



Expression Patterns of Eighteen Genes Involved in Crucial Cellular Processes in the TP53 Pathway in Multiple Myeloma

Gozde OZTAN^{1*}, Ilknur SUER^{2,3}, Aynur DAGLAR ADAY³, Mesut AYER⁶,
Sukru OZTURK³, Kivanc CEFLE³, Mustafa Nuri YENEREL⁵, Halim ISSEVER⁴, Sukru
PALANDUZ³

¹ Department of Medical Biology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

² Department of Medical Genetics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

³ Department of Internal Medicine, Division of Medical Genetics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

⁴ Department of Public Health, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

⁵ Department of Hematology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

⁶ Department of Hematology, Başakşehir Çam and Sakura City Hospital, Istanbul, Türkiye

Highlights

- In this article, expression profiling of genes in the p53 pathway was performed.
- Custom RT² Profiler PCR Array method was used in the study.
- The study aimed to elucidate the molecular mechanisms of MM.
- Early MM diagnosis and innovative treatment techniques will benefit from the results.

Article Info

Received: 23 Sep 2023

Accepted: 13 Feb 2024

Keywords

Multiple myeloma
TP53 pathway
RT² profiler PCR array
Gene expression

Abstract

Multiple myeloma (MM) is a malignant disease that causes abnormal immunoglobulin synthesis by bone marrow plasma cells. The relationship between MM and the TP53 pathway has not been fully elucidated in the literature. Investigation of the effect of the expression of genes in the TP53 pathway on the molecular pathogenesis and prognosis of multiple myeloma disease. We assessed the expression of 18 genes in the TP53 pathway in 48 MM patients and 31 healthy subjects by RT²-profiler PCR array technique, and investigated their possible association with the presence of cytogenetic aberrations. Twelve of the 18 genes (*APAF1*, *ATM*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *E2F1*, *MCL1*, *MDM2*, *MDM4*, *PTEN*) expression levels were found to be statistically up-regulated in MM patients compared to controls. The *CDK4*, *CDKN1A* and *MCL1* genes were found to have remarkable diagnostic power distinguishing MM and healthy controls (AUC=0.89;AUC=0.86;AUC=0.77, respectively and $p<0.001$ for all three) via using Receiver operating characteristic (ROC) analysis. Overexpression of *CDK4* and *CDKN1A*, which are involved in the cell cycle, and *MCL1*, which is an important gene in the anti-apoptotic process, were found to be excessively increased in MM patients compared to controls in terms of mRNA fold change. In addition, the high sensitivity of these genes found in the ROC analysis results suggests that they may be suggested as potential biomarkers for MM.

1. INTRODUCTION

An increase in plasma cells produced from B lymphocytes in the bone marrow is a hallmark of multiple myeloma (MM) [1]. Recurrent chromosomal and genetic alterations, including chromosomal translocations, copy number abnormalities, and point mutations, are common in MM [2]. A multistage disease progression model is based on clinical evaluation. Through "extramedullary disease," monoclonal gammopathy of unknown significance (MGUS) causes MM and smoldering MM. Methods of genetic analysis aid in understanding MM pathogenesis [3].

The p53 protein has several roles in tumor suppression; it is a transcription factor that regulates hundreds of genes. A changed p53 ubiquitin ligase called *MDM-2* breaks down the protein in response to different signals that make it transcribe genes. When DNA is damaged, p53 is activated for transcription, which decides whether to enter cell cycle arrest and fix the DNA or to kill the injured cell by one of five feasible

*Corresponding author, e-mail: gozdeoztan@istanbul.edu.tr

paths. One common sort of stress that triggers transcription is DNA damage. The p53 protein, which plays an essential function in repairing DNA strand breaks, keeps an eye out for the aberrant DNA recombination that leads to B cell translocations in the development of MM [4].

TP53 gene anomalies, which are uncommon at diagnosis, become more common with disease progression, indicating that the *TP53* gene plays a crucial role in the development of MM. According to many clinical studies, loss of *TP53* is strongly associated with poor prognosis in MM patients. Investigation of the role of *TP53* as a diagnostic biomarker in MM is limited due to the heterogeneity of the disease and the small number of patients [5].

Exposure to DNA damage, hypoxia, viruses, heat stress, mitogenic, and carcinogenic stimuli turns on the TP53 pathway [6]. A change or loss in the *TP53* gene is not often found in people with MM when they are first diagnosed, but these problems get worse as the disease gets worse [7]. The cell cycle requires the simultaneous activation of cyclin-dependent kinases (CDK) dimers. MGUS and MM are early-oncogenic due to cyclin D dysfunction [8]. The *TP53* gene is mutated or signaling pathways are canceled in tumors, which causes the loss of p53's vital role in the cell. *TP53* mutations/deletions are uncommon in MM, which may benefit from its treatment [9]. Upregulation of *MDM2* in MM has been observed in many cancers. *MDM2* fluorescence signals were enhanced in 8% of MM patients with diploid chromosome 12 or trisomy 12 [6].

In terms of all malignant neoplasms, MM accounts for one percent [10]. In terms of hematological malignancies, MM is the second most frequent kind. While the global incidence rate is ~2 per 100,000 people, it varies significantly [11]. MM seems to be somewhat more common in males and has an exceedingly low reported incidence in those under the age of 30 (0.02% to 0.3%). Although rare family instances do arise, MM is generally not seen as a hereditary illness [12].

The frequency of this condition rises with age and peaks in the 70s. It is the most frequent primary malignant bone tumor (47%), and it is the second most prevalent hematological malignancy after lymphomas. It accounts for 10% of all cases. Diagnosis of MM is most common in those 65–74 years of age; instances in those less than 40 years of age are very rare. While it is less prevalent in Asian groups, it is somewhat more common in men than women. On the other hand, data from a study in indicates a greater frequency in females and whites. It is twice as common among Afro-descendants compared to Caucasians [10]. In 2020, while the number of new cases in Turkey was 2680 (1.1%), the number of deaths due to MM was recorded as 1970 (1.6%). The 5-year prevalence across all ages was 6886 per 100,000 (<https://gco.iarc.fr/today/home>).

For this reason, studying the expression of genes involved in TP53-related pathways can help us learn more about the molecular causes and clinical outcomes of MM. In our study, 18 genes related to the TP53 signaling pathway were selected via the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<https://www.kegg.jp/pathway/hsa04115>) to better define TP53 changes in determining the prognosis of MM disease. The research included 18 custom-designed genes that are involved in DNA repair, cell cycle, apoptosis, proliferation, and differentiation, each of which has been previously described [13].

Our goal was to examine the potential role that genes in the TP53 pathway play in the initiation of MM pathogenesis. We selected 18 of the genes [ATM serine/threonine kinase (*ATM*), BCL2 Associated X-protein (*BAX*), Cyclin-dependent kinase-4 (*CDK4*), Cyclin-Dependent Kinase Inhibitor-2A (*CDKN2A*), Checkpoint Kinase-2 (*CHEK2*), E2F Transcription Factor-1 (*E2F1*), Cyclin -Dependent Kinase Inhibitor-1A (*CDKN1A*), Ataxia Telangiectasia and Rad3 related (*ATR*), Mouse double minute-2 homolog (*MDM2*), Mouse Double Minute-4 (*MDM4*), Retinoblastoma-1 (*RBI*), Caspase 9 (*CASP9*), Tumor Protein P53 (*TP53*), B-cell lymphoma-2 (*BCL2*), Apoptotic Peptidase Activating Factor 1 (*APAF1*), E2F Transcription Factor-3 (*E2F3*), Myeloid Cell Leukemia-1 (*MCL1*), Phosphatase and tensin homolog (*PTEN*)] in the TP53 pathway.

2. MATERIAL AND METHODS

2.1. Patient Specimens

Bone marrow samples taken from 48 MM patients and 31 healthy people between 2016 and 2019 from the hematology clinics of the Istanbul Faculty of Medicine and the Istanbul Haseki Training and Research Hospital were used. Samples were collected from newly diagnosed and untreated patients. After the pathology results were clarified samples were included in the study. Physical characteristics, laboratory findings (biochemistry, pathology and genetic results) and MM staging have been recorded. Our investigation used the diagnostic criteria established by the International Myeloma Working Group (IMWG) to identify cases with MM [14].

Thirty-one male (64.6%) and 17 female (35.4%) patients, whose median age was 65.33 years (range: 41–83 years), were included in the study. The data shown in Table 2 includes diagnostic data and results from the cytogenetic analysis and FISH. A median age of 54.10 years was observed among 14 males (45%) and 17 females (55%) as controls (range: 18-76 years). Ethics committee approval was obtained for our study (Istanbul Medical Faculty Clinical Research Ethic Committee approval number/date: E-29624016-050.99-876968/May 9, 2022).

2.2. Sample Size Calculation

The minimal sample size was calculated as $n=24$ individuals for each group, with a patient/control ratio of 2, in order to find the difference significant with an estimated 3-unit standard deviation in the $\Delta\Delta C_t$ values of the analyzed genes between the MM and the control group. According to post-hoc power analysis, the required sample sizes were accepted as Power 0.80, Type I error of 0.05, Type II error of 0.20, and 48 patients and 31 controls were included in the study.

2.3. Cytogenetic Evaluation

Samples of bone marrow from MM patients were analyzed cytogenetically. A short culture period of 24/48 hours was followed by conventional cytogenetic analysis for all samples. We report the results of an analysis of 20 metaphase spreads in accordance with the International System of Human Cytogenetic Nomenclature [15].

2.4. Fluorescence in Situ Hybridization (FISH)

Samples obtained from patients with MM were subjected to FISH analysis. FISH probes for the MM panel identifying deletions at 13q14 (CytoCell 13q14.3 Deletion), 17p13.1 (CytoCell P53 (TP53) Deletion), and 11q22.3 (CytoCell ATM Deletion) and translocations at t(4;14)(p16.3;q32.33) (CytoCell IGH/FGFR3 Plus Translocation, Dual Fusion Probe Set), t(14;16)(q32.33;q23.1) (CytoCell IGH/MAF v2 Translocation, Dual Fusion Probe Set), and t(11;14)(q13;q32.33) (CytoCell IGH/MYEOV Plus Translocation, Dual Fusion Probe Set) (CytoCell, Cambridge, UK) were used. Nuclei in interphase were counterstained with DAPI (CytoCell, Cambridge, UK). Fluorescence microscope evaluation of FISH signals was conducted (Zeiss Axioskop 2 Plus, Germany). Karyotyping and FISH software were used to capture the images (MetaSystems ISIS FISH imaging system, Germany). Each slide was evaluated alongside 200 interphase nuclei.

2.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted from MM and control samples using the QiaAmp RNA Blood Mini Kit according to the manufacturer's instructions (catalog no:52304; Qiagen, Germany). The NanoDrop 2000c (Thermo Fisher Scientific, USA) was used to measure RNA quality and quantity (A260/A280: 1.8-2.0). In the study, 0.5 μ g of total RNA was utilized to reverse-transcribe (RT) each sample. Qiagen RT²HT First Strand Kit (cat no. 330404; Qiagen, USA) using 0.5 μ g of mRNA. Then, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed.

2.6. RT² Profiler PCR Array

An RT² Profiler PCR Array (Qiagen; Custom Cat no: CAPH13446) was used to detect 18 genes associated with cell cycle, apoptosis, proliferation, DNA repair and differentiation mediated by the TP53 protein. It was conducted using a custom RT² Profiler PCR Array with 96 wells in F format (CAPH_133446F; cat no. 330131/12 plate). Each MM patient and control group was evaluated using a custom RT² Profiler PCR Array, which measured 18 genes along with a reference gene (*ACTB*). Qiagen RT² SYBR Green qPCR mastermix kit (catalog no. 330502), 10.5 µl ddH₂O, and 1 µl cDNA were prepared on the LightCycler 480 II RT-PCR instrument (Roche Diagnostics, Germany) with 25 µl of 12.5 µl SYBR green master mix, 10.5 µl ddH₂O, and 1 µl cDNA (Table 1). The PCR process was performed once.

Table 1. PCR cycling conditions

Cycles	Duration	Temperature	Program name	Comment
1	10 min.	95 ⁰ C	Heat Activation	This heating process activates HotStart DNA Taq polymerase.
45	15 sec. 1 min.	95 ⁰ C 60 ⁰ C	PCR Cycle	Fluorescence data collection takes place.
1	15 sec. -	60 ⁰ C 95 ⁰ C	Melting curve	It has been confirmed that the cDNA is the target region.

2.7. Data Analysis

A Second Derivative Max Method was used in this software for analyzing data with the LightCycler 480 system. The Cp value is the point where the greatest value of the second derivative occurs in an amplification curve, therefore characterizing the PCR reaction (Qiagen, USA). The RT² Profiler PCR Array Data Analysis 3.5 application was used to assess the Ct values for each sample [13]. Differential expression was analyzed by 2^{-ΔΔCt} method using *ACTB* for normalization. Gene expression levels are also measured as fold changes based on the RT² Profiler PCR Array data.

2.8. String Analysis

An automated text-mining approach was used to extract information from scientific literature. There were databases containing complexes and pathways annotated with interactions like STRING. An analysis of co-expression and conserved genomic context was conducted to estimate computational interactions [16]. Gene set enrichment analysis, subset visualization as interaction networks, and full genome-wide dataset loading are all possible with the STRING tool [17].

2.9. Gene Ontology (GO) Enrichment Analysis

GO is a freely available web resource that annotates genes with their biological system and pathway functions and stores this information in a computationally sound manner [18]. Genes are described using biological terminology (GO terms) based on the properties of the proteins they encode, according to the GO database. Terms like "cellular component," "biological process," and "molecular function" fall into three broad categories [19]. ShinyGO, an R/Bioconductor program, uses a big route database. A tool called ShinyGO 0.77 can be used to make graphs of GO enrichment results and gene features. It can also connect to KEGG and STRING databases [20].

2.10. Statistical Analysis

Data was analyzed using SPSS version 21 and a Qiagen custom RT² Profiler PCR Array. Analyses using the Student's t-test were performed on parametric data that followed a normal distribution. Log-transforming values (logFC) were also analysed using Qiagen GeneGlobe RT² Profiler PCR Array. The

sensitivity and specificity of the genes in distinguishing the MM group from the control group were calculated using ROC (receiver operating characteristics) analysis.

3. RESULTS

3.1. Clinical Characteristics of MM Patients

The participants in this research consisted of a total of 48 people who were diagnosed with MM and 31 people who were considered to be healthy. In MM, male/female ratio was 31/17; the ratio of male to female was 1.82. In the control group, male/female ratio was 14/17 (the ratio of male to female was 0.82). The MM and control groups had mean ages of 65.33 ± 9.93 and 54.10 ± 13.14 , respectively. The median, minimum, and maximum ages were respectively 65.33 (41-83) and 54.10 (18-76). Both the patient and control groups had comparable gender distributions ($p=0.08$). The average age of patients was greater than that of controls ($t=4.32$; $p<0.001$).

There were a total of 36 MM patients, with 18 at stage 1A (37.5%), 18 at stage 2A (37.5%), and 12 at stage 2B (25.0%). When the distribution of Igs according to stage was examined, a significant difference was found in IgM. IgM levels were similar between stage 1 and stage 2A, but IgM levels were significantly lower in stage 2B compared to stages 1A and 2A ($X^2=6.835$; $p=0.03$) (Table 2).

Table 2. Ig distributions by stage

Stage code		IgA	IgG	IgM
1A	Mean	310.91	2046.636	48.782
	Std. Deviation	400.26	1451.35	46.32
	Median	94	1502	39
	Minimum	19	612	5
	Maximum	1060	4450	172
2A	Mean	354.25	1898.175	30.725
	Std. Deviation	559.93	2031.67	24.53
	Median	79	804.7	27.5
	Minimum	13	120	8
	Maximum	1456	4450	76.8
2B	Mean	556.67	2918.17	12.50
	Std. Deviation	1288.651	3016.788	7.287
	Median	34	1870.5	9.5
	Minimum	15	186	5
	Maximum	3187	7380	23
Significance		IgA	IgG	IgM
	Chi-Square	1.763	0.94	6.835
	<i>p</i>	0.414	0.625	0.033

Std: Standard

We evaluated the FISH results of patients with and without deletions of 13q14, 17p13, 11q22.3, and translocation of t(4;14)(p16.3; q32.33), t(14;16)(q32.33; q23.1) and t(11;14)(q13; q32.33) chromosomal aberrations. Of the 17 patients whose FISH results were present, 4 patients had t(11;14)(q13; q32.33) (23.5%), and 1 patient had monosomy 8, monosomy 13, del13q14.3, del13q34 and trisomy 11q13 (5.8%). No abnormality was detected in 12 patients (70.5%). Patient diagnostic data and cytogenetic / FISH results are demonstrated in Table 3.

Table 3. Data from patient diagnoses and cytogenetic/FISH analyses

Number	Gender	Age	Prognosis	Stage	Cytogenetic and FISH Results	WBC (x10 ⁹ /L)	Hb (g/dl)	PLT (x10 ⁹ /L)
1	M	64	IgG/κ MM	DSS-2B	46,XY IGH/CCND1 t(11;14)	3.31	9.20	95.00
2	F	71	IgG/κ MM	DSS-1A	46, XX IGH/CCND1 t(11;14)	6.86	10.6	231.00
3	M	53	IgA/ λ MM	DSS-1A	46,XY	8.06	15.00	230.00
4	M	83	IgG/κ MM	DSS-2A	46,XY, FISH normal	4.34	10.20	244.00
5	M	77	IgG/κ MM	DSS-2A	46,XY, Monosomy 8, monosomy 12, del13q14.3, del13q34, trisomy 11q13 (+)	1.97	8.2	149.00
6	F	54	λ light chain myeloma	DSS-1A	46, XX, FISH normal	9.71	10.90	133.00
7	M	70	IgG/κ MM	DSS-1A	46,XY, FISH normal	5.68	11.7	174.00
8	M	47	λ light chain myeloma	DSS-2A	46,XY	4.72	10.6	246.00
9	M	66	MM	DSS-1A	46,XY	9.07	13.70	261.00
10	M	51	MM	DSS-2A	46,XY, FISH normal	6.99	11.9	182.00
11	F	78	IgA/κ MM	DSS-2A	46,XX, FISH normal	5.31	6.21	193.00
12	M	83	IgG/κ MM	DSS-2B	46,XY, FISH normal	4.86	8.4	125.00
13	M	65	IgG/ λ MM	DSS-2B	46, XY	4.87	9.7	111.00
14	F	81	IgA/κ MM	DSS-2A	46,XX, FISH normal	3	7.6	191.00
15	M	53	λ light chain myeloma	DSS-2B	46,XY	6.70	7.3	227.00
16	M	75	λ light chain myeloma	DSS-1A	46,XY	4.20	11	217.00
17	F	70	IgG/κ MM	DSS-1A	46,XX	11.6	11.20	282.00
18	M	63	IgG/κ MM	DSS-1A	46,XY	4.78	12.1	139.00

19	M	71	MM	DSS-1A	46, XY	5.40	13.30	218.00
20	M	60	IgG/κ MM	DSS-3A	46,XY, FISH normal	8.14	8.00	312.00
21	F	49	λ light chain myeloma	DSS-2A	46,XX, FISH normal	4.73	10	176.00
22	F	71	MM	DSS-2A	46,XX	4.17	8.62	268.00
23	F	62	MM	DSS-2A	46, XX	3.50	7.60	233.00
24	F	71	MM	DSS-2A	46, XX	7.10	10.80	211.00
25	M	68	IgA/ λ MM	DSS-2B	46,XY	5.08	10.1	172.00
26	F	51	IgA/κ MM	DSS-2B	46, XX	7.64	7.20	175.00
27	F	69	IgG/ λ MM	DSS-2A	46,XX, IGH/CCND1 t(11;14)	8.21	10.4	223.00
28	M	65	IgG/ λ MM	DSS-2B	46,XY	14.55	9.2	228.00
29	M	67	MM	DSS-1A	46, XY	4.46	12.50	153.00
30	M	78	IgA/ λ MM	DSS-1A	46,XY	11.78	14.40	298.00
31	M	83	IgG/ λ MM	DSS-1A	46,XY, FISH normal	7.60	12.4	398.00
32	M	68	IgD/ λ MM	DSS-2B	46,XY, IGH/CCND1 t(11;14)	15.6	9.00	359.00
33	M	41	IgD/κ MM	DSS-2B	46,XY, FISH normal	18.20	8.2	141.00
34	F	62	IgA/κ MM	DSS-2A	46,XX	6.90	8.50	250.00
35	M	81	IgG/κ MM	DSS-1A	46,XY, FISH normal	4.70	11.70	132.00
36	M	54	MM	DSS-1B	46,XY	7.10	10.80	128.00
37	F	60	IgG/λ MM	DSS-2A	46,XX, hyperdiploidy	4.10	6.2	190.00
38	F	66	IgG/λ MM	DSS-2A	46,XX, t(8;14) (q24;q32), t(4;14) p16;q32), hypodiploidy	8.33	11.90	198.00
39	M	67	IgG/ λ MM	DSS-1A	46,XY	3.44	11.6	126.00
40	F	72	MM	DSS-2A	46,XX	5.50	8.4	171.00
41	F	67	IgG/ λ MM	DSS-1A	46,XX	7.60	11.90	162.60

42	M	57	κ light chain myeloma	DSS-1A	46,XY	18.67	12.10	300.00
43	M	60	MM	DSS-2B	46,XY	6.00	9.26	132.00
44	F	63	MM	DSS-1A	46, XX	15.24	10.00	158.00
45	M	64	MM	DSS-2A	46, XY	9.52	7.26	201.00
46	M	61	MM	DSS-2A	46, XY	9.94	10.01	178.00
47	M	61	MM	DSS-1A	46, XY	8.40	12.20	210.00
48	M	63	MM	DSS-2B	46, XY	5.66	9.30	195.00

FISH: Fluorescence in situ hybridization DSS: Durie Salmon staging system del: deletion

IGH/CCND1: immunoglobulin heavy chain gene/cyclin D1 WBC: white blood cells Hb: hemoglobin PLT: Platelets

3.2. Results of Differentially Expression Analysis

Gene expression levels of *APAF1*, *ATM*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *E2F1*, *MCL1*, *MDM2*, *MDM4*, *PTEN* were found to be statistically significant. Twelve of the genes studied had elevated expression levels in MM patients compared to controls. Among these, the highest fold changes were detected in *BAX* and *CDKN2A* genes with 12.27 and 19.48 folds, respectively. On the other hand, statistically significant alteration was not detected in terms of *ATR*, *CHEK2*, *E2F3*, *RB1*, *TP53* and *BCL2* gene expressions between groups (Table 4).

Table 4. Comparison of patient and control group mean ΔCt and fold change values ($p < 0.05$)

Gene	ΔCt		$\Delta \Delta Ct$		$2^{-\Delta \Delta Ct}$		Fold Change	p value
	MM	Control	MM	Control	MM	Control	MM/Control	
APAF1	25.89	25.75	6.23	7.66	0.013309	0.004944	2.69	0.036751
ATM	27.89	28.25	8.23	10.16	0.003336	0.000873	3.82	0.012776
ATR	27.96	29.29	8.30	11.20	0.003176	0.000425	7.48	0.074824
BAX	24.25	26.29	4.59	8.21	0.041563	0.003388	12.27	0.024502
CASP9	27.72	27.99	8.06	9.90	0.003751	0.001045	3.59	0.045504
CDK4	26.83	27.77	7.17	9.69	0.006953	0.001212	5.74	0.000660
CDKN1A	26.15	27.23	6.49	9.15	0.011146	0.001761	6.33	0.000042
CDKN2A	29.99	32.71	10.30	14.58	0.000795	0.000041	19.48	0.013985
CHEK2	28.04	29.88	8.38	11.80	0.002997	0.000281	10.68	0.197278
E2F1	29.64	30.15	9.05	12.06	0.001890	0.000234	8.08	0.045762
E2F3	27.57	27.34	7.91	9.26	0.004161	0.001635	2.55	0.130064
MCL1	22.56	21.96	2.90	3.87	0.133643	0.068225	1.96	0.000034
MDM2	25.55	25.85	5.89	7.76	0.016870	0.004607	3.66	0.014514
MDM4	27.95	28.40	8.29	10.32	0.003204	0.000785	4.08	0.003958
PTEN	24.63	24.26	4.97	6.17	0.031838	0.013848	2.30	0.004188
RB1	26.16	26.69	6.49	8.60	0.011092	0.002574	4.31	0.144422
TP53	25.74	26.33	6.08	8.24	0.014763	0.003304	4.47	0.254569
BCL2	24.29	27.65	4.63	9.57	0.040404	0.001317	30.68	0.238986

ΔCt : Delta cycle threshold MM: Multiple myeloma

The expressions of the selected genes according to $\Delta \Delta Ct$ values were demonstrated in Figure 1.

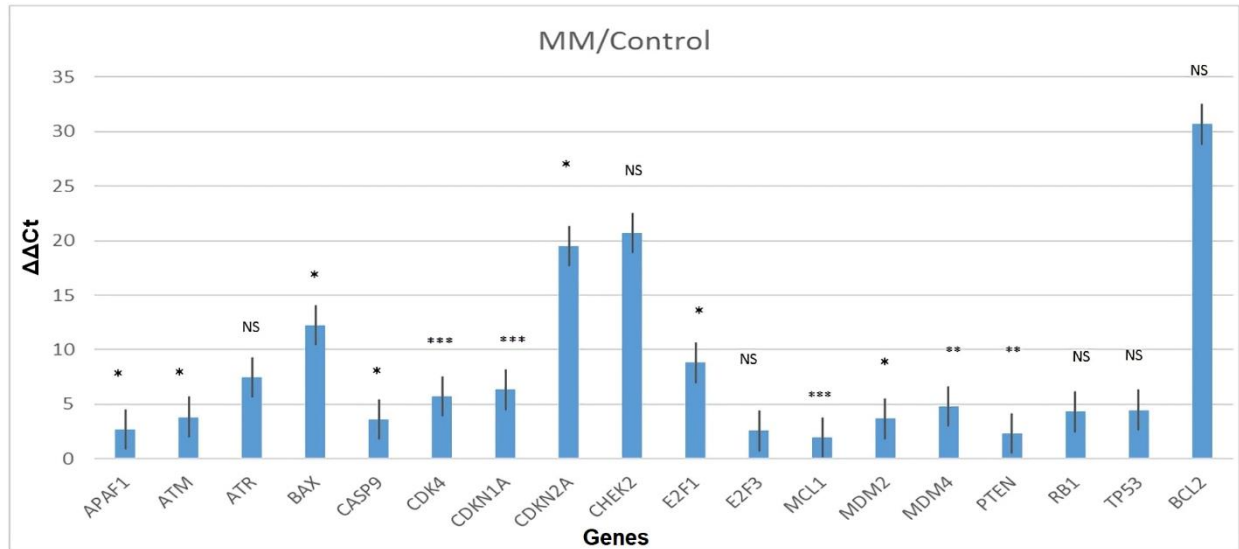


Figure 1. The ratio of fold change and significance values of the genes examined in the MM/Control group. A fold-change ($2^{-\Delta\Delta C_T}$) is the difference in normalized gene expression ($2^{-\Delta C_T}$) between the Test Sample and the Control Sample. Values of fold-change greater than one suggest an up-regulation, whereas values of fold-change less than one indicate a down-regulation.. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS: Non-Significant.

A dendrogram heat map was created for the differential expression of 18 genes in two groups (Figure 2).

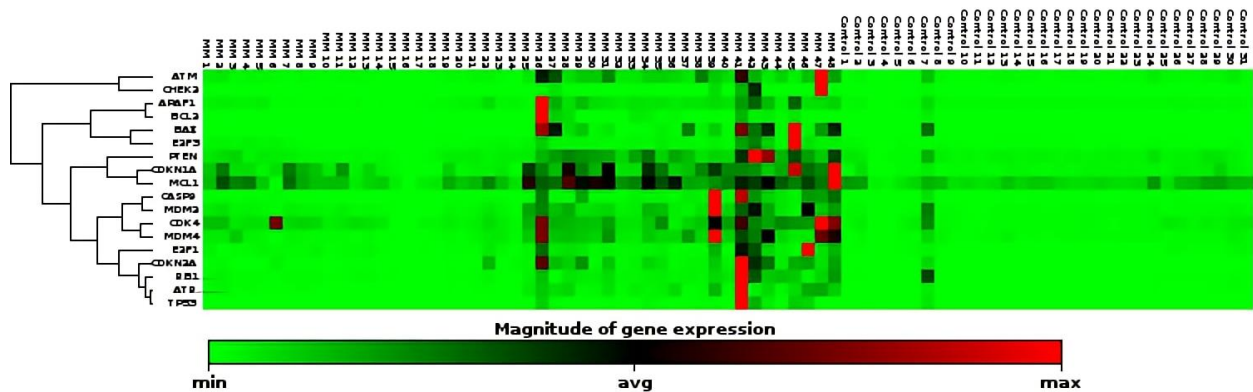


Figure 2. Clustergram results obtained with RT2 Profiler PCR Array

Clustergram analysis and heatmap plot of gene expression data is represented one-dimensionally with color codes (genes). Expression levels of genes are shown with a heatmap plot (clustergram), and genes are clustered according to their expression patterns. The heat map graphically shows the folded up- or down-regulation of expression data between the two groups (patient and control) examined in the RT²-PCR Array. The amount of change in gene expression is represented by the color saturation. Patient samples with low gene expression (ratio: <1) are shown in green squares. Black squares represent equivalent gene expression (ratio close to 1). Red squares indicate higher (ratio: >1) gene expression than control levels.

According to ROC analysis data, three of the genes with marked increases in mRNA expression were found to have a robust potential to differentiate between MM and the control group. Those genes were *CDK4*, *CDKN1A* and *MCL1* with area under the curve (AUC) values of 0.89, 0.86 and 0.77, respectively and $p<0.001$ for all three (Figure 3).

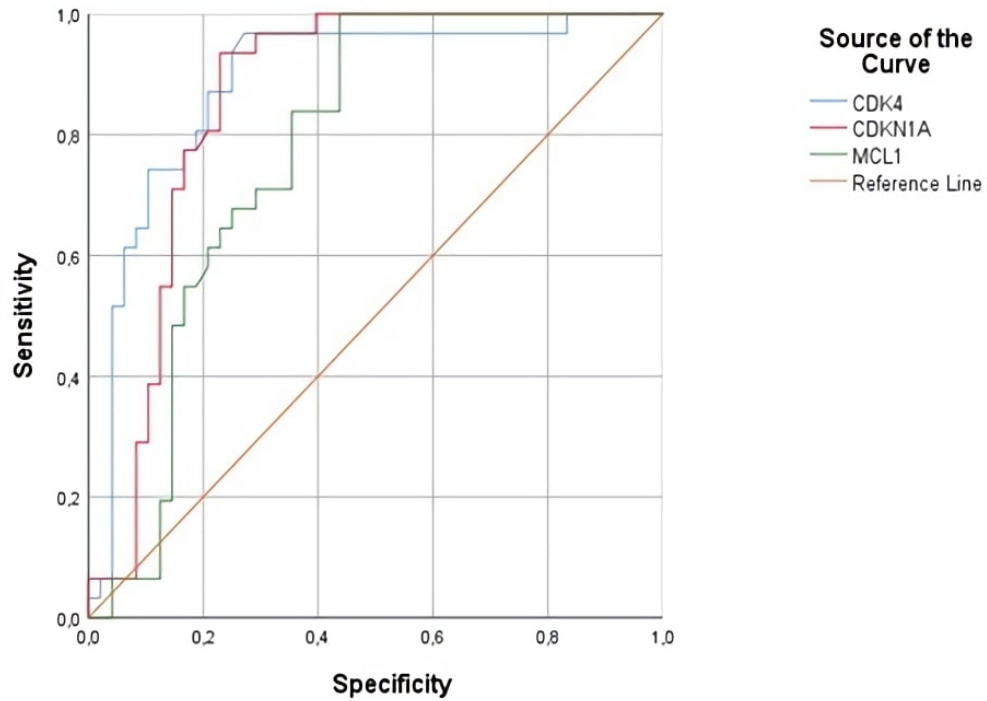


Figure 3. The ROC analysis graph showing CDK4, CDKN1A, and MCL1 genes, which have the most significant increase in gene expression levels and have the potential to make a strong distinction between the MM and control groups.

ROC Curves of the CDK4, CDKN1A, and MCL1 genes with the highest fold change ($p < 0.001$) (Reference line: control group). The highest total area under the curve (AUC) of the CDK4, CDKN1A, and MCL1 genes in MM patients and healthy controls was AUC = 0.89, 0.86, and 0.77, respectively; ($p < 0.001$ for all three genes). This indicated that CDK4, CDKN1A, and MCL1 had a good ability to accurately distinguish between MM samples and healthy control samples.

By using String's web-based tool, we investigated the likely interactions of these 12 genes and found that the resulting network ($p < 1.0e-16$) was enriched with interactions (Figure 4).

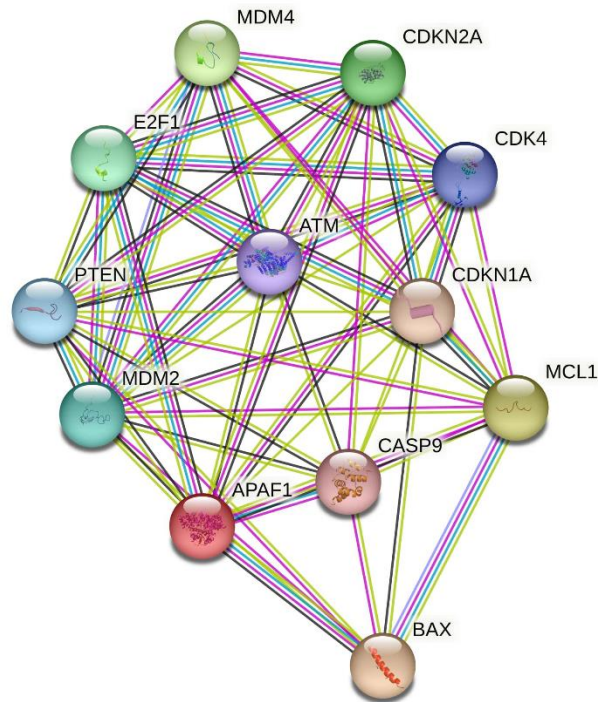


Figure 4. A graphical illustration of the interactions that take place between the 12 genes that are differently expressed in MM patients (STRING v.11.5).

This view shows the predicted association network for a particular protein group. Network nodes proteins; the edges represent predicted functional relationships. In proof mode, an edge can be drawn with a different colored line used to predict up to seven relationships. Fusion evidence is red, neighborhood evidence is green, cooccurrence evidence is blue, experimental evidence is purple, text mining evidence is yellow, database evidence is light blue, and co-expression evidence is black.

An enrichment analysis is performed for Gene Ontologies, pathways, and domains. We associated the *APAF1*, *ATM*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *E2F1*, *MCL1*, *MDM2*, *MDM4*, *PTEN* genes as functional categories (GO biological process, GO cellular component and GO molecular function) through the ShinyGO 0.77 database by enrichment analysis. This hypergeometric test yields a notional P-value, which is then used to compute the false discovery rate (FDR). Divide the genes involved in a pathway by the background fraction to get the fold enrichment. Large paths tend to have lower FDRs owing to their statistical power. This statistic indicates a pathway's gene overrepresentation. An analysis of the biological process using GO enrichment showed that the genes involved were significantly enriched (Table 5).

Table 5. ShinyGO 0.77 Gene Ontology Enrichment Analysis (Biological Process)

Enrichment FDR	Genes (n)	GO terms	Fold Enrichment
8.4E-08	<i>BAX</i> , <i>APAF1</i> , <i>CASP9</i>	GO:0008635	888.2
6.0E-07	<i>BAX</i> , <i>CDKN2A</i> , <i>ATM</i>	GO:1903624	478.2
1.1E-06	<i>CDKN1A</i> , <i>CDKN2A</i> , <i>ATM</i>	GO:0090399	388.6
4.1E-05	<i>BAX</i> , <i>ATM</i>	GO:1903626	690.8
2.1E-11	<i>BAX</i> , <i>E2F1</i> , <i>CDKN1A</i> , <i>MDM2</i> , <i>ATM</i> , <i>MDM4</i>	GO:0006977	188.4
3.3E-11	<i>BAX</i> , <i>E2F1</i> , <i>CDKN1A</i> , <i>MDM2</i> , <i>ATM</i> , <i>MDM4</i>	GO:0044819	168
3.4E-11	<i>BAX</i> , <i>E2F1</i> , <i>CDKN1A</i> , <i>MDM2</i> , <i>ATM</i> , <i>MDM4</i>	GO:0044819	165.8
4.3E-14	<i>BAX</i> , <i>E2F1</i> , <i>CDKN1A</i> , <i>CDK4</i> , <i>MDM2</i> , <i>CDKN2A</i> , <i>ATM</i> , <i>MDM4</i>	GO:2000134	144.2
1.4E-04	<i>BAX</i> , <i>CDKN2A</i>	GO:1902510	376.8
7.0E-06	<i>CDKN1A</i> , <i>MDM2</i> , <i>ATM</i>	GO:0071480	194.3

4.6E-12	<i>BAX, E2F1, CDKN1A, MDM2, CDKN2A, ATM, MDM4</i>	GO:0030330	119.9
2.4E-04	<i>CDK4, MDM2</i>	GO:0045472	276.3
3.1E-07	<i>BAX, CDKN1A, MDM2, ATM</i>	GO:0010332	145.4
1.1E-05	<i>MDM2, CDKN2A, ATM</i>	GO:0043516	163.6
3.0E-04	<i>CDKN2A, ATM</i>	GO:0043517	243.8
5.0E-07	<i>BAX, CASP9, MCL1, ATM</i>	GO:0043525	127.5
3.4E-10	<i>BAX, E2F1, CDKN1A, CASP9, MCL1, ATM</i>	GO:0008630	108.1
3.3E-04	<i>CDKN2A, ATM</i>	GO:0030889	230.3
1.0E-12	<i>BAX, E2F1, CDKN1A, CDK4, MDM2, CDKN2A, ATM, MDM4</i>	GO:1902806	85
1.4E-05	<i>E2F1, CDKN1A, CDK4</i>	GO:0030851	148

FDR cut-off is taken as 0.05. In terms of biological processes, the top one is "activation of cysteine-type endopeptidase activity involved in the apoptotic process by cytochrome c," (FDR: 8.4E-08) which includes genes like *BAX*, *APAF1*, and *CASP9* (Figure 5).

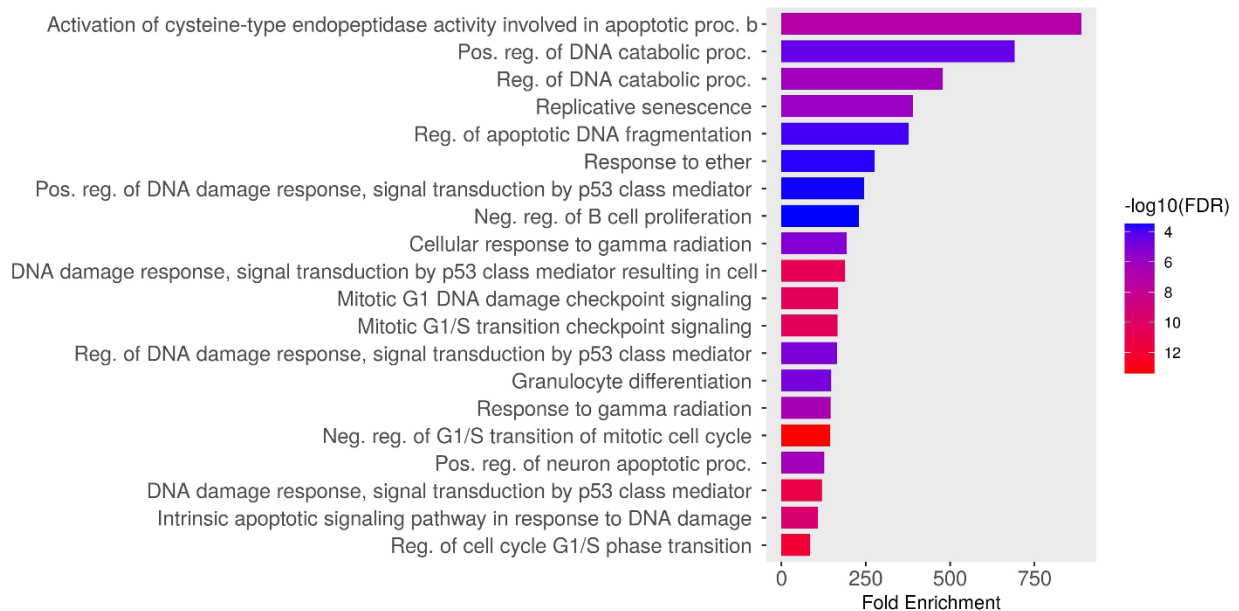


Figure 5. Diagram of a biological process identifying a biological target for gene products.

For the gene ontology enrichment analysis, the best pathways were selected according to FDR out of the twelve genes found to be associated with MM, and then the obtained pathways were ranked according to fold enrichment.

4. DISCUSSION

The most distinctive hallmark of MM is the presence of plasma cells in the bone marrow [21]. The genomic abnormalities are common in MM [22]. There is a strong correlation between TP53 abnormalities and poor prognosis in MM. The *TP53* gene mutation is rare at diagnosis in MM [23]. Detection of driver mutations that cause oncogenesis is crucial in determining the response to therapy for MM. So, mRNA-based studies provide information about the oncogenic pathways in MM [24].

MM patients with a deletion of the 17p13-del (17p) chromosomal region have a poor prognosis. The *TP53* gene encodes the P53 protein. Transactivating its downstream genes, P53 causes cell cycle arrest via *CDKN1A* and apoptosis through *BAX*, P53-up-regulated modulator of apoptosis (*PUMA*), and phorbol-12-myristate-13-acetate-induced protein 1 (*NOXA*). Advanced MM is associated with *TP53* mutations and their function in disease development [25].

TP53 can inhibit anti-apoptotic proteins like *BCL2*, *BCL-xL*, and *MCL1* while upregulating pro-apoptotic genes like *Puma*, *Noxa*, *Apaf1*, and *Bax* [26]. A caspase-3 activation in response to *TP53*-mediated neuronal cell death is mediated by *Bax* [27]. *BAX* and *APAF1* expression is increased by *TP53*-induced expression of *p53*, increasing the *TP53*-dependent apoptotic response [28]. The data of our study suggested that epigenetic mechanisms may also play a role in increasing the expression of apoptosis-related genes (*APAF1*, *BAX*, *CASP9*, etc.), which are known to be regulated by this gene, although *TP53* expression did not differ in MM and controls. There is evidence that myeloma cells may survive through the overexpression of pro-survival proteins, particularly *MCL-1*. Possible drugs that could prolong a person's survival or even treat multiple myeloma may lie in factors that control the growth, survival, and complex interactions of myeloma cells with the bone marrow microenvironment [29].

E2F1 overexpression stimulates the stabilization and accumulation of *p53* by directly processing the *CDKN2A* transcript, *p14ARF*, in a *p53*-dependent pathway. *CHEK2* and *p53* phosphorylation and apoptosis are induced by *E2F1* without *p14ARF*. A cyclin A binding domain in *E2F1* induces *p53*'s apoptotic activity when DNA damage is observed, which is directly linked to *E2F1* [30]. *MDM2* overexpression promotes the expansion and survival of MM cells. A therapeutic strategy targeting *MDM2* in MM may be beneficial in the treatment of MM [31].

Furthermore, multiple myeloma is associated with *MDM2* overexpression, which leads to *p53* turnover and maintains low levels. *p53* inactivation is also associated with *MDM4*, a homologue of *MDM2* on chromosome 1q. An independent and substantial prognostic factor is 1q amplification, which indicates a high-risk illness [29].

The increase in function or loss of inhibition of *CDK4* or *CDK6* activity is a common aberration in cancer. Overexpression of *CDK4* (*CDK6*) leads to unrestrained growth of CD138+ bone marrow myeloma cells in living things, especially when the tumor grows quickly and comes back. Retinoblastoma protein *Rb* is inactivated, and the *INK4* family of *CDK* inhibitors is antagonized by *CDK4* and *CDK6*, which, in association with the D-type cyclin, increase cell cycle entrance and advancement through G1. Inhibiting *CDK4/6* may be an effective way to manage the cell cycle in MM [32].

We hypothesized in this study that the deregulation of 18 *TP53* pathway genes may contribute to MM development. Thus, the expression levels of the chosen genes were compared between 48 patients and 31 controls. In our study, 1.5 fold change was determined as cut-off value. The *APAF1*, *ATM*, *BAX*, *CASP9*, *CDK4*, *CDKN1A*, *CDKN2A*, *E2F1*, *MCL1*, *MDM2*, *MDM4*, and *PTEN* genes had differentially significant elevated mRNA expression values in MM patients compared to controls. Among them, the most highly upregulated gene was *CDKN2A* in MM patients, which showed a 19.48-fold change and the *BAX* was the second highly upregulated gene with a 12.27-fold change. In addition, ROC analysis results showed that *CDK4*, *CDKN1A* and *MCL1* genes may have a strong ability to distinguish MM from controls. In terms of biomarker potential, *CDK4* and *CDKN1A* were interpreted as excellent and *MCL1* as good.

The plasma cells of individuals with MM have been shown to have varying expression of the antiapoptotic genes *BCL2* and *BCL-xL*. Renner et al. [33] studied *BAX* expression levels in patients with reactive plasmacytosis and in patients with MM and found an increase ($p < 0.05$) in both *BAX* and *BCL2* expression in MM patients compared with patients with reactive plasmacytosis. The increased *BAX* expression level in our study is concordant with the study of Renner et al. According to this, in the literature, it is concluded that the expression studies on most genes associated with apoptosis in multiple myeloma are in line with the results of our study and that the genes are overexpressed.

Additionally, Skrtic et al. found that the MM stage had low expression of *BCL2*, *MCL1*, and *BAX* in the Revised International Staging System 1 (rISS1) compared to rISS3, while the MM stage had low expression of *BCL2* and *MCL-1* in rISS1 compared to ISS2. The findings revealed an abnormal expression of *BCL2* family members, which might be relevant to MM therapy delivery techniques. They found that abnormal *BAX* expression in MM cells may make apoptosis resistance stronger by triggering prerequisite or hormesis-like responses in stressful environments [34].

CDKN2A is a tumor suppressor gene that produces a protein that sequesters the cyclin D component from the CDK4 and CDK6 kinases [35]. Consistent with the data we found in our study group, it has been shown by Zhan et. al. that *CDKN1A*, a specific cyclin-dependent kinase inhibitor, is upregulated in MM compared to normal plasma cells [36]. As a result of a meta-analysis study, a few genes, including the *CDKN1A* and *CDKN2A* genes, have been suggested as biomarkers for MM [37]. An antiapoptotic protein encoded by the *MCL1* gene that has been linked to medication resistance and a poor prognosis in MM [38].

Adamia et al. [39] studied a group of MM patients to determine their CDK4/6 transcript expression levels. They confirmed that clonal plasma cells expressed more CDK4/6 than healthy donor plasma cells as the tumor moved from MGUS to MM. CDK4/6 activation and overexpression contributed to MM pathogenesis and had clinical relevance.

In the current study, we determined that the expressions of *BAX*, *CDKN2A*, *CDKN1A*, *CDK4* and *MCL1*, which are genes involved in the production of proteins that play a role in the control of both the cell cycle and apoptosis mechanisms are elevated in MM. Based on this, we suggest that cell proliferation, which is the biggest problem in MM, may be inhibited through specific targeted therapies. Our findings may shed light on the genetic process behind the TP53 pathway, which in turn may help us better understand the etiology of MM. Further studies may reveal the cellular functions of these genes in biological processes associated with MM pathogenesis.

5. CONCLUSION

Our data show that TP53 mRNA levels are comparable across MM patients and healthy controls. Due to proteasomal degradation by MDM2, p53 is normally produced in a functionally latent state. Post-translational modifications, including phosphorylation and acetylation, promote the accumulation of p53 in the nucleus upon DNA damage [40]. Phosphorylation, acetylation, ubiquitination, sumoylation, and glycosylation of p53 may alter MM patients despite no variation in mRNA or protein levels. As a continuation of our investigation, western blotting, immunoprecipitation, and mass spectrometry may be used to estimate protein levels linked to our TP53 pathway genes.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES

- [1] Ilknur, S., Aynur, A., Sariman, M., Mesut, A., Hindilerden, I.Y., Ekmekci, S.S., Abaci, N., Palanduz, S., Çefle, K., Öztürk, S., "Dysregulation of MS4A3 and PRDX5 gene expression in multiple myeloma patients", *International Journal of Hematology and Oncology*, 31(4): 205-213, (2021).
- [2] Flynn, E., Bisht, K., Sridharan, V., Ortiz, M., Towfic, F., Thakurta, A., "Prognosis, Biology, and Targeting of TP53 Dysregulation in Multiple Myeloma", *Cells*, 9(2): 287, (2020).
- [3] López-Corral, L., Corchete, L.A., Sarasquete, M.E., Mateos, M.V., García-Sanz, R., Fermiñán, E., Lahuerta, J.J., Bladé, J., Oriol, A., Teruel, A.I., Martino, M.L., Hernández, J., Hernández-Rivas, J.M., Burguillo, F.J., San Miguel, J.F., Gutiérrez, N.C., "Transcriptome analysis reveals molecular profiles associated with evolving steps of monoclonal gammopathies", *Haematologica*, 99(8): 1365-72, (2014).
- [4] Levine, A.J., Carpten, J.D., Murphy, M., Hainaut, P., "Exploring the genetic and molecular basis of differences in multiple myeloma of individuals of African and European descent", *Cell Death & Differentiation*, 31: 1-8, (2024).

- [5] Ye, F., Wang, T., Liu, A., Li, Y., Li, N., Wang, H., Chen, W., "Clinical Significance of TP53 Abnormalities in Newly Diagnosed Multiple Myeloma", Turkish Journal of Hematology, 38(4): 246-253, (2021).
- [6] Herrero, A.B., Rojas, E.A., Misiewicz-Krzeminska, I., Krzeminski, P., Gutierrez, N.C., "Molecular Mechanisms of p53 Deregulation in Cancer: An Overview in Multiple Myeloma", International Journal of Molecular Sciences, 17(12): 2003, (2016).
- [7] Stühmer, T., Chatterjee, M., Hildebrandt, M., Herrmann, P., Gollasch, H., Gerecke, C., Theurich, S., Cigliano, L., Manz, R.A., Daniel, P.T., Bommert, K., Vassilev, L.T., Bargou, R.C., "Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma", Blood, 106(10): b3609-17, (2005).
- [8] Maes, A., Menu, E., Veirman, K., Maes, K., Vand Erkerken, K., De Bruyne, E., "The therapeutic potential of cell cycle targeting in multiple myeloma", Oncotarget, 8(52): 90501-90520, (2017).
- [9] Saha, M.N., Yang, Y., Chang, H., "Targeting p53 by small molecule p53 activators in multiple myeloma", Journal of Hematology & Oncology, 5(1): A7, (2012).
- [10] Guedes, A., Becker, R.G., Teixeira, L.E.M., "Multiple Myeloma (Part 1) - Update on Epidemiology, Diagnostic Criteria, Systemic Treatment and Prognosis", Revista Brasileira de Ortopedia (Sao Paulo), 58(3): 361-367, (2023).
- [11] Mateos, M.V., Ailawadhi, S., Costa, L.J., Grant, S.J., Kumar, L., Mohty, M., Aydin, D., Usmani, S.Z., "Global disparities in patients with multiple myeloma: a rapid evidence assessment", Blood Cancer Journal, 13(1): 109, (2023).
- [12] Kazandjian, D., "Multiple myeloma epidemiology and survival: A unique malignancy", Seminars in Oncology, 43(6): 676-681, (2016).
- [13] Öztan, G., Aktan, M., Palanduz, S., İşsever, H., Öztürk, S., Nikerel, E., Uçur, A., Bağatır, G., Bayrak, A., Çefle, K., "Relationship between Chromosomal Aberrations and Gene Expressions in the p53 Pathway in Chronic Lymphocytic Leukemia", Balkan Journal of Medical Genetics, 23(1): 15-24, (2020).
- [14] Rajkumar, S.V., Dimopoulos, M.A., Palumbo, A., Blade, J., Merlini, G., Mateos, M.V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P., Landgren, O., Paiva, B., Dispenzieri, A., Weiss, B., LeLeu, X., Zweegman, S., Lonial, S., Rosinol, L., Zamagni, E., Jagannath, S., Sezer, O., Kristinsson, S.Y., Caers, J., Usmani, S.Z., Lahuerta, J.J., Johnsen, H.E., Beksac, M., Cavo, M., Goldschmidt, H., Terpos, E., Kyle, R.A., Anderson, K.C., Durie, B.G., Miguel, J.F., "International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma", The Lancet Oncology, 15(12): e538-48, (2014).
- [15] Gonzalez Garcia, J.R., Meza-Espinoza, J.P., "Use of the International System for Human Cytogenetic Nomenclature (ISCN)", Blood, 108(12): 3952-3, (2006).
- [16] Szklarczyk, D., Gable, A.L., Nastou, K.C., Lyon, D., Kirsch, R., Pyysalo, S., Doncheva, N.T., Legeay, M., Fang, T., Bork, P., Jensen, L.J., von Mering, C., "The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets", Nucleic Acids Research, 49(D1): D605-D612, (2021).

- [17] Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., Jensen, L.J., von Mering, C., "The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible", *Nucleic Acids Research*, 45(D1): D362-D368, (2017).
- [18] Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., "Gene ontology: tool for the unification of biology. The Gene Ontology Consortium", *Nature Genetics*, 25(1): 25-9, (2000).
- [19] Kopke, G., Anklam, K., Kulow, M., Baker, L., Swalve, H.H., Lopes, F.B., Rosa, G.J.M., Döpfer, D., "The identification of gene ontologies and candidate genes for digital dermatitis in beef cattle from a genomewide association study", *International Journal of Veterinary Sciences Research*, 6(1): 027-037, (2020).
- [20] Ge, S.X., Jung, D., Yao, R., "ShinyGO: a graphical gene-set enrichment tool for animals and plants", *Bioinformatics*, 36(8): 2628-2629, (2020).
- [21] Solimando, A.G., Da Vià, M.C., Bolli, N., Steinbrunn, T., "The route of the malignant plasma cell in its survival niche: Exploring "Multiple myelomas", *Cancers*, 14(13): 3271, (2022).
- [22] Sadaf, H., Hong, H., Maqbool, M., Emhoff, K., Lin, J., Yan, S., Anwer, F., Zhao, J., "Multiple myeloma etiology and treatment", *Journal of Translational Genetics and Genomics*, 6(1): 63-83, (2022).
- [23] Teoh, P.J., Chng, W.J., "p53 abnormalities and potential therapeutic targeting in multiple myeloma", *BioMed research international*, 2014: 717919, (2014).
- [24] Puła, A., Robak, P., Mikułski, D., Robak, T., "The significance of mRNA in the biology of multiple myeloma and its clinical implications", *International Journal of Molecular Sciences*, 22(21): 12070, (2021).
- [25] Drozdokova, D.H., Gursky, J., Minarik, J., Uberall, I., Kolar, Z., Trtkova, K.S., "CDKN1A Gene Expression in Two Multiple Myeloma Cell Lines With Different P53 Functionality", *Anticancer Research*, 40(9): 4979-4987, (2020).
- [26] Ahmadi, S.E., Rahimi, S., Zarandi, B., Chegeni, R., Safa, M., "MYC: a multipurpose oncogene with prognostic and therapeutic implications in blood malignancies", *Journal of Hematology & Oncology*, 14(1): 121, (2021).
- [27] Fortin, A., Cregan, S.P., MacLaurin, J.G., Kushwaha, N., Hickman, E.S., Thompson, C.S., Hakim, A., Albert, P.R., Cecconi, F., Helin, K., Park, D.S., Slack, R.S., "APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death", *Journal of Cell Biology*, 155(2): 207-16, (2001).
- [28] Lees, A., Sessler, T., McDade, S., "Dying to Survive-The p53 Paradox", *Cancers (Basel)*, 13(13): 3257, (2021).
- [29] Bansal, R., Rakshit, S., Han, W., Kumar, S.K., "Modulation of Apoptosis Pathways in the Biology and Treatment of Multiple Myeloma", *Oncology & Haematology*, 17(1): 48-54, (2021).
- [30] Putzer, B.M., "Targeting E2F1 death signaling: opposing role in cancer control and neurodegeneration", *Discovery Medicine*, 6(33): 123-127, (2009).

- [31] Teoh, G., Urashima, M., Ogata, A., Chauhan, D., DeCaprio, J.A., Treon, S.P., Schlossman, R.L., Anderson, K.C., "MDM2 protein overexpression promotes proliferation and survival of multiple myeloma cells", *Blood*, 90(5): 1982-92, (1997).
- [32] Huang, X., Di Liberto, M., Louie, T., Jayabalan, D.S., Ely, S., Niesvizky, R., Chen-Kiang, S., "Selective Inhibition of CDK4 and CDK6 Primes Chemoresistant Myeloma Cell for Cytotoxic Killing through Induction of Cell Cycle-Coupled Mitochondrial Dysfunction and Apoptosis", *Blood*, 112(11): 3678, (2008).
- [33] Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J.C., Lichtenstein, A., "Expression of BAX in plasma cell dyscrasias", *Clinical cancer research*, 6(6): 2371-2380, (2000).
- [34] Skrtic, A., Jaksic, O., Ostojic, S., Antica, M., Crnkovic, P., Skelin, J., Jurenec, S., Habijanec, B., Kristo, D.R., "Association between BCL2, BCL2 E17, MCL1 and BAX Protein Expression, Bone Marrow Microenvironment Histological Features, Clinical Presentation, Therapeutic Outcome, and the Overall Survival in Newly Diagnosed Multiple Myeloma", *Blood*, 138(1): 4716, (2021).
- [35] Dilworth, D., Liu, L., Stewart, A.K., Berenson, J.R., Lassam, N., Hogg, D., "Germline CDKN2A mutation implicated in predisposition to multiple myeloma", *Blood, The Journal of the American Society of Hematology*, 95(5): 1869-1871, (2000).
- [36] Zhan, F., Hardin, J., Kordsmeier, B., Bumm, K., Zheng, M., Tian, E., Sanderson, R., Yang, Y., Wilson, C., Zangari, M., Anaissie, E., Morris, C., Muwally, F., van Rhee, F., Fassas, A., Crowley, J., Tricot, G., Barlogie, B., Shaughnessy, J., Jr., "Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells", *Blood*, 99(5): 1745-57, (2002).
- [37] Katiyar, A., Kaur, G., Rani, L., Jena, L., Singh, H., Kumar, L., Sharma, A., Kaur, P., Gupta, R., "Genome-wide identification of potential biomarkers in multiple myeloma using meta-analysis of mRNA and miRNA expression data", *Scientific Reports*, 11(1): 10957, (2021).
- [38] Wei, A.H., Roberts, A.W., Spencer, A., Rosenberg, A.S., Siegel, D., Walter, R.B., Caenepeel, S., Hughes, P., McIver, Z., Mezzi, K., Morrow, P.K., Stein, A., "Targeting MCL-1 in hematologic malignancies: Rationale and progress", *Blood Reviews*, 44: 100672, (2020).
- [39] Adamia, S., Bhatt, S., Wen, K., Chyra, Z., Fell, G.G., Tai, Y.T., Pioso, M., S. Abiatari, I., Letai, A., Dorfman, D.M., Hideshima, T., Anderson, K.C., "Combination therapy targeting Erk1/2 and CDK4/6i in relapsed refractory multiple myeloma", *Leukemia*, 36(4): 1088-1101, (2022).
- [40] Ozaki, T., Nakagawara, A., "Role of p53 in Cell Death and Human Cancers", *Cancers (Basel)*, 3(1): 994-1013, (2011).