Original Article

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The Correlation between OCT4B1 and Apoptosis in Human Cancer Cell Lines

M.R. Mirzaei (PhD)*[1], G.H. Hasanshahi (PhD)[1], M.R. Hajizadeh (PhD)[1], M. Mahmoodi (PhD)[1]
M. Nazari (BSc)[2], M. Chahardoli (BSc)[2]

1.Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, I.R.Iran.
2.Department of Biochemistry, Biophysics, and Genetics, Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, I.R.Iran

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Abstract

Background & Objectives: OCT4B1, as one of the variants of OCT4 gene, is expressed at higher levels in cancer tissues and cancer cell lines, compared to other OCT4 variants. Recent studies have revealed the anti-apoptotic role of OCT4B1. The aim of this study was to evaluate the effect of OCT4B1 suppression on the expression profile of anti-apoptotic genes in three human tumor cell lines.

Methods: In this in-vitro study, three human tumor cell lines including AGS (stomach adenocarcinoma), 5637 (bladder cancer), and U87MG (brain tumor) were purchased from the National Cell Bank of Iran (Pasteur Institute) and cultured in test and control groups. In order to suppress OCT4B1 expression, the cultures were transfected, using siRNA technology and lipofection method. After confirming the suppression process, total RNA was extracted and cDNA was synthesized. Finally, the expression rates of anti-apoptotic genes were determined, using specific primers and real-time PCR technique.

Findings: Our data revealed an almost similar pattern of alteration in the expression profile of anti-apoptotic genes in all three cell lines. Also, BCL2, BRAF, and BFAR genes exhibited the most significant down-regulation by 20.87, 18.33, and 15.11 folds, respectively. The expression of at least 20 genes (out of 26 genes) decreased, while the expression rates of CASP2, IGF1R, TNF, and MCL1 were up-regulated or remained unchanged. Also, the expression of CFLAR gene was up-regulated in U87MG and down-regulated in other cell lines (5637 and AGS).

Conclusion: Based on our findings, OCT4B1 suppression by blocking the expression of anti-apoptotic genes may result in the induction of apoptosis in cancer cell lines.

Keywords: Anti-apoptotic Gene Family, Cancer Cell Lines, OCT4B1.

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Introduction

Many theories have been proposed to explain the origin of cancer. The most recent and popular theory is known as “cancer stem cells”, according to which adult stem cells (which are present in almost all body

* **Corresponding Author; M.R. Mirzaie (PhD)**
Address: Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, I.R.Iran.
Tel: +98 23 915234003-5
E-mail: mirzaeemr@gmail.com

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tissues, with a ratio of 1:100,000) or somatic cells (which turn into reprogrammed somatic cells via genetic mutations) are the origin of cancer developement (1). According to this theory, changes in gene expression pattern are the main factor for the transformation of cells into cancer cells. Given the similarities between stem cells and cancer cells, important genes, which control the "immortalization" or "stemness" of the cells, should be taken into acount. Octamer-binding transcription factor 4 (OCT4), NANOG, SOX2, KLF4, and nucleostemin,which are mainly activated in stem cells (embryonic, cancer, and tissue cells) are the most important of these genes (2). OCT4, which belongs to the POU transcription factor family (containing a DNA-binding domain), is expressed in stem cells and can encode at least three variants (OCT4A, OCT4B, and OCT4B1) via alternative splicing (3). The expression of this gene can maintain cell immortality, and its down-regulation results in cell differentiation (4).

In studies of cancer cell lines and tumor tissues, a new variant of OCT4, called OCT4B1, was identified (5). Unlike the other two variants, this variant is expressed to a greater extent in cancer cell lines and tumor tissues. Further research also showed that this variant is of an anti-apoptotic nature (6,7). The transformation of a normal cell into a cancer cell with the ability to create a cancerous tissue requires numerous cellular and molecular changes, which induce apoptotic escape. Therefore, the suppression of apoptotic process is a phenomenon that can be observed in many cancers (8). In fact, apoptotic mechanism is a very complex and sophisticated process that involves an energy-dependent cascade of molecular events, which facilitate programmed cell death by the activation of caspase proteins and apoptosome formation.

Today, researchers have described two major apoptotic pathways: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway. However, there is evidence that these two pathways are interconnected and that molecules in one pathway can influence those in the other pathway (9). Genes involved in apoptosis are classified as twelve families including: NF-ligand family, tumor necrosis factor receptor (TNFR) family, Bcl-2 family, Caspase family, Inhibitor of Apoptosis (IAP) family, tumor necrosis factor receptor-associated factor (TRAF) family, CARD family, death domain family, death effector domain family, CIDE domain family, p53 family, anti-apoptotic genes, and DNA damage response family (9-14). The anti-apoptotic gene family is perhaps the most important of all. As several studies have indicated, OCT4B1 is expressed at higher levels in cancer cell lines and cancer tissues, compared to most other OCT4 variants.

This variant, due to an unknown reason, has attained immortality, which prevents its entry into apoptosis. The aim of this study was to investigate the role of OCT4B1 in the expression pattern of anti-apoptotic gene family. For this purpose, the expression of this variant in three cancer cell lines (AGS, U87MG, and 5637) was inhibited using siRNA technology, and the inhibitory impact of this variant on the expression of 26 genes in the anti-apoptotic family was inquired.

Methods

In this experimental study, three cancer cell lines including AGS (gastric adenocarcinoma), 5637 (bladder cancer), and U87MG (brain tumor) were examined as representatives of cancer cell lines.

Cell culture and the expression pattern of OCT4 variants: The selected cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute) and were cultured in 1640 RPMI medium, containing 10% bovine serum (FBS), L-glutamine (2mM), penicillin (100u/ml), and streptomycin (100µg/ml) in a CO_2 incubator at 37°C, with 90% humidity and 5% carbon dioxide.

Before transfection, the expression rates of OCT4 variants in the cell lines were evaluated. Thus, following cell culture procedures, the cells were dispatched from the medium to obtain a density of about 70%. After the cells were washed with phosphate-buffered saline (PBS), total RNA was

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isolated by cell destruction (using TRIzol); RNA precipitates were isolated by alcohol, according to kit instructions. DNA was dispatched using TURBO DNase method. The accuracy of the refined RNA was inspected via light absorption methods by spectrophotometer and electrophoresis on 1% agarose gel. Immediately after RNA extraction, all mRNA molecules were transcribed to cDNA, using cDNA synthesis kit (Invitrogen) and transferred to a freezer (-20°C). The expression rates of OCT4 variants were calculated, using specific primers for each variant (table 1) and real-time PCR method. Afterwards, 200ng of cDNA and 2pg/mc of each primer were added to master mix reaction solution (SYBR Green, Pars Toos, Iran), with a final volume of 20ml. The reaction conditions were as follows: One cycle at 95°C for 15 min, 45 cycles at 95°C for 30 seconds, one cycle at 60°C for 30 seconds (for OCT4B1 at 61°C for 20 seconds), and one cycle at 72°C for 30 seconds. The reaction was repeated three times, and beta-actin was used as an internal control agent. The expression of each variant was measured, using 2-ΔΔCT formula.

OCT4B1-siRNA design and transfection: To supress OCT4B1 variant, two siRNA versions against OCT4B1 (exon 2b) were used. Additionally, a non-specific siRNA (scrambled siRNA), deemed to act as a control agent with no similar sequences in human genome, was used (table 2). For siRNA modeling, siRNA design software was utilized (available at htt://jura.wi.mit.edu/) and the desired sequences were generated by Takapouzist Company (representative of Germany's MWG) (6). For the transfection procedure, the nominated cell lines used in both case and control groups were cultured in 25 ml flasks in similar conditions, with a density of 10^5 cells per ml; then, after reaching a density of 30-50%, the cells were transfected. Five ml of siRNA (25mM) and 4.5 ml of RNAimax solution were diluted in 250 ml of Opti-MEM medium, stored at room temperature for 10 minutes.

Table 1. The sequence of primers in OCT4 and β-action gene variants

Gene Symbol	Gene bank number	Designed Oligo	Relative sequence	Fragment length
OCT4A	NM-002701	F	CGCAAGCCCTCATTTCAC	111
		R	CATCACCTCCACCACCTG	
OCT4B	NM-203289	F	CAGGGAATGGGTGAATGAC	177
		R	AGGCAGAAGACTTGTAAGAAC	
OCT4B1	EU518650	F	GGTTCTATTTGGTGGGTTCC	128
		R	TTCTCCCTCTCCCTACTCCTC	
ß-actin	NM-001101	F	AGGCACCAGGGCGTGAT	184
		R	GCCCACATAGGAATCCTTCTGAC	

Table 2. The sequence and specifications of siRNA, used for the suppression of OCT4B1 and scrambled siRNA for the control group

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
Scrambled	Sense	GCGGAGAGGCUUAGGUGUAdTdT
	Anti-sense	UACACCUAAGCCUCUCCGCdTdT

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The final compound was added to cell culture flasks with a final volume of 2.5ml; the flasks were incubated for 72 hours. To determine the suppression of genes at 24, 48 and 72 h intervals, the cells were evaluated in the two groups by isolating the total cell RNA and synthesizing cDNA. Then, OCT4B1 expression rate in both test and control groups was evaluated using real-time PCR and specific primers. In order to evaluate the occurrence of apoptosis in the transformed cells, the incidence of apoptosis in the studied cell lines was determined, using Annexin-v-FLUOS kit. In a nutshell, 48 hours after transfection, 200ml of cultured medium containing the studied cells (control and test groups) was transferred to the eppendorf tube; after centrifugation, the cell pellet was dissolved in the binding buffer. After 5 minutes, 1 ml of annexin and 1ml of Propidium Iodide (PI) were added to each tube and incubated for 5 min in the dark; finally, the apoptotic cells were analyzed by flow cytometry (Beakman Counter). The degree of apoptosis was determined as the percentage of FITC/PI positive cells.

To determine the expression of anti-apoptotic gene family, 48 hours after transfection, the total RNA of control and test groups (associated with the cell lines) was purified and cDNA was synthesized. By using real-time PCR and specific primers (table 1), the expression rates of the mentioned cells were determined. Gene expression analysis was performed by real-time PCR (CFX96 manager software Bio-Rad, USA), Micorsoft Excel, and SPSS version 18. Additionally, Repeated Measures Analysis of Variance (ANOVA) and Duncan's test were performed. $p \leq 0.05$ was considered statistically significant.

Results

The results showed that prior to celluar transfection, all three variants of OCT4 (OCT4A, OCT4B, and OCT4B1) were expressed in the studied cell lines; in almost all three cell lines, the OCT4B1 expression was dominant (fig 1). In order to suppress the OCT4B1 expression, we utilized two siRNA versions, which were designed and synthesized based on the specific sequence of this variant (exon 2b). The results showed that both versions were capable of suppressing the expression of this variant, while the efficacy of version II was slightly greater than version I. Therefore, the second version of siRNA was used for continuing the research. The highest rate of down-regulation in this variant occurred 48 hours after transfection (fig 2).

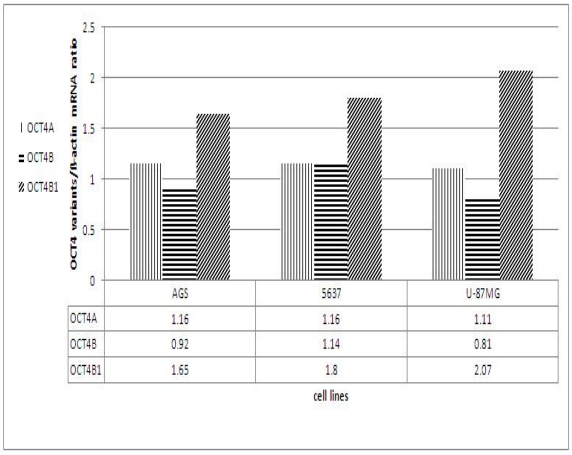

Figure 1. The expression of OCT4 variants in three studied cell lines

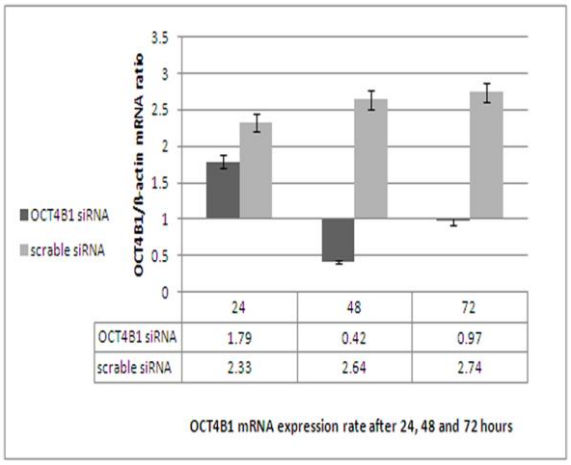

Figure 2. Changes in the expression of OCT4B1 after celluar transfection (24, 48, and 72 h intervals) in the AGS cell line

The study of apoptosis in the cell lines: Flow-cytometric analysis of cell lines showed that 48 hours after transfection, approximately 30% of cells in the test group (transfected with OCT4B1 siRNA) entered

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the apoptosis, compared to the control group (transfected with scrambled siRNA).

The expression results of anti-apoptotic gene famil: The pattern of changes in gene expression was similar in all three cell lines in a way that 20 and 4 genes showed signs of double down-regulation and up-regulation in all three cell lines, respectively. Only two genes including BIRC4 (XIAP) and CFLAR exhibited no significant change in the expression pattern. Considering the cell lines, the only gene with a different expression pattern was CFLAR, which manifested a trifle overexpression in U87MG cell line (a 1.07-fold increase) and a slight down-regulation in the other cell lines (AGS and 5637) (1.23- and 1.2-fold reduction, respectively).

Among genes with down-regulation, seven genes including BCL2, BFAR, BIRC2, BIRC6, BIRC8, BNIP2, and BRAF were down-regulated by more than five folds (maximum of 20.87 times) in all three cell lines (table 3).

Table 3. Characteristics and the level of expression in the anti-apoptotic family, following the suppression of OCT4B1 variants in the three studied cell lines

Symbols	Description of genes	Cell line/fold changes		
		AGS	5637	U87MG
AKT1	V-akt murine thymoma viral oncogene homolog 1	-3.16	-1.35	-1.42
BAG1	BCL2-associated athanogene	-4.26	-3.89	-3.27
BAG3	BCL2-associated athanogene 3	-4.96	-3.89	-8.16
BCL2	B-cell CLL/lymphoma 2	-20.87	-13.01	-20.26
BCL2A1	BCL2-related protein A1	-4.43	-2.54	-5.01
BCL2L1	BCL2-like 1	-4.78	-3.17	-1.84
BCL2L10	BCL2-like 10 (apoptosis facilitator)	-2.33	-2.22	-3.46
BCL2L2	BCL2-like 2	-4.91	-3.95	-4.17
BFAR	Bifunctional apoptosis regulator	-15.11	-10.02	-12.86
NAIP	NLR family, apoptosis inhibitory protein	-5.58	-2.81	-3.55
BIRC2	Baculoviral IAP repeat-containing 2	-8.88	-5.13	-6.22
BIRC3	Baculoviral IAP repeat-containing 3	-5.8	-9.81	-3.42
XIAP	X-linked inhibitor of apoptosis	1.37	1.62	1.65
BIRC6	Baculoviral IAP repeat-containing 6	-7.04	-5.87	-8.35
BIRC8	Baculoviral IAP repeat-containing 8	-6.76	-5.3	-7.91
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	-5.86	-8.28	-3.55
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	-5.52	-10.35	-8
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-6.39	-4.3	-4.24
BRAF	Murine sarcoma viral (v-raf) oncogene homolog B1	-18.33	-10.73	-12.23
CASP2	Caspase 2, apoptosis-related cysteine peptidase	5.28	3.3	3.51
CFLAR	CASP8 and FADD-like apoptosis regulator	-1.23	-1.2	1.07
FAS	FAS (TNF receptor superfamily, member 6)	-2.61	-2.19	-1.29
IGF1R	Insulin-like growth factor 1 receptor	3.14	1.88	7.3
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	1.47	2.77	5.55
TNF	Tumor necrosis factor	6.03	8.37	3.38
CD27	CD27 molecule	-11.74	-3.63	-4.29

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Discussion

The obtained results showed that OCT4B1 suppression in the studied cell lines leads to a decreased expression of anti-apoptotic gene family. The changes in gene expression were similar in all three cell lines. Overall, 20 out of 26 genes showed down-regulation, among which 14 genes including BAG3, BCL2, BCL2A1, BFAR, NAIP, BIRC2, BIRC3, BIRC6, BIRC8, BNIP1, BNIP2, BNIP3, BRAF, and CD27 had a five-fold down-regulation in at least one of the three studied cell lines. These genes actes as negative controllers of apoptotic pathway, and their increased expression was the reason behind the inhibition of apoptosis (7,15-17).

BCL2 protein family is the most important group of genes involved in the apoptotic process. These proteins, which have been identified in more than 20 different types, all share one Bcl-2 homology (BH) domain (15). In fact, BCL2 gene, known as the main factor for the activation of intrinsic apoptotic pathway, operates by releasing cytochrome C from the outer mitochondrial membrane.

Several studies have shown that in cancer cell lines and tissues, the expression of BCL2 family proteins increases, which might be the reason why these cells do not enter the apoptotic pathway. However, as the results of this study indicated, the expression of BCL2 genes including BCL2, BCL2A1, BCL2L1, BCL2L10, and BCL2L2 reduced 1.84 (BCL2L1) to 20.87 times (BCL2); this affirms the anti-apoptotic nature of this variant. In other words, OCT4B1 variant is one of the target and effective genes in the expression of BCL2 gene family. This variant is expressed more frequently in cancer cells, resulting in the up-regulation of BCL2 genes and decreased incidence of apoptotis.

BAG proteins, known mainly as co-chaperon proteins, are characterized by a great similarity to heat shock protein 70 (18). Wang and colleagues showed that these proteins exist in a variety of cancer cell lines and tissues, bearing an anti-apoptotic effect (19). In this study, the expression rates of two genes, belonging to BAG1 and BAG3 groups, were evaluated in different cell lines. As it was shown, the expression of these genes was down-regulated in the three examined cell lines. Moreover, Chua et al. showed that an increased expression of BFAR induces anti-apoptotic activities. This protein is an endoplasmic-reticulum-associated protein and plays an anti-apoptotic role in both intrinsic and extrinsic pathways (20). In the present study, the expression of this gene reduced more than 10 times in all three cancer cell lines after OCT4B1 suppression.

BIRCs are a group of important IAPs, with a common domain protected by baculovirus. Through this domain, these proteins are linked to target domain proteins (caspase proteins) and inhibit apoptosis (21). Moreover, NAIP (NLR family) is a protein which belongs to the same group as BIRC and is also known as BIRC1. This protein prevents apoptosis by inhibiting caspase-3, caspase-7, and caspase-9 (22). Watihayati and colleagues showed that NAIP deletion is significantly associated with spinal muscular atrophy, which is an autoimmune disorder interrupting appropriate apoptosis (23). NAIP is mainly expressed in nervous cells, although it has been recently detected in other body tissues, cancer cell lines, and cancer tissues (24).

BIRCs studied in this research included BIRC1 (NAIP), BIRC2, BIRC3, BIRC4, BIRC6, and BIRC8, which exhibited a down-regulation by 10 times, with the exception of BIRC4 (X-linked inhibitor of apoptosis), which showed a similar pattern to that of the control group. Three of the studied BNIP genes including BNIP1, BNIP2, and BNIP3, which form a novel group of proteins similar to BCL2,… Also, BRAF and 27CD showed a down-regulation, following OCT4B1 suppression. As mentioned earlier, of 26 studied genes, four genes (TNF, MCL1, IGF1R, and CASP2) showed an unexpected up-regulation. This could be either related to the unknown and often complex relationship between genes and apoptotic pathway or differences in the performance of these genes in different cells. Therefore, further research is required on cancer cell lines, cancer tissues, and expression of gene families involved in apoptotic

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pathways. In this study, the expression pattern of these genes revealed that OCT4B1 is an important anti-apoptotic factor. In other words, the inhibited expression of this variant results in the down-regulation of anti-apoptotic genes, which are often inhibitors of apoptosis. Therefore, in cancer cell lines and tissues, in which OCT4B1 expression is more significant compared to other OCT4 variants, the increased expression of reference genes (anti-apoptosis gene family) results in the inhibition of apoptosis. It can be concluded that OCT4B1 may be an important factor for helping cells skip programmed cell death. Therefore, this variant should be considered in future studies for understanding the molecular mechanisms of cancer development and the design of cancer treatment methods.

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