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ORIGINAL ARTICLE

RELATIONSHIP BETWEEN CHROMOSOMAL ABERRATIONS AND GENE EXPRESSIONS IN THE p53 PATHWAY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a neoplasm characterized by excessive accumulation of B lymphocytes in the peripheral blood, bone marrow and lymph nodes. We assessed the expressions of 22 genes in the p53 pathway in 30 CLL patients and 15 healthy subjects by a RT[2] Profiler PCR (polymerase chain reaction) Array technique and their relation to cytogenetic aberrations detected by fluorescent *in situ* hybridization (FISH). Our Student's *t*-test results indicated that *ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN2A*, *CHEK1*, *CHEK2*, *E2F3*, *MCL1*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *P53* and BCL2 genes were statistically significant (p <0.001). For six genes (*APAF1*, *CDKN1A*, *E2F1*, *GADD45A*, *PTEN* and *PTX3*) were not statistically significant. The *ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *CHEK1*, *CHEK2*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *P53*, *E2F1*, *GADD45A* and *BCL2* genes were found to be upregulated by the $2^{-\Delta\Delta Ct}$ (relative fold change in gene expression) method. The highest upregulation was detected in *CDKN2A* and *BCL2* genes, 10.22- and 8.51-fold, respectively. On the other hand, the *PTX3* gene with a fold regulation of 1.84 was found to the highest downregulation. Overall, the *CDNK2A BCL2* and *PTX3* genes are related to the mechanism of the disease in the p53 pathway and may be an important predictor of the prognosis of the disease. The *BCL2* gene may be associated with increased risk of developing CLL. We suggest that the *PTX3* gene may be considered as a marker associated with CLL disease. The *CDKN2A* gene expression seems to play a protective role in CLL.

Keywords: Chronic lymphocytic leukemia (CLL); gene expression; p53 Pathway; Prognosis; RT[2] Profiler PCR (polymerase chain reaction)Array.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a neoplasia that occurs with the accumulation of CD5 +B cells. These neoplastic cells infiltrate peripheral blood, lymph nodes, bone marrow and lymphoid tissues. The prognosis of CLL disease is variable and the average age of diagnosis is 70 years [1]. Chronic lymphocytic leukemia disease is widely seen in Western societies. The CLL cells consist of monoclonal B lymphocytes expressing the CD23, CD19, CD5 antigens on their surface; in addition CD20, CD79b, FMC7 and Ig antigens are expressed in minute amounts [2]. The CLL patients have a high survival rate and 33.0% of patients do not need treatment. The initial phase of indolent follows the progression of the disease and the aggressive form of the disease that requires treatment [3]. More than 50.0% of CLL cases are diagnosed in the early stage of the disease, usually with lymphocytosis on routine blood screening. Repetitive gene mutations, including *NOTCH1* and *SF3B1*, elevation of serum thymidine kinase (TK) or β2-microglobulin (β2-MG) levels and downregulation of immunoglobulin heavy chain genes (*IGHV*) have prognostic significance [4].

Currently, two well-known clinical staging systems are used to classify patients according to risk groups based on clinicopathological characteristics [5]. Two widely accepted methods of hematologically based staging, Rai and

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Binet systems, have been used in both routine patients and in clinical trials, and have some similarities. The Rai classification is based on the presence or absence of lymphadenopathy and peripheral blood lymphocytosis, platelet count, hemoglobin (Hb) level. In the Binet staging system, CLL is categorized according to the number of lymphoid tissues, the presence of anemia and thrombocytopenia [6]. Fluorescence *in situ* hybridization (FISH) detects chromosomal anomalies in more than 80.0% of CLL cases, most of which are chromosomal deletions [7]. The most frequent chromosomal abnormalities are deletions of the *TP53* gene located on 17p13 (8.0%), trisomy 12 (16.0%), 11q22.3-q23.1 deletions (19.0%) and 13q14 deletions (53.0%). Patients with 17p deletion (17p-) and 11q deletion (11q-) are associated with poor prognosis and short survival. The 13q deletion (13q-) is associated with a slower progressive form of the disease and a better prognosis [8].

The *P53* gene protects the integrity of the genome. The *p53* gene is functionally ineffective in all cancers and has a role in preventing tumorigenesis [9]. The P53 pathway responds to stresses in cell division and replication. Stress signals are also transmitted to the p53 protein [10]. In response to the P53 activation, P53-mediated downstream events consist of cell cycle arrest and apoptosis. DNA damage can activate p53 with the inclusion of DNA double-strand break and DNA repair intermediates following chemical damage to DNA. In this case, there is an increase in p53 level of the cell and activation of p53 as a transcription factor [11].

Determining the prognosis of CLL patients is important for survival and remission. Prognostic parameters should be considered in the genetic evaluation of the process of the disease. By determining the differences in gene expression in CLL cases, it will be possible to use them as markers. There are few studies in the literature regarding the overall evaluation of genes in the p53 pathway. Thus, we designed the current study to perform expression profiling of 22 genes for the first time in CLL. We aimed to obtain new data with respect to the pathogenesis of the disease and infer new molecular markers related to prognosis of CLL. Our results are possibly significance for clarifying the currently unknown molecular pathogenesis of CLL. In this study, the most frequently selected two housekeeping genes (HKG), were used as candidates for normalizing RT^2 Profiler polymerase chain reaction (PCR) Array data in CLL. These genes are β-actin (*ACTB*) and β2-microglobulin (*β2-MG*).

In the present study, we performed expression profiling of 22 genes (*ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *CHEK1*, *CHEK2*, *E2F1*, *GADD45A*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *TP53*, *BCL2*, *APAF1*, *E2F3*, *MCL1*, *PTEN*, *PTX3*) in the p53 pathway, which are related to apoptosis, proliferation, cell cycle, cell growth, differentiation and DNA repair processes. We also looked for a possible relation between gene expression alterations and cytogenetic abnormalities.

MATERIALS AND METHODS

Patient Specimens. Peripheral blood samples were collected from 30 patients with CLL and 15 healthy subjects at the İstanbul Medical Faculty Hospital (İstanbul, Turkey). Demographical characteristics, physical findings, laboratory results (complete blood count, peripheral smear, immunoglobulin values, FISH and flow cytometry results), Rai and Binet staging, medications and treatment indications were recorded. The diagnosis of CLL was defined by the criteria of the International Workshop on CLL (IWCLL) 2008. Sixteen male (53.3%) and 14 female (46.6%) patients, whose median age was 63.8 ± 9.48 years (range 46-86 years), were included in the analyses. Table 1 shows the patients' diagnostic data and FISH results. There were seven males (46.6%) and eight females (53.3%) included as controls whose median age was 56.53 ± 6.80 years (range 44-70 years). Our study was approved by the local ethics committee (İstanbul University, İstanbul Medical Faculty Ethic Committee of Clinical Research Approval, number: 2015/920; date: May 7 2015).

Cytogenetic evaluation with respect to 13q and 17p deletions and trisomy 12 was performed with the FISH technique as a part of routine studies. Patients both with and without chromosomal aberrations (deletion of 13q, 17p regions, also trisomy 12 [+]) were included in the study. We evaluated the prognosis according to good and bad prognoses. The 17p or 11q deletions of prognostic factors were examined to determine treatment approach and survival. We selected the 17p deletion from two deletions because it was suggested to investigate that deletion in patients with active or progressive disease symptoms and treatment indication.

Interphase Fluorescence *In Situ* Hybridization (I-FISH). Interphase FISH analyses were done on peripheral blood obtained from CLL patients. The CLL panel contained commercial FISH probes for the detection of deletions at 13q14, 13q34, 17p13 and trisomy 12 (Cytocell, Cambridge, Cambridgeshire, UK). Gibco PB-MAX™ Karyotyping Medium (Gibco Life Technologies, Carlsbad, CA, USA) was used on cultured peripheral blood cells fixed in 3:1 Carnoy's fixative. Standard cytogenetic procedures were applied for the samples. Interphase nuclei were counterstained with DAPI (Cytocell). Fluorescence *in situ* hybridization signals were evaluated under a fluorescence microscope (Zeiss Axioskop 2 plus; Carl Zeiss CMP GmbH, Göttingen, Germany). The images were cap-

Table 1. Patient diagnostic data and FISH results.

n	Sex-Age	Prognosis	Rai Stage	Binet Stage	FISH Results				WBC (10^9/L)	Hb (g/dL)	Platelet (10^9/L)
					del(13q)	del(17p)	+12	Normal[a]			
1	F-75	bad	1	B	[–]	[+]	[–]	[–]	12.6	12.7	166.0
2	M-71	good	0	A	[–]	[–]	[–]	[+]	23.0	12.0	203.0
3	M-57	good	0	A	[–]	[–]	[–]	[+]	24.8	15.1	262.0
4	M-56	good	3	A	[–]	[–]	[–]	[+]	71.4	13.2	49.0
5	F-59	bad	2	B	[–]	[+]	[–]	[–]	7.9	10.9	284.0
6	F-74	bad	2	B	[–]	[+]	[–]	[–]	108.6	10.3	190.0
7	F-63	bad	2	A	[–]	[+]	[–]	[–]	35.4	13.4	185.0
8	M-80	bad	2	A	[–]	[+]	[–]	[–]	3.6	12.9	104.0
9	F-62	bad	0	A	[–]	[–]	[+]	[–]	190.2	12.0	192.0
10	M-67	bad	2	A	[–]	[–]	[+]	[–]	142.6	11.6	81.0
11	F-65	bad	0	A	[–]	[–]	[+]	[–]	5.2	12.1	144.0
12	F-74	bad	2	A	[–]	[–]	[+]	[–]	179.7	11.9	321.0
13	F-86	bad	3	A	[–]	[–]	[+]	[–]	213.0	8.6	113.0
14	M-60	good	3	A	[–]	[–]	[–]	[+]	51.7	9.4	137.0
15	M-46	good	2	A	[–]	[–]	[–]	[+]	15.0	14.4	184.0
16	M-54	good	0	A	[–]	[–]	[–]	[+]	41.9	13.8	352.0
17	F-61	good	1	A	[–]	[–]	[–]	[+]	11.8	12.1	148.0
18	F-67	good	0	A	[–]	[–]	[–]	[+]	195.3	10.8	188.0
19	F-53	good	2	B	[–]	[–]	[–]	[+]	39.8	11.4	135.0
20	M-67	good	0	A	[+]	[–]	[–]	[–]	74.6	12.4	183.0
21	M-62	good	2	A	[+]	[–]	[–]	[–]	121.4	14.5	294.0
22	M-68	good	0	A	[+]	[–]	[–]	[–]	37.7	13.9	197.0
23	F-46	good	1	A	[+]	[–]	[–]	[–]	59.4	12.3	91.0
24	M-58	good	2	B	[+]	[–]	[–]	[–]	201.4	11.1	101.0
25	M-66	good	0	A	[+]	[–]	[–]	[–]	43.9	12.3	88.0
26	M-61	good	4	C	[+]	[–]	[–]	[–]	110.0	13.9	39.0
27	M-51	good	2	A	[+]	[–]	[–]	[–]	98.5	14.3	250.0
28	F-78	good	3	B	[+]	[–]	[–]	[–]	13.7	11.0	324.0
29	F-63	good	0	A	[+]	[–]	[–]	[–]	113.3	11.5	178.0
30	M-64	good	0	A	[+]	[–]	[–]	[–]	432.0	15.3	206.0

FISH: fluorescence *in situ* hybridization; WBC: white blood cell count; Hb: hemoglobin.

[a] Non-del(13q), del(17p), +12.

tured on a computer with karyotyping and FISH software (MetaSystems ISIS FISH imaging system; MetaSystems GmbH, Altlussheim, Germanay). Two hundred interphase nuclei were analyzed for each sample. The cutoff values were 5.0% for D13S319 (13q14.2, red)/13qter (13q34, blue)/12cen (trisomy 12, green) deletion. Enumeration probe have two red, two blue and two green signals in a normal cell and 5.0% for 17p13.1 (P53, red) probe has two red and two green signals in a normal cell.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from whole blood samples using QiaAmp RNA Blood Mini Kit (cat #52304; Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. RNA quality and quantity were checked using a NanoDrop 2000c (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The A260/A280 ratio of RNA was 1.8-2.0. The same amount of total RNA was used for reverse transcription of each sample in the study. It was started with 0.5 µg (500 ng) total RNA in accordance with a 96-well plate format. The cDNA was synthesized from 0.5 µg of mRNA for the reverse transcription and quantitative PCR (RT-qPCR), using an RT^2 First Strand Kit (cat #330404;

Qiagen GmbH) following the manufacturer's protocol. The RT² Profiler PCR Array protocol was continued.

RT² Profiler PCR Array. Expression of 22 genes from isolated RNA related to p53-mediated signal transduction detected in DNA repair, apoptosis, cell cycle, proliferation and differentiation processes, were profiled using RT² Profiler PCR Array (Qiagen; Custom cat #CAPH 13446). Real-time PCR-Custom RT² Profiler PCR Array's F format-96-well (CAPH_133446F; cat #330131/ 12 plate) was used in this study. Custom RT² Profiler PCR Array was performed to measure the expression levels of 22 genes beside the reference genes (*ACTB* and β*2-GM*) for each well of the patient or control group; a total of 25 µL of 12.5 µL SYBR green master mix, 10.5 µL ddH_2O and 1 µL cDNA were prepared to using RT² SYBR Green qPCR Mastermix kit (cat #330502; Qiagen) on the Light Cycler 480 II RT-PCR Instrument (Roche Diagnostics GmbH, Mannheim, Germany). The 12.5 µL SYBR green master mix and 12.5 µL RNase-free water were added for each of the HKG (*ACTB* and β*2-MG*) and positive PCR control.

Data Analysis. For the analysis of data with Light Cycler 480 system (Roche Diagnostics GmbH), the Second Derivative Max Method was used. This method was defined as the crossing point of the PCR reaction and this crossing point value is the maximum value of the second derivative of the amplification curve. We used commercially available primers for all genes (Qiagen GmbH). Threshold cycle (Ct) values for each sample were evaluated using web-based RT² Profiler PCR Array Data analysis 3.5 [12].

The $2^{-\Delta\Delta Ct}$ method is used as a relative quantification strategy for qPCR data analysis. This method directly measures relative gene expression levels between different samples using the Ct values produced by the qPCR system. Gene expression levels were also defined as fold change. Relative expressions were calculated in accordance with the $2^{-\Delta\Delta Ct}$ method. Relative quantification related the PCR signal of the target transcript in a patient group to the signal of another sample such as a control group. The values of positive PCR control (Ct^{PPC}) wells were examined. The mean Ct^{PPC} ratio in each RT² Profiler PCR Array should be 20 ± 2. The ΔCt was used for each gene in all plate using Ct values for the gene of interest (GOI) and the HKG ($\Delta Ct: Ct_{GOI}-Ct_{HKG}$). When replication was performed, the mean ΔCt values of each gene (each well) were calculated.

Statistical Analysis. In the evaluation of the data, Qiagen Custom RT² Profiler PCR Array data analysis methods were used. Student's *t*-test analysis was performed by a web-based program. Significant differences in expression levels by Student *t*-test analysis are shown as log-transforming values (logFC) to identify fold regulation. A p value of <0.05 was considered to indicate a statistically significant result.

RESULTS

Clinical Characteristics of CLL Patients. Thirty patients with chronic lymphocytic leukemia (with and without chromosomal aberrations) and 15 healthy adults as control group were included in the study. Fourteen CLL patients were female (46.6%) and 16 were male (53.3%); the male/female ratio was 1.14. Eight individuals in the control group were female (53.3%) and seven were male (46.6%). The mean ages of the CLL and control groups were 63.8 ± 9.4 and 56.5 ± 6.8, respectively. Median and minimum-maximum ages were 63.8 (46-86) and 56.5 (44-70), respectively. The age of diagnosis was 58.1 ± 10.2 in the CLL group. There was no statistically significant difference between the two groups with respect to gender distribution (p >0.05). According to the Rai staging, 11 patients were in stage 0 (36.6%), one in stage I (3.33%), 10 in stage II (33.3%) and two were in stage III (6.66%). In the Binet staging system, 17 patients were in stage A (56.6%), eight in stage B (26.6%), and five in stage C (16.6%).

All patients were evaluated by the FISH technique and RT² Profiler PCR Array method. The distribution of the patients with respect to prognosis (based on FISH findings) and complete blood count results in the CLL group are shown in Table 1. The mean difference values of the patient and control group according to average ΔCt and fold change are shown in Table 2.

Results of FISH. Patients with both deletions and trisomy (del 17p, del 13q and trisomy 12) and normal [non-del(13q), del(17p), trisomy 12] were included in the study. As a result of the FISH assessment in the CLL group: 11 had 13q [–] (36.7%), five had 17p [–] (16.7%), nine without deletion (normal) (3.00%), five had trisomy 12 (16.7%).

We evaluated the prognosis according to good and bad prognoses. Good prognosis was found in 20 patients and poor prognosis in 10 patients. The 17p or 11q deletions of prognostic factors were examined to determine treatment approach and survival. We selected the 17p deletion from two deletions because it was suggested to investigate that deletion in patients with active or progressive disease symptoms and treatment indication. According to the interphase-FISH results, the cutoff value was higher than 5.0% in 21/30 CLL patients. These findings were considered clonal when the percent of cells with any given chromosome aberration exceeded the normal cutoff value (5.0%).

Analysis of Differentially Expressed Genes. The RT² Profiler PCR Array data were expressed as fold change. The 2^{-Ct} method is the normalized gene expression in the patient sample divided by 2^{-Ct} in the control sample. The average ΔCt values of *ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN2A*, *CHEK1*, *CHEK2*, *E2F3*, *MCL1*, *MDM2*,

MDM4, *PCNA*, *RB1*, *P53* and *BCL2* genes were found to be statistically different than the controls. However, *APAF1*, *CDKN1A*, *E2F1*, *GADD45A*, *PTEN* and *PTX3* genes were not statistically significant (Table 2). The RT^2 Profiler PCR Array data analysis showed that *ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDNKN1A*, *CDKN2A*, *CHEK1*, *CHEK2*, *E2F1*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *P53*, *GADD 45A* and *BCL2* genes were upregulated. On the other hand, *APAF1*, *E2F3*, *MCL1*, *PTEN*, *CDKN2A* and *PTX3* genes were downregulated (Table 2).

were calculated using a Student's *t*-test on the $2^{-\Delta Ct}$ values. The RT^2 Profiler PCR Array data analysis version 3.5 was used for comparisons between experimental groups.

String v10 analysis (https://string-db.org) was used to create gene-gene and network interactions. Our initial results using RT^2 Profiler PCR Array analysis in CLL patients revealed 18 differently expressed genes. Network analysis used for the probable interactions of these 18 genes through the String web-mediated tool showed that the interactions of the resulting network were enriched (*p*

Table 2. The mean difference values of the patient and control group according to average ΔCt and fold change.

Gene	Average Ct		Average ΔCt		$2^{-\Delta Ct}$		Fold Change		*p* Value	Fold Regulation
	CLL	Control	CLL	Control	CLL	Control	CLL/ Control	95% CI		CLL/ Control
APAF1	26.57	25.28	7.72	7.54	0.004695	0.005368	0.87	0.78-0.96	0.072729	–1.14
ATM	27.85	27.91	9.01	10.17	0.001936	0.000869	2.23	1.67-2.79	<0.001	2.23
ATR	29.00	29.41	10.17	11.67	0.000871	0.000307	2.83	2.23-3.43	<0.001	2.83
BAX	25.61	25.87	6.78	8.13	0.009116	0.003578	2.55	2.15-2.95	<0.001	2.55
CASP9	27.35	27.58	8.52	9.84	0.002723	0.001091	2.50	2.06-2.94	<0.001	2.50
CDK4	26.92	27.96	8.09	10.22	0.003672	0.000839	4.38	3.20-5.56	<0.001	4.38
CDKN1A	27.59	26.82	8.75	9.08	0.002315	0.001852	1.25	0.97-1.53	0.066	1.25
CDKN2A	30.50	32.77	11.67	15.02	0.000307	0.000030	10.22	7.14-13.3	<0.001	10.22
CHEK1	31.37	31.58	12.54	13.83	0.000168	0.000068	2.45	1.98-2.92	<0.001	2.45
CHEK2	29.45	30.42	10.62	12.68	0.000638	0.000152	4.18	3.02-5.34	<0.001	4.18
E2F1	31.44	30.88	12.60	13.14	0.000161	0.000111	1.45	1.04-1.86	0.012741	1.45
E2F3	28.88	27.09	10.05	9.34	0.000944	0.001541	0.61	0.53-0.69	<0.001	–1.63
GADD45A	31.94	31.14	13.10	13.39	0.000114	0.000093	1.22	0.90-1.54	0.121	1.22
MCL1	23.34	21.85	4.51	4.11	0.043950	0.057965	0.76	0.66-0.86	<0.001	–1.32
MDM2	25.77	25.60	6.93	7.86	0.008184	0.004312	1.90	1.56-2.24	<0.001	1.90
MDM4	27.43	28.00	8.60	10.26	0.002577	0.000818	3.15	2.32-3.998	<0.001	3.15
PCNA	25.75	26.36	6.92	8.62	0.008266	0.002545	3.25	2.64-3.86	<0.001	3.25
PTEN	25.53	24.05	6.50	6.31	0.011036	0.012615	0.87	0.78-0.96	0.062542	–1.14
RB1	26.51	27.04	7.67	9.29	0.004894	0.001595	3.07	2.40-3.74	<0.001	3.07
P53	25.76	26.32	6.92	8.58	0.008229	0.002619	3.14	2.47-3.81	<0.001	3.14
PTX3	32.50	30.53	13.67	12.79	0.000077	0.000141	0.54	0.37-0.71	0.006057	–1.84
BCL2	25.94	27.94	7.11	10.20	0.007231	0.000850	8.5`	5.46-11.56	<0.001	8.51

CLL: chronic lymphocytic leukemia.

Normalized expression of genes was compared between CLL patients and healthy controls (Figure 1). *CHEK1*, *CDKN2A*, *CDK4*, *PCNA* are known as cell cycle and cell growth, proliferation and differentiation genes; *CHEK2*, *ATR*, *ATM*, *RB1* are cell cycle genes; the *MDM4* gene is cell growth, proliferation and differentiation gene; the *CASP9* gene is an apoptotic gene; the *BCL2* gene is apoptotic (anti-apoptosis) and cell growth, proliferation and differentiation gene; *P53* and *BAX* are apoptotic (induction of apoptosis) and cell cycle genes. The *p* values <1.0e-16) (Figure 2). A significant enrichment was detected in a subgroup of genes involved in the mitotic cell cycle G1/S transition regulation [GO: 2000045], cell cycle negative regulation [GO: 0045786], mitotic cell cycle G1/S transition negative regulation [GO: 2000134], cell cycle phase transition regulation [GO: 1901987] and cell cycle phase transition negative regulation [GO: 1901988] in the biological process by Gene Ontology (GO) (http://www.geneontology.org) enrichment analysis. In the analysis used for molecular function, enrichment of a gene subset

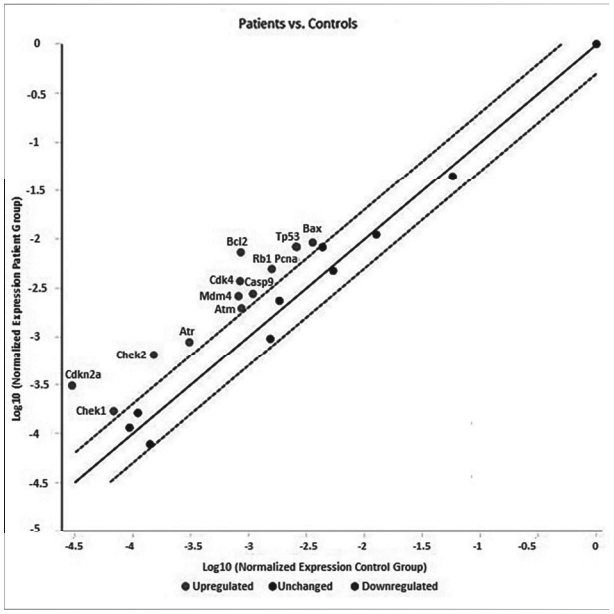

Figure 1. Scatterplot comparing the normalized expression of genes between the CLL patients and healthy controls. Genes involved in CLL cell cycle and cell growth, proliferation and differentiation and apoptosis are shown. The central line indicates unchanged gene expression. Red dots represent genes with a fold regulation of >2.

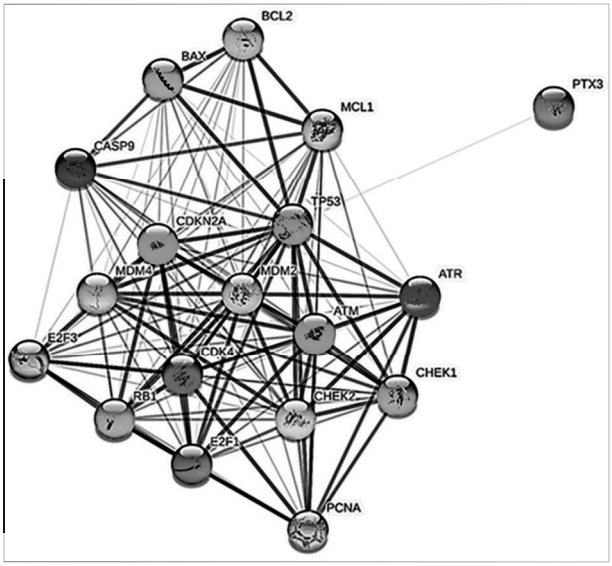

Figure 2. Illustration of interactions between the 18 differentially-expressed genes in CLL patients (String v10, confidence view).

was determined in specific binding to the protein domain [GO: 0019904], specific binding to the disordered domain [GO: 0097718], binding to the BH3 domain [GO: 0051434], binding to the identical protein [GO: 0042802] and protein binding [GO: 0005515]. In the cellular component analysis, it was revealed that the nuclear part [GO: 0044428], nucleoplasm [GO: 0005654], intracellular organelle lumen [GO: 0070013], nucleus [GO: 0005634] and chromosome [GO: 0005694] demonstrated a significative enrichment in a subset of genes.

DISCUSSION

Chronic lymphocytic leukemia is a neoplastic disease characterized by the accumulation of small lymphocytes in blood, bone marrow and lymphoid tissues. The CLL disease is the most common form of leukemia in older persons in Western countries [13]. Recent advances allowed clinicians to have a relatively better understanding of the genetic mechanisms related to CLL development and their clinical use as new biomarkers. In 2000, Döhner *et al.* [14] described a hierarchical prognostic model based on FISH findings. Accordingly, five risk categories were defined: those patients with the 17p53 deletion had the worst prognosis followed by 11q22-q23del, tri12, 13q14del and normal karyotype [14]. Indeed, the *TP53* gene defects (deletion or mutation) is one of the two genetic parameters used in the international prognostic index for CLL (the other is the mutational status of the *IGHV* gene) [15].

In 80.0% of CLL cases with 17pdel, a mutation with complete loss of function is observed in the remaining allele. However, 4.0-5.0% of cases have a mutation without a deletion. Such sole monoallelic mutations at the gene level also effect outcome [16,17]. However, the *TP53* aberrations are seen in only 3.0-8.0% of *de novo* cases and 40.0-50.0% of refractory cases. Therefore, in this study, we hypothesized that not only mutations, but also expression defects of other genes in the TP53 pathway may be playing a role in disease pathogenesis [18]. We used Qiagen RT^2 Profiler PCR Array for the amplification and expression detection of 22 genes in the TP53 pathway for 30 patients and 15 healthy controls. We also compared the level of gene expression in patients with good and poor prognosis (grouped according to FISH findings). Genes that differed more than HKG (*ACTB* and β*2-MG*) between the two groups were considered significant. When we compared the CLL group with the control group, we found that there were significant differences in mRNA levels. In our study, we determined 22 mRNAs that were differentially expressed by more than one-fold up or down after RT^2 Profiler PCR Array. Many of these mRNAs encode apoptotic, cell cycle, cell proliferation, growth and differentiation proteins.

For 16 genes (*ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN 2A*, *CHEK1*, *CHEK2*, *E2F3*, *MCL1*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *P53* and *BCL2)* the Student's *t*-test corrected *p* value was statistically significant ($p < 0.001$). The Student's *t*-test corrected *p* value of *APAF1*, *CDKN1A*, *E2F1*, *GADD45A*, *PTEN* and *PTX3* genes was not statistically significant. We observed upregulation of 17 mRNA lev-

els, including *ATM*, *ATR*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *CHEK1*, *CHEK2*, *MDM2*, *MDM4*, *PCNA*, *RB1*, *P53*, *E2F1*, *GADD45A* and *BCL2* in our study. Two of them, *CDKN2A* (p <0.001) and *BCL2* (p <0.001), were upregulated by more than 10.22- and 8.51-fold (Figure 1).

The alteration of gene expression included the downregulation of *E2F3*, *MCL1*, *APAF1*, *PTEN* and *PTX3* mRNA expression. The highest downregulation, that was about 1.84-fold, was observed for the *PTX3* mRNA expression in CLL patients. We can especially say that these three genes (*CDNK2A*, *BCL2* and *PTX3*) in the p53 pathway are linked to the mechanism of the disease and may have a significant effect on the diagnosis and prognosis of the disease.

The *BCL2* gene has been shown to be overexpressed in the absence of gene rearrangement in most CLL cases. *BCL2*, is a protooncogen, has apoptosis regulatory function. *BAX*, the opposite of BCL2 protein activity, is a homologous protein that accelerates cell death rate. An increase in *BCL2/BAX* ratios was detected in CLL patients compared to the control group. It has been reported that drug-induced apoptosis in B-CLL cells is associated with reversing the *BCL2/BAX* ratio. Increased *BCL2* mRNA level and increased *BCL2/BAX* ratio were also reported by Pepper *et al.* [19].

Vucicevic *et al.* [20] reported marked overexpression of *BCL2* mRNA in a cohort of CLL. In addition, *BAX* mRNA and *BCL2/BAX* ratio were determined to be significantly higher in CLL samples than the controls. The authors pointed out that abnormal *BCL2* and *BAX* expression may have an impact on the pathogenesis and clinical course of CLL [20]. The increased *BCL2/BAX* ratio (CLL/ control fold change: 8.51/2.55 = 3.33) in our study is in parallel with the studies mentioned above. Given that *BCL2* has an anti-apoptotic effect, while *BAX* is implicated in apoptosis, the altered ratio of expression of the two genes as described above favors cell survival, which is in agreement with the nature of CLL where the life-span of the cells are abnormally long. The $p16^{Ink4a}$ (*CDKN2A*) is a tumor suppressor gene, which is inactivated in human neoplasms by mutations, hypermethylations and homozygous deletions [21].

The *CDKN2A* gene methylation has been identified in multiple myeloma patients (61.0%), B cell precursor acute lymphoblastic leukemia (ALL) patients (75.0%), B cell non-lymphomas patients (14.0%). In studies, the p16 promoter methylation was not observed in non-Hodgkin lymphomas.

Tsirigotis *et al.* [21] revealed that genetic changes in the *CDKN2A* gene are rare in CLL patients. Further studies are needed to explain the role of the *CDKN2A* gene promoter methylation in the pathogenesis of CLL.

Ogawa *et al.* [22] aimed to determine the exact behavior and frequency of inactivation of the *p16* gene in various hematopoietic tumor types and to reveal the importance of human hematopoietic malignancies in development. The *p16* gene inactivation triggers tumor formation. The *p16* gene deletion is frequently observed in lymphoid malignancies. Although *p16* deletions were observed in all subtypes of ALL, in the study of Ogawa *et al.* [22], the *p16* deletion ratio appears to be lower in T-ALL than in B-ALL.

The *CDKN2A* gene might be upregulated in CLL patients compared to controls in our study due to the higher number of patients in the early stage and the limited number of patients included in the study. Although decreased expression of the *CDKN2A* gene is associated with an increased risk in CLL and the other human cancers in the literature. According to the study of Romagosa *et al.* [23], $p16^{Ink4a}$ protein also plays a role in apoptosis and angiogenesis; therefore, these functions may be associated with overexpression of cancer. They observed $p16^{Ink4a}$ as over-expressed in endometrial, colorectal and basal cell carcinoma. Accordingly, our study is consistent with the study of Romagosa *et al.* [23], showing that the *CDKN2A* gene may facilitate differential diagnosis of tumors that progressively increase the *CDKN2A* expression. Overexpression of the *CDKN2A* gene suggests that it may have a prognostic role in the tumors in which the irregular *CDKN2A* gene is overexpressed.

Pentraxin 3 (*PTX3*) is an essential component of congenital immunity and acts as a prognostic biomarker to control infections. The *PTX3* gene has been known as an extrinsic oncosuppressor in cancer and plays an important role in hematological malignancies and allogeneic hematopoietic stem cell transplantation [24]. It appears that expression of *PTX3* in CLL was not investigated in previous studies. There are studies on the *PTX3* gene in other cancer types. The *PTX3* gene induces tumor progression in malignancies and also functions as a tumor suppressor in human neoplasms. Ke *et al.* [25] found that low *PTX3* immuno-histochemical expression is associated with good prognosis in gliomas.

Liu *et al.* [26] analyzed the prognostic significance of serum PTX3 levels in patients with colorectal cancer (CRC). An increase in serum PTX3 levels correlates with poor prognosis in postoperative CRC patients. Serum PTX3 level can be used as an independent prognostic biomarker in colorectal cancers [26].

The *PTX3* gene has been shown to act as an extrinsic oncosuppressor gene of cancer-related inflammation (CRI) and reduce CRI. Loss of *PTX*3 was observed in cancer types such as CRC and leiomyosarcoma other than CLL [27]. The *PTX3* gene has biological roles such as apoptosis and immune defense. Therefore, in our study, the downregulation of the *PTX3* gene in CLL patients compared to

controls may slow down apoptosis of leukemic cells in patients and thus increase the CLL cells. These results may shed light on the pathogenesis of CLL disease.

We discussed genes with significantly different expression based on ΔCt value in CLL patients. At the last stage of the our study, based on the results obtained from RT^2 Profiler PCR Array Data analysis, gene expressions were evaluated by the 2^{-Ct} method. According to RT^2 Profiler PCR Array Data analysis, *PTX3* and *CDNK2A* genes can be used to guide CLL pathogenesis. The *CDNK2A* gene, which had the highest upregulation by more 10.22-fold [95% confidence interval (95% CI): 7.14-13.3] and the *PTX3* gene expression had the highest downregulation, which was 1.84-fold (95% CI: 0.37-0.71) in CLL patients/control group. We focused first on the expression profile of 22 key genes to the p53 pathway in this study. On the other hand, other gene(s) associated with the p53 pathway, together with the genes investigated in our study may shed light on the genetic mechanism of the p53 pathway. In this study, the effects of gene expression changes in the p53 pathway in the prognosis and molecular pathogenesis of CLL were investigated. In conclusion, our results imply that there is marked expression disturbances of many genes in the p53 pathway. This suggests that mechanisms other than genomic alterations affecting the *TP53* gene *per se* may be operative in the disease pathogenesis.

As a continuation of this study, changes in protein levels associated with our genes in the p53 pathway will be investigated. In future studies, it is important to evaluate the localization and function of proteins in the cell by researching RNA transcripts on this pathway with various protein analysis methods. Advanced researches are necessary to explain by which mechanisms such expression abnormalities occur, such as abnormal methylation and up- or downregulation of particular miRNAs.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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REFERENCES

1. Moreno C, Montserrat E. Genetic lesions in chronic lymphocytic leukemia: What's ready for prime time use? Haematologica. 2010; 95(1): 12-15.
2. Demir V, Kahraman S, Katgı A, Pişkin Ö, Özsan GH, Demirkan F, *et al.* Kronik lenfositik lösemi hastalarının genel klinik değerlendirilmesi. J DEU Med Fac. 2012; 26(1): 9-19.
3. Libisch MG, Casás M, Chiribao ML, Moreno P, Cayota A, Osinaga E, *et al.* GALNT11 as a new molecular marker in chronic lymphocytic leukemia. Gene. 2014; 533(1): 270-279.
4. Langerbeins P, Groß-Ophoff-Müller C, Herling CD. Risk-adapted therapy in early-stage chronic lymphocytic leukemia. Oncol Res Treat. 2016; 39(1-2): 18-24.
5. Amaya-Chanaga CI, Rassenti LZ. Biomarkers in chronic lymphocytic leukemia: Clinical applications and prognostic markers. Best Pract Res Clin Haematol. 2016; 29(1): 79-89.
6. Jones D. Neoplastic Hematopathology: Experimental and Clinical Approaches. Houston, TX, USA: Humana Press, 2010.
7. Dunphy CH. Molecular Pathology of Hematolymphoid Diseases. Chapel Hill, NC, USA: Springer, 2010.
8. Bottoni A, Calin GA. MicroRNAs as main players in the pathogenesis of chronic lymphocytic leukemia. Microrna. 2013; 2(3): 158-164.
9. Shengkan J, Levine AJ. The p53 functional circuit. J Cell Sci. 2001; 114(23): 4139-4140.
10. Harris SL, Levine AJ. The p53 pathway: Positive and negative feedback loops. Oncogene. 2005; 24(17): 2899-2908.
11. Levine AJ. p53, The cellular gatekeeper for growth and division. Cell. 1997; 88(3): 323-331.
12. RT2 Profiler PCR Array Data Analysis. 2017 (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php).
13. Goldin LR, Slager SL. Familial CLL: Genes and environment. Hematology Am Soc Hematol Educ Program. 2007: 339-345.
14. Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000; 343(26): 1910-1916.
15. The International CLL-IPI working group. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): A meta-analysis of individual patient data. Lancet Oncol. 2016; 17(6): 779-790.
16. Te Raa GD, Kater AP. TP53 dysfunction in CLL: Implications for prognosis and treatment. Best Pract Res Clin Haematol. 2016; 29(1): 90-99.
17. Zenz T, Kröber A, Scherer K, Häbe S, Bühler A, Benner A, *et al.* Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: Results from a detailed genetic characterization with long-term follow-up. Blood. 2008; 112(8): 3322-3329.

18. Zenz T, Mertens D, Döhner H, Stilgenbauer S. Importance of genetics in chronic lymphocytic leukemia. Blood Rev. 2011; 25(3): 131-137.
19. Pepper C, Hoy T, Bentley DP. Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. Br J Cancer. 1997; 76(7): 935-938.
20. Vucicevic K, Jakovljevic V, Sretenovic J, Tosic N, Kostic T, Glumac I, *et al.* Expression of the Bcl2 gene in chronic lymphocytic leukaemia patients. Ser J Exp Clin Res. 2015; 16(3): 187-191.
21. Tsirigotis P, Pappa V, Labropoulos S, Papageorgiou S, Kontsioti F, Dervenoulas J, *et al.* Mutational and methylation analysis of the cyclin-dependent kinase 4 inhibitor (p16INK4A) gene in chronic lymphocytic leukemia. Eur J Haematol. 2006; 76(3): 230-236.
22. Ogawa S, Hangaishi A, Miyawaki S, Hirosawa S, Miura Y,Takeyama K, *et al.* Loss of the cyclin-dependent kinase 4-inhibitor (p16; MTS1) gene is frequent in and highly specific to lymphoid tumors in primary human hematopoietic malignancies. Blood. 1995; 86(4): 1548-1556.
23. Romagosa C, Simonetti S, López-Vicente L, Mazo A, Lleonart ME, Castellvi J, *et al.* p16Ink4a Overexpression in cancer: A tumor suppressor gene associated with senescence and high-grade tumors. Oncogene. 2011; 30(18): 2087-2097.
24. Greco R, Sala E, Crucitti L, Morelli M, Stanghellini MTL, Vago L, *et al.* Pentraxin 3 as a novel diagnostic and prognostic biomarker for acute GvHD and fungal infections in adult allogeneic HSCT recipients. Blood. 2016; 128(22): 4600.
25. Ke H-H, Hueng D-Y, Tsai W-C. Low expression of pentraxin 3 and nuclear factor-like 2 implying a relatively longer overall survival time in gliomas. Chin J Physiol. 2019; 62(1): 35-43.
26. Liu B, Zhao Y, Guo L. Increased serum pentraxin-3 level predicts poor prognosis in patients with colorectal cancer after curative surgery, a cohort study. Medicine. 2018; 97(40): e11780.
27. Bonavita E, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, *et al.* PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. Cell. 2015; 160(4): 700-714.