* were significantly correlated with each other both in blood samples and in tissue samples. The strongest correlation was detected between *BCL2* and *CASP2* in tissue samples ($r = 0.86$, $P < 0.0001$). In blood samples, expression of *PVT1* was inversely correlated with *BCL2* (r=-0.48, $P < 0.001$) (Fig. 3).

Besides, we weigh up the diagnostic power of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes in blood and tissue specimens using the Bayesian Generalized Linear Model (Fig. 4).

BCL2 and *CASP2* had the highest diagnostic values for separation of patients' gingival tissues from normal ones (AUC values = 0.80 and 0.78, respectively). The performance of *HULC* in the separation of patients' blood samples from controls was superior to other genes (AUC value = 0.73). Combination of transcript levels of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* improved the AUC to 0.84 and 0.72 in tissue and blood, respectively (Table 3).

Discussion

Periodontitis is a multifaceted condition with several underlying mechanisms among them is dysregulation of apoptosis. This condition is associated with activation of immune-related mechanisms including Periodontitis activates the NLRP3 inflammasome [16]. In addition, Transglutaminase 2 [6] and periodontal biotype [17]

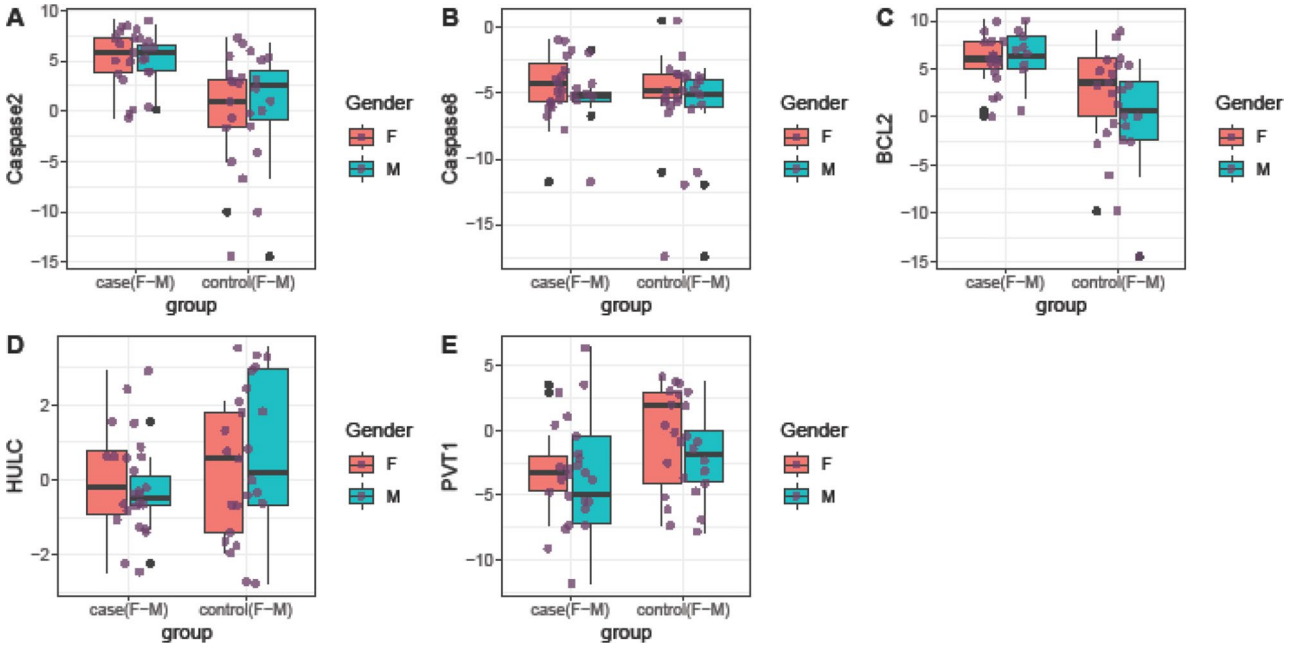

Fig. 1 Quantities of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes in affected versus control tissues. Expression of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes was calculated using Cq and PCR efficiency. These values were first log-transformed. Mean value of gene expression was compared between two groups using t-test. P values < 0.05 were considered as significant (F: female, M: male)

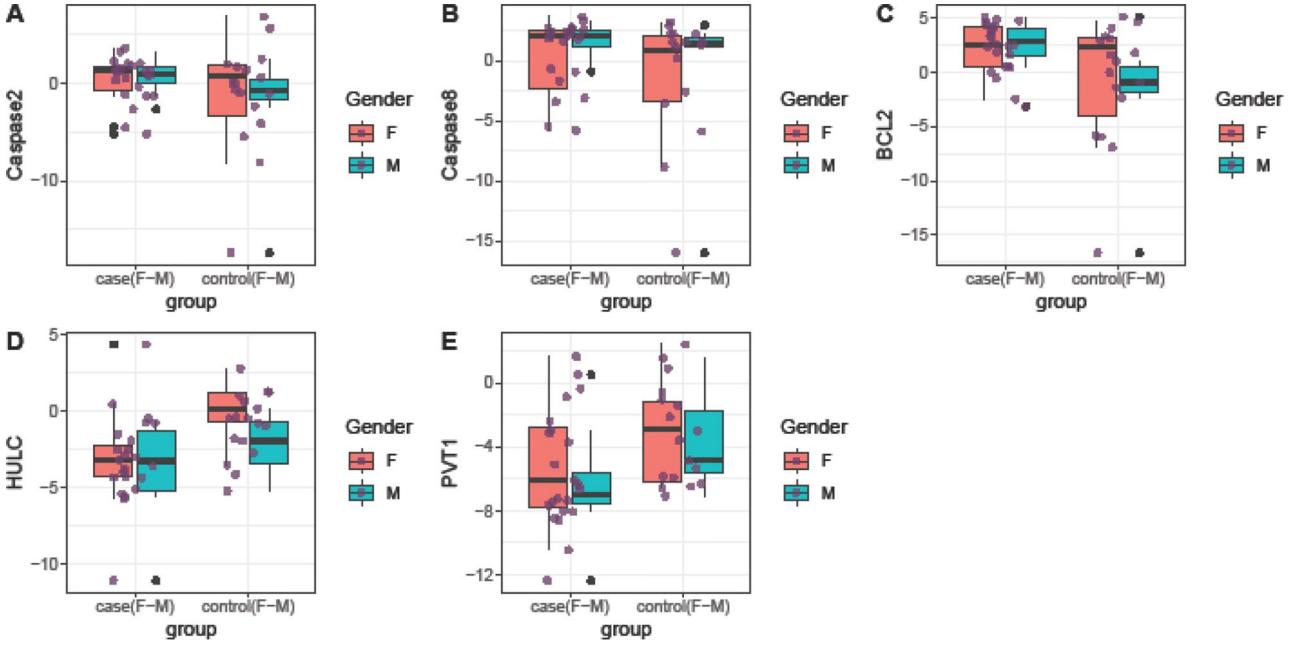

Fig. 2 Expression quantities of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes in blood of cases versus controls. Expression of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes was calculated using Cq and PCR efficiency. These values were first log-transformed. Mean value of gene expression was compared between two groups using t-test. P values < 0.05 were considered as significant (F: female, M: male)

have important roles in this condition. In the current investigation, we measured expression of five apoptosis-related genes in patients with periodontitis. Expressions of *CASP2* and *BCL2* were higher in gingival tissues of patients. On the other hand, *HULC* was less expressed in blood samples of patients. CASP2 protein is located in the mitochondria and promotes apoptosis [13]. Another study has demonstrated up-regulation of CASP2 in human periodontal ligament fibroblasts after mechanical stretch [18]. Moreover, *CASP2* has been predicted to be targeted by miR-125a-5p and miR-34a, two differentially expressed miRNAs in apical periodontitis [19].

Table 2 Expression of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes in patients versus normal persons (RME: ratio of mean expression)

	CASP2					CASP8					BCL2					HULC					PVT1				
Sample	SE	RME	P Value	95% CI		SE	RME	P Value	95% CI		SE	RME	P Value	95% CI		SE	RME	P Value	95% CI		SE	RME	P Value	95% CI	
Tissue																									
Total	1.21	24.56	0.00	2.17	7.06	0.83	1.74	0.34	-0.89	2.48	1.21	32.28	0.00	2.55	7.48	0.47	0.66	0.21	-1.56	0.35	1.11	0.25	0.08	-4.24	0.24
F	1.94	30.53	0.03	0.65	9.21	1.26	1.08	0.93	-2.58	2.79	2.06	9.69	0.14	-1.31	7.86	0.65	0.91	0.83	-1.51	1.24	1.61	0.18	0.14	-5.98	0.95
M	1.65	20.59	0.01	0.96	7.76	1.01	1.96	0.35	-1.13	3.07	1.56	69.03	0.00	2.88	9.34	0.64	0.49	0.12	-2.37	0.28	1.92	0.23	0.29	-6.22	2.01
Blood																									
Total	1.42	2.91	0.29	-1.43	4.52	1.37	3.18	0.24	-1.17	4.51	1.43	7.75	0.05	-0.02	5.93	0.79	0.21	0.01	-3.83	-0.62	1.06	0.20	0.04	-4.45	-0.14
F	1.62	1.31	0.82	-3.14	3.91	1.53	2.48	0.40	-1.93	4.54	1.55	3.74	0.24	-1.48	5.29	0.87	0.15	0.01	-4.52	-0.90	1.38	0.23	0.14	-4.99	0.76
M	2.61	9.57	0.25	-2.94	9.46	2.60	5.23	0.39	-3.85	8.63	2.74	22.27	0.14	-1.90	10.86	1.43	0.30	0.25	-4.86	1.41	1.78	0.15	0.15	-6.60	1.11

Expression of *BCL2* was higher in affected tissues compared with controls. Our finding supports the results of a previous research which indicated involvement of anti-apoptotic mechanisms in the pathogenesis of periodontal inflammation through up-regulation of BCL2 [20].

Finally, *HULC* was less expressed in blood samples of total patients as well as female patients compared with corresponding controls. This lncRNA inhibits cell apoptosis, augments expression of BCL2 and caspase-3 and decreases levels of cleaved-caspase-3 [12]. Although other assessed apoptosis-related genes had similar expression levels in the blood of patients versus controls, down-regulation of this lncRNA might represent disturbance in apoptosis regulation in the bloodstream of these patients. However, valuation of expression of other apoptosis-related lncRNAs and mRNA coding genes is needed to identify the overall activity of this pathway in the circulation of affected patients.

BCL2 and *CASP2* had the best diagnostic value for separation of diseased gingival tissues from normal ones. *HULC*, as the only differentially expressed gene in the bloodstream of patients with periodontitis could separate patients from controls with a moderate power. Combination of transcript levels of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* enhanced AUC to 0.84 and 0.72 in tissue and blood, respectively. Therefore, these genes could effectively distinguish affected tissue samples from normal one, yet their performance as peripheral markers is not optimal. The data presented above expands the knowledge about dysregulation of apoptosis-related genes in the course of periodontitis and implies the importance of this pathway in the pathophysiology of this disorder. Aberrant expression patterns of these genes may indicate their potential contribution to the disease process. This observation is in line with our hypothesis regarding abnormal function of apoptosis-related pathways in periodontitis.

Conclusion

Taken together, we reported abnormal levels of some apoptosis-related genes in diverse biological samples of patients with periodontitis. However, their exact roles should be clarified in future.

Limitations

The limitations of our study are small sample size, lack of functional validations and lack of proteomics analyses. The relatively limited number of samples included in our analysis may decrease the statistical power of obtained results and limit the generalizability of the findings. A larger cohort is necessary to validate the observed trends and warrant broader applicability across other populations. Our study mainly focuses on expression assays, without in vitro or in vivo functional studies. Future work

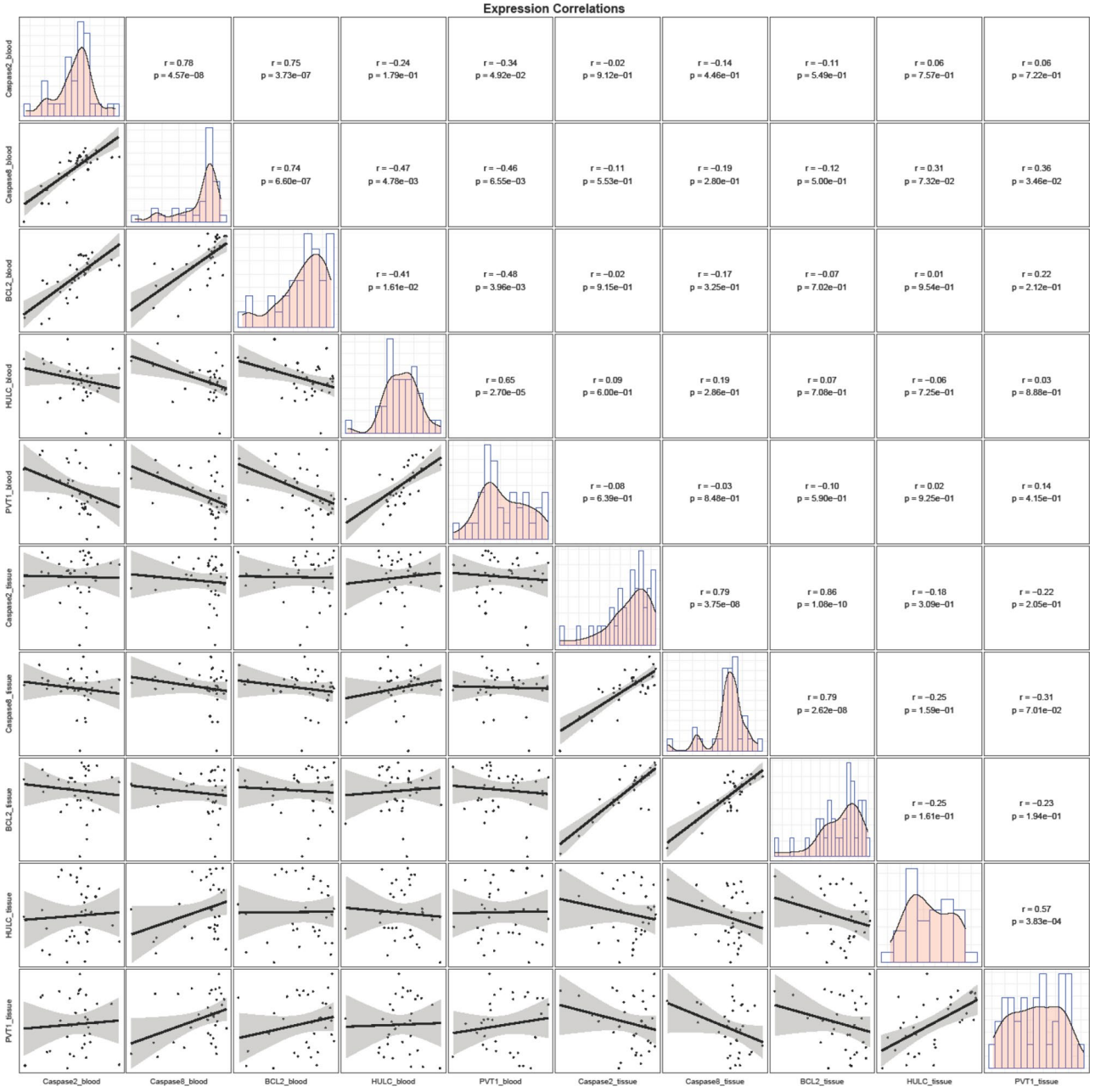

Fig. 3 Correlation between gingival and blood levels of *CASP2*, *CASP8*, *BCL2*, *HULC* and *PVT1* genes. The distribution of parameters is shown on the diagonals. Lower segment of diagonals shows the bivariate scatter plots. Correlation coefficient and P value are shown on the superior parts of the diagonal. Correlation coefficient values more and less than zero indicate positive and inverse correlations, respectively

should include mechanistic studies to approve the biological relevance of the observed dysregulation of genes. Finally, while our study utilized transcriptomic approach, proteomic profiling was not performed. Since mRNA levels do not always correlate with protein expression, integrating proteomics data could provide a more broad understanding of the underlying molecular mechanisms.

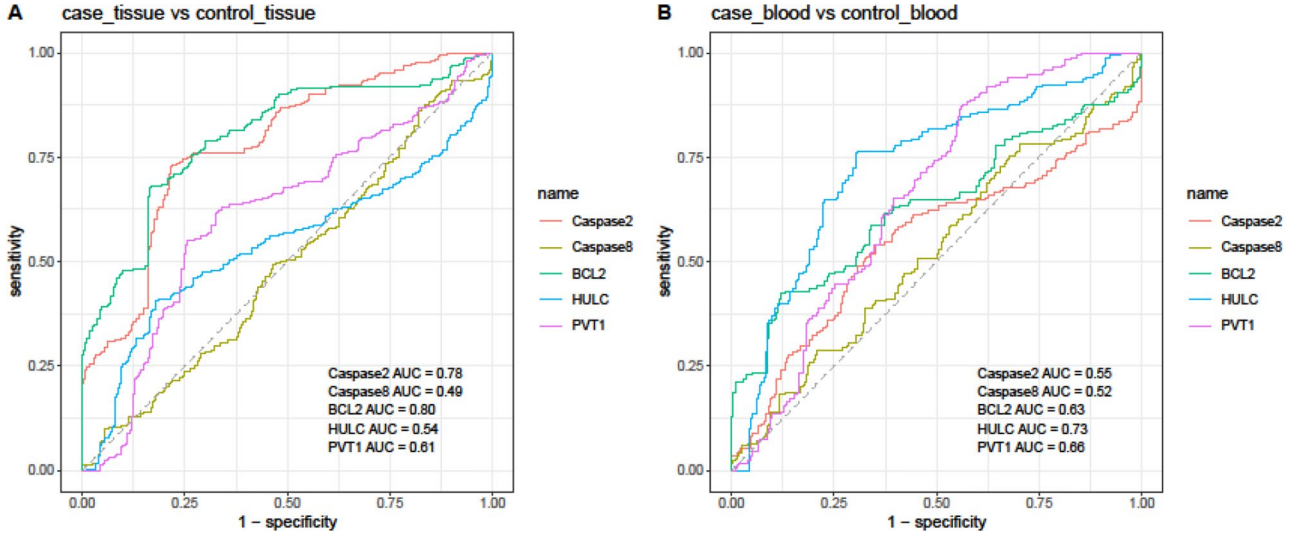

Fig. 4 ROC curves plotted using the Bayesian Generalized Linear Model (Area under curve (AUC) values for each gene are shown. Higher AUC values show better performance in distinguishing cases from controls)

Table 3 Statistics related to ROC curve analyses in tissue and blood samples

	CASP2			CASP8			BCL2			HULC			PVT1			All		
Sample	**AUC**	**Sensitivity**	**Specificity**	**AUC**	**Sensitivity**	**Specificity**	**AUC**	**Sensitivity**	**Specificity**	**AUC**	**Sensitivity**	**Specificity**	**AUC**	**Sensitivity**	**Specificity**	**AUC**	**Sensitivity**	**Specificity**
Tissue	0.78	0.73	0.78	0.49	0.10	0.94	0.80	0.68	0.84	0.54	0.41	0.82	0.61	0.55	0.74	0.84	0.93	0.58
Blood	0.55	0.54	0.65	0.52	0.29	0.79	0.63	0.42	0.88	0.73	0.76	0.69	0.66	0.88	0.43	0.72	0.75	0.74

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Author contributions
SGF wrote the manuscript. SK revised it. AS designed and supervised the study. LG, FH, EB, and SS performed the experiment. NN and MJ analyzed the data. All authors read and approved the submitted manuscript.

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Data availability
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
All methods were carried out in accordance with in accordance with the Declaration of Helsinki. All experimental protocols were approved by ethical committee of Shahid Beheshti University of Medical Sciences. Informed consent forms were signed by all patients.

Consent for publication
Informed consent for publication was obtained in written form from all patients.

Competing interests
The authors declare no competing interests.

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