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Research Article

OCT4B1 Suppression down Regulated BCL2 Gene Family Expression in Human Tumor Cell Lines - a

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ABSTRACT

Background and Aims: The OCT4B1, an important OCT4 variant is expressed more than other variants in both human cancer cell lines and tissues. New finding showed this variant has anti-apoptotic potency in mentioned cells and tissues. BCL2 family is one of the twelve gene families involved in apoptosis pathway with negative control in apoptosis recurrence. The aim of the present study was to investigate the effects of OCT4B1 silencing on several genes of BCL2 family in human tumor cell lines.

Materials and Methods: three human tumor cell lines; AGS (gastric adenocarcinoma), 5637 (bladder tumor) and U-87MG (brain tumor) were transfected with specific OCT4B1 siRNA and a scrambled sequence as control, using Lipofectamine 2000 comerical kit. Following, The expression rate of BCL2 gene family transcripts were evaluated in parallel with beta-actin (as housekeeping gene) using Real-Time PCR technique.

Results: Expressional profile of the studied BCL2 transcripts in three cell lines is almost similar. Nineteen of twenty one studied genes in BCL2 family showed down-regulation, forteen gene were decreased in expression more than 3 and three genes (BAD,BCL2 and BNIP3L) more than 10 folds. BCLAF1 showed up-regulation (in U87MG and 5637 tumor cell lines) and MCL1 showed unchanged gene expression.

Conclusions: According to these results, it may be concluded that OCT4B1 suppression can lead to apoptosis in tumor cell lines via down-regulation of several BCL2 transcripts. Thus, OCT4B1 suppression effects on BCL2 may be considered as promising target genes in future studies in cancer research and therapy.

Key words: OCT4B1; BCL2 Gene Family; Tumor Cell Lines

INTRODUCTION

OCT4 is an important and critical genes involved in both embryonic and adult stem cell proliferation and differentiation [1]. OCT4 belong to a family of transcription regulatory genes containing the PUO DNA binding domain, located on human chromosome 6 that potentially encodes at least three different variants (A, B and B1) via mRNA alternative splicing [2]. OCT4 is expressed in stem cells and induced cell survival (immortalization), while, silencing or down regulation of this gene caused cell differentiation [3-5]. According to the "cancer stem cell" theory of cancer, cancers are originated from either adult stem cells (tissue stem cells, presented in almost all tissues) or reprogrammed tissue somatic cells [6-8]. New finding revealed that there are similar pattern of gene expression in both cancer and normal stem cells [7, 9]. So, investigation of genes collaborated in both normal and cancer stem cells and especially finding the differences of gene's variants expression pattern in mentioned cells, were considered by cancer researchers. Because of OCT4 alternative splicing and producing of at least three different variants by specific function, were candidate genes and investigation showed that a new variant of OCT4, known as OCT4B1, is expressed in both normal stem cells (embryonic and adult) and cancer cell lines/ cancer tissues [10, 11]. While OCT4A variant expressed mainly in embryonic stem cells and silenced followed differentiation and OCT4B variant expressed in a wide variety of cells (stem and somatic cells) with no function in cell pluripotency [12]. New study revealed the OCT4B1 variant has anti-apoptotic potency in cancer cell lines [1, 9]. One hypothesis suggests that it can affect the expression of essential genes involved in cell survival and cell death, especially programmed cell death (apoptosis) to induce the self-renewal state of the cell.

Apoptosis is a specialized biochemical pathway for removal of the aggressive cells including cancer cells [13]; however, within the cancer tissues and cancer cell lines, apoptosis would be decreased or suppressed [14]. Delineation of both cellular and molecular mechanisms that contribute to these suppression processes could be considered as promising targets in cancer therapy.

There are at least eleven gene families related to apoptotic pathway, of which BCL2 gene family is most important of these families [15, 16, 17]. Most of the *BCL2* family genes have been found to play a central regulatory role in apoptosis induction [18]. High level of BCL2 family proteins are considered primarily responsible for inhibiting apoptosis in lot of cancer types [19].

Mammalian BCL2 protein family consists of at least 30 related proteins, characterized by the presence of up to four relatively short sequence motifs (less than 20 amino acid residues in length) termed BCL2 homology (BH) domains [20]. BCL2 family is divided into three different subclasses based on structural and functional features: Prosurvival or Antiapoptotic Family Members, Proapoptotic Family Members and BH3-Only Family Members [21, 22, 23, 24]. Normal cellular homeostasis appears to be dependent on the balance between pro- and anti-apoptotic members of BCL2 family. BCL2 gene is over-expressed in almost all types and subtypes of leukemia, indicating the importance of this molecule in disease pathogenesis and evolution [18].

The aim of this study was to explore the expression profile of several BCL2 family genes in three human tumor cell lines including AGS (gastric adenocarcinoma), 5637 (bladder tumour) and U-87MG (brain tumour) followed OCT4B1 suppression.

MATERIALS AND METHODS

Human tumor cell lines and cell culture

The human tumor cell lines; AGS (gastric adenocarcinoma), 5637 (bladder tumour) and U-87MG (brain tumour) were prepared from pasture cancer institute (IRAN) and cultured in RPMI-1640 (Gibco) medium containing 10% fetal bovine serum and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) at 37°C in a humidified incubator containing 5% CO2.

RNA extraction, cDNA synthesis and Real-time PCR

Total RNA was extracted from cultured cells (10^6 cells/ml) using TRIzol reagent (Invitrogen, UK). The contaminated DNA in the RNA samples was removed by TURBO DNase I (Fermentase, Lithuania). The RNA fidelity purity and integrity were measured by spectrophotometer (260/280 NM ratio) and gel electrophoresis (1% agarose gel). The first strand cDNA was synthesized at 42°C for 60

min using 100 pmol oligo-dT primer, 200 units of MMuLV reverse transcriptase and 1µg of total RNA according to the manufacturer's instructions (Fermentase, Lithuania). Specific primers were designed for OCT4 variants (A, B and B1), interested BCL2 family genes transcripts and ß-actin, using Gene Runner (version 3.02) and Allele ID (version 4.0) software (Table 1). Quantitative real-time PCR was performed by addition of SYBR green master mix (Qiagene, USA), 200ng of the cDNA, and 2 pgr/µl of the appropriate primer pairs (Table 2). The following cycling program was set on the BIO-RAD CFX96 instrument (Bio-Rad Company, USA): one cycle at 95°C for 15 minutes, 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds (OCT4B1, 61°C for 40 seconds), and 72°C for 30 seconds. Real-time PCR was carried out in triplicate and ß-actin was used as a housekeeping internal control for normalization of amplified target genes. The proportional amounts of PCR products were determined using the $2^{-\Delta\Delta Ct}$ formula. The dissociation stages, melting curves and quantitative analyses of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA).

All of the PCR products were electrophoresed and visualized using a 1% agarose gel containing 0.5 mg/ml ethidium bromide to visualize the size and uniqueness of amplified products.

OCT4B1 knock-down in tumor cell lines

To silencing the OCT4B1 variant, two specific siRNA were utilized based on the specific OCT4B1 sequences. One irrelevant or scrambled siRNA (with no complementary target sequence in the human genome) were transfected under the same conditions as controls. The siRNA were designed by a selection program (Whitehead Institute for Biomedical Research, htt://jura.wi.mit.edu/) and the process of synthesis was performed by MWG Company (Germany) (Table 2).

Table 1: BCL2 genes family up/down regulated after OCT4B1 suppression in 5637, AGS and U87MGtumor cell lines.

Symbols	Description of genes	Cell Lines/ Fold changes		
		AGS	**5637**	**U87M**
BAD	BCL2-associated agonist of cell death	**-15.35**	**-12.24**	**-17.56**
BAG1	BCL2-associated athanogene	**-5.84**	**-4.26**	**-2.94**
BAG3	BCL2-associated athanogene 3	**-5.48**	**-3.41**	**-9.72**
BAG4	BCL2-associated athanogene 4	**-7.46**	**-6.93**	**-12.03**
BAK1	BCL2-antagonist/killer 1	**-13.69**	**-17.88**	**-9.93**
BAX	BCL2-associated X protein	**-19.77**	**-16.93**	**-8.00**
BCL2	B-cell CLL/lymphoma 2	**-17.83**	**-15.28**	**-17.04**
BCL2A1	BCL2-related protein A1	**-6.93**	**-4.48**	**-8.42**
BCL2L1	BCL2-like 1	**-3.45**	**-7.03**	**-4.28**
BCL2L10	BCL2-like 10 (apoptosis facilitator)	**-6.89**	**-4.38**	**-5.03**
BCL2L11	BCL2-like 11 (apoptosis facilitator)	**-12.83**	**-9.84**	**-11.20**
BCL2L2	BCL2-like 2	**-6.30**	**-2.49**	**-2.66**
BCLAF1	BCL2-associated transcription factor 1	**4.40**	**3.92**	**4.01**
BID	BH3 interacting domain death agonist	**-7.12**	**-5.90**	**-8.03**
BIK	BCL2-interacting killer (apoptosis-inducing)	**-3.82**	**-5.04**	**-3.71**
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	**-7.83**	**-9.50**	**-3.62**
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	**-8.79**	**-13.71**	**-9.33**
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	**-4.73**	**-3.67**	**-6.03**
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	**-12.89**	**-14.74**	**-13.72**
HRK	Harakiri, BCL2 interacting protein	**-6.73**	**-7.30**	**-6.28**
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	**3.80**	**2.38**	**3.71**

Table 2: sequences and criteria of designed siRNAs.

siRNA name	Target	Sequences
Version I	Target	AAGGAGTATCCCTGAACCTAG
	Sense	(GGAGUAUCCCUGAACCUAG)dTdT
	Anti-sense	(CUAGGUUCAGGGAUACUCC)dTdT
Version II	Target	AAGAGGTGGTAAGCTTGGATC
	Sense	(CAGUGGUAAGCUUGGAUC)dTdT
	Anti-sense	(AAUCCAAGCUUACCACCUC)dTdT
Scramble	Sense	GCGGAGAGGCUUAGGUGUAdTdT
	Anti-sense	UACACCUAAGCCUCUCCGCdTdT

For siRNA transfection, lipofection method was used; briefly, a suspension of freshly cultured cells was prepared (1×10^5 cells/ml/well) in the RPMI1640 culture media without antibiotics and seeded in six well plates in two groups (test and control). During logarithmic growth phase (confluences 30-50%), cells were transfected with 50 nmol/ml synthetic siRNA (in the control group, scrambled sequence siRNA), using lipofectamin 2000 (Invitrogen, USA) and Opti-MEM media, according to the manufacturers' instructions. In brief, 5µl of siRNA (25µM) and 4.5 µl RNAi-MAX reagents were diluted in 250 µl Opti-MEM and incubated for 10 min at room temperature. The mixture was then added to the cells in a final volume of 2.5ml, and then the cells were incubated at 37°C in a humid atmosphere of 5% CO_2 for 72 hours.

MTT Assay

The cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on the basis of the ability of viable cells to metabolize MTT. MTT is a water-soluble tetrazolium salt which can be metabolized into a water-insoluble formazan product by mitochondrial succinate dehydrogenase in viable cells [25]. Briefly, $7x10^4$ cells/ml/well were seeded onto a 96-well plate at a final volume of 200 µL. Subsequent to cell transfection, the resultant supernatant was replaced by 200 µL of pre-warmed RPMI 1640 (without phenol red) and continued by addition of 10 µL of 5 mg/ml MTT to each well. The plate was incubated at 37°C for 3.5 h in the dark until a purple precipitate was visible under light microscopy. Finally, 100 µL of DMSO (dimethyl sulphoxidase) was added to each well and 15 min after the absorbance (at 570 nm) was read after with a reference filter of 620 nm.

Apoptosis analysis

The ratio of apoptotic cell in OCT4B1 siRNA transfected cells (test group) against the scrambled sequence siRNA transfected cell (control group) was detected 48 hours after transfection. Single cell suspension was carried out, and the Annexin V- FLOUS kit (Roch, Germany) was used for detection of the exposed phosphatidylserin on cell surfaces, as described elsewhere [26]. Then, 48 hours after transfection, cells (1×10^6) were centrifuged and resuspended in 200µl of binding buffer. Annexin V-FLOUS (1µl) and propidium iodide (1µl) were added to tubes after 5 minutes and were further incubated

for 5 minutes at room temperature (in the dark) and then analyzed by Beckman-Coulter Elite flow cytometer. Annexin V-FLOUS binding was detected using a FITC signal detector (FL1) and propidium iodide staining by the phycoerythrin emission signal detector (FL3). The ratio of FITC/PI positive cells (apoptosis) was calculated from flow histograms [27].

BCL2 gene transcripts profiling

Followed suppression of OCT4B1 variant, mRNA synthesis of twenty one transcripts in BCL2 genes family was detected. Total RNA from test and control group were extracted, cDNA synthesized and Quantitative Real Time PCR was performed as described. The following cycling program was set on a BIO-RAD CFX96 system (Bio-Rad Company, USA): one cycle at 95°C for 15 min, 40 cycles at 95°C for 30 S, 58-61°C for 30 S and 72°C for 30 S. Real-time PCR was carried out in triplicate and ß-actin was assessed as a housekeeping gene for the normalization of amplification signals of the target genes. The proportional amounts of PCR products were determined using the $2^{-\Delta\Delta Ct}$ formula. Relative expression is determined by comparison of interested gene expression in test and control group using data from the real-time cycler and the ΔΔCT method. The dissociation stages, melting curves and quantitative analyses of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA). All PCR products were visualized by electrophoresis on a 1% gel containing 0.5 mg/ml ethidium bromide to check the size of PCR products.

RESULTS

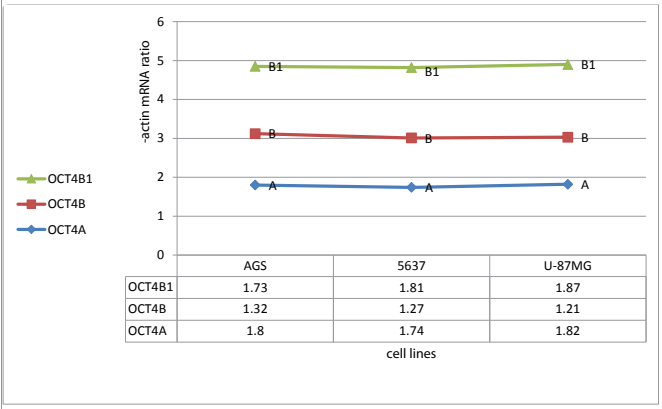
Suppression of OCT4B1variant in AGS, 5637, and U87MG cell lines

After confirming the expression of OCT4B1 in three different cell lines (Figure 1), we employed two specific siRNAs to suppress OCT4-B1 expression in the aforementioned cell lines. Compared to the cells transfected with a scrambled siRNA, as the control group, the expression of OCT4B1 was sharply suppressed at 24, 48 and 72 hours following OCT4B1: siRNA transfection. As it is shown in (Figure 2) for AGS cells, the highest decline in OCT4B1 expression occurred at 48 hours post-transfection, using OCT4B1: siRNA version 1. For that reason, the next experiments were carried out by siRNA version 1 and for 48 hours post-transfection time, in triplicate. The same observations were made for the other tested cell lines (data not shown).While the morphology of the 5637, and U87MG cell lines was not distinguishable for cells transfected with OCT4B1 vs. scrambled siRNAs, a remarkable morphological change including the presence of multinucleate giant cells was observed in AGS cells transfected with OCT4B1:siRNA (data not shown).

Elevated apoptosis and decreased viability of studied tumor cell lines treated with specific OCT4B1 siRNA

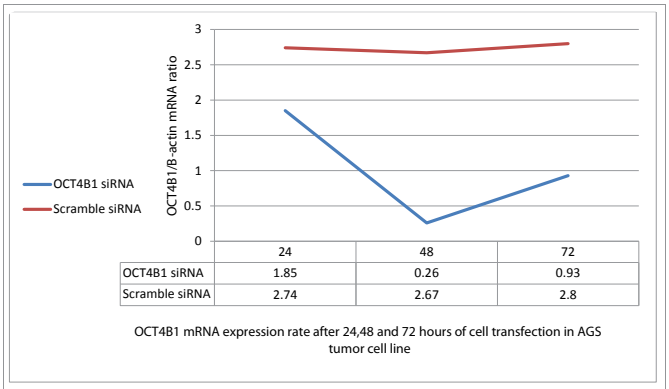
Flow cytometry analysis of the cells stained with Annexin V and PI demonstrated that two days after siRNA transfection up to 33% of the test group cells (transfected by specific OCT4B1 siRNA) underwent apoptosis, in comparision to control cells (transfected by scrambled sequence siRNA)(Figure 3).

MTT assay also indicated reduced cell activity after OCT4B1 siRNA transfection compared to cells transfected by scrambled sequence siRNA and mock transfected control cells.(Figure not shown).



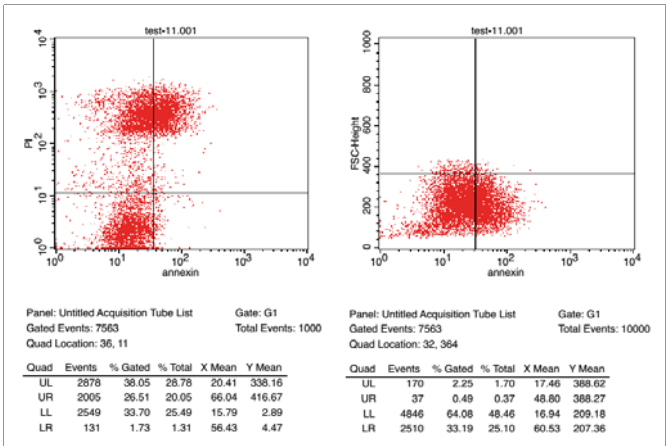
	AGS	5637	U-87MG
OCT4B1	1.73	1.81	1.87
OCT4B	1.32	1.27	1.21
OCT4A	1.8	1.74	1.82

Figure 1: Demonstrates level of OCT4 variants expression in studied tumor cell lines. Y axis shows OCT4B1 variance mRNA expression level compared to ß-actin (as housekeeping control gens) and X axis indicates three studied tumor cell lines (AGS, 5637 and U87MG).



	24	48	72
OCT4B1 siRNA	1.85	0.26	0.93
Scramble siRNA	2.74	2.67	2.8

Figure 2: Demonstrates OCT4B1 expression level after 24, 48 and 72 hours of siRNA transfection. Y axis shows OCT4B1 variance mRNA expression compared to ß-actin (housekeeping as control gene) and X axis indicates AGS tumor cell lines, Test; transfected cells with OCT4B1 siRNA and Control; transfected cells with scramble siRNA.



Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	2878	38.05	28.78	20.41	338.16
UR	2005	26.51	20.05	66.04	416.67
LL	2549	33.70	25.49	15.79	2.89
LR	131	1.73	1.31	56.43	4.47

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	170	2.25	1.70	17.46	388.62
UR	37	0.49	0.37	48.80	388.27
LL	4846	64.08	48.46	16.94	209.18
LR	2510	33.19	25.10	60.53	207.36

Figure 3: Flow cytometric analysis of OCT4B1 suppressed in AGS tumor cell line. Test; transfected cells with OCT4B1 siRNA and Control; transfected cells with scramble siRNA. The cells falling in the lower right square in scatter diagram are considered as apoptotic cells. Apoptosis analyzed by using Annexin-V-FLOS assay kit, Annexin V-FLOUS binding was detected using a FITC signal detector (FL1) and propidium iodide staining by the phycoerythrin emission signal detector (FL3).

Quantitative Real-time PCR results

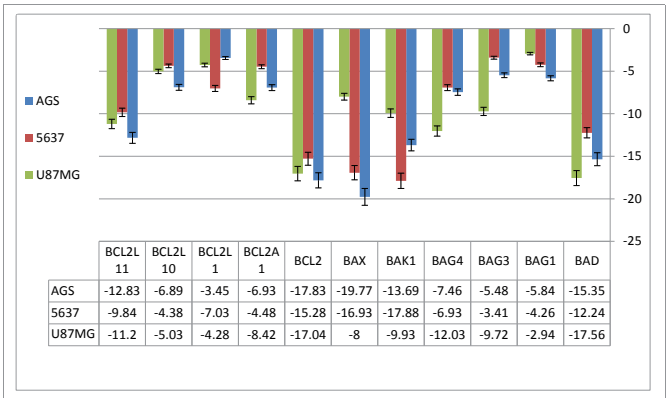
Our data revealed that all OCT4 three variants were expressed in the studied cell lines (Figure 1). Expressional profile of the studied BCL2 transcripts in three cell lines is almost similar (Table 2, Figures 4a and 4b). Nineteen of twenty one studied genes in BCL2 family showed down-regulation, from these, 14 gene were decreased in expression more than 3 and three genes (BAD,BCL2 and BNIP3L) more than 10 folds. BCLAF1 showed up-regulation (in U87MG and 5637 tumor cell lines) and MCL1 showed unchanged gene expression.

DISCUSSION

Based on anti-apoptotic effect of OCT4B1[1, 28] and "cancer stem cell" hypothesis of cancer, we aimed to explore the gene expression alteration of 21 genes of BCL2 family after suppression of OCT4B1 variant in three tumor cell lines; AGS (gastric adenocarcinoma), 5637 (bladder tumour) and U-87MG (brain tumour).

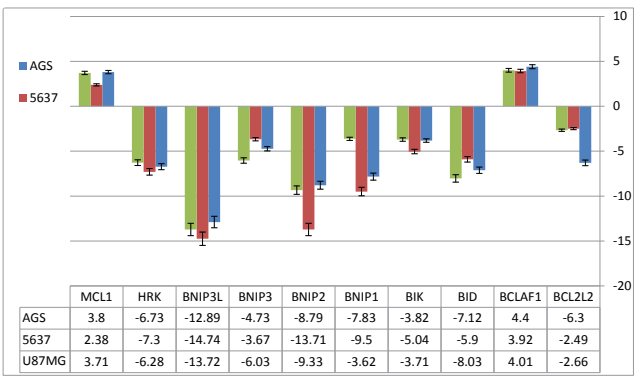
The results demonstrated that OCT4B1 variant is highly expressed in all aforementioned cell lines. Interestingly, OCT4B1 suppression generated almost similar pattern of gene expression alterations of studied genes in all three cell lines, suggesting general targets of OCT4B1 within BCL2 family, one of the critical gene families that control apoptosis pathway.

In general, progression of tumor growth is characterized by a net increase in the number of tumor cells. This could be due to increased proliferation and/or decreased apoptosis, or both. A coordination and balance between cell proliferation and apoptosis is crucial for normal development [28]. Bcl-2 gene family is the best known genes for its ability to suppress apoptosis.



	BCL2L11	BCL2L10	BCL2L1	BCL2A1	BCL2	BAX	BAK1	BAG4	BAG3	BAG1	BAD
AGS	-12.83	-6.89	-3.45	-6.93	-17.83	-19.77	-13.69	-7.46	-5.48	-5.84	-15.35
5637	-9.84	-4.38	-7.03	-4.48	-15.28	-16.93	-17.88	-6.93	-3.41	-4.26	-12.24
U87MG	-11.2	-5.03	-4.28	-8.42	-17.04	-8	-9.93	-12.03	-9.72	-2.94	-17.56

Figure 4a: Demonstrates BCL2 gene expression, 48 hours after siRNA transfection in studid tumor cell lines. Y axis showed fold gene regulation and X axis showed 11 genes of BCL2 gene family.



	MCL1	HRK	BNIP3L	BNIP3	BNIP2	BNIP1	BIK	BID	BCLAF1	BCL2L2
AGS	3.8	-6.73	-12.89	-4.73	-8.79	-7.83	-3.82	-7.12	4.4	-6.3
5637	2.38	-7.3	-14.74	-3.67	-13.71	-9.5	-5.04	-5.9	3.92	-2.49
U87MG	3.71	-6.28	-13.72	-6.03	-9.33	-3.62	-3.71	-8.03	4.01	-2.66

Figure 4b: Demonstrates BCL2 gene expression, 48 hours after siRNA transfection in studid tumor cell lines. Y axis showed folds gene regulation and X axis showed 10 genes of BCL2 gene family.

As expected, OCT4B1 silencing caused a significant decrease in cell survival and a significant elevation in apoptosis rate of the transfected cells. Quantitative real-time PCR method were used to detect gene expression alteration of cells transfected with OCT4B1 siRNA compared same cell lines transfected by irrelevant (scramble) siRNA and results showed more than 90% (19 out of 21) of studied genes were decreased in expression (down-regulation). The highest alteration take place in BCL2, BCL2L11, BAD, BAX and BNIP3L genes with down-regulation more than 10 folds (Table 2).

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