

More Related Gene Pathways to Vincristine-Induced Death Events in a Human T-Acute Lymphoblastic Leukemia Cell Line

Azam Rashidbaghan¹, Ali Mostafaie*¹, Yaghoub Yazdani², Kamran Mansouri³

Abstract

Background: Acute lymphoblastic leukemia (ALL) is common in children but rare in adults. Vincristine (VCR) is one of the drugs used at the beginning of treatment. Some genes are resistant to VCR in B-ALL.

Methods: Here, we examined the effect of VCR on gene expression changes in a T-ALL cell line, Jurkat. The MTT method was used to determine the IC₅₀ in Jurkat cells treated with different concentrations of VCR for 48 and 72 hours. Total RNA was isolated from the cells and cDNA was prepared. The Human Cancer Drug Target PCR Array kit was used to evaluate the 84 gene expression changes in Jurkat cells. Protein-protein interaction was analyzed by STRING software.

Results: We identified 66 differentially expressed genes as comparison to untreated cells. The response to VCR-induced apoptotic events was remarkable in the pathways of heat shock protein, topoisomerases, protein kinases, cathepsins and cell cycle. In other pathways, there were resistant genes as well as sensitive genes to VCR treatment. Some proteins like HSP90AA1 and ESR1 had determining associations with other proteins.

Conclusions: The results suggest VCR target genes in T-ALL cells may be beneficial biomarkers for ALL treatment and can be used to select appropriate synergistic drugs for VCR.

Keywords: ALL, Gene expression profile, Jurkat, PCR array, Vincristine.

Introduction

ALL is a malignancy of lymphoid cells and is common in children with an incidence of 80% (1). ALL is rare but fatal in adults (2). New progress for the treatment of ALL has increased survival in children (3). However, the success of these therapies in adults decreases in older ages (2). VCR, corticosteroids and anthracycline are the primary drugs used in chemotherapy of ALL by induction therapy for complete healing of the disease (1). Some patients with ALL (mainly B-ALL) exhibit resistance to anti-leukemic drugs such as VCR (4) and also *in vitro* resistance to VCR and other drugs has been shown as lethal concentration (LC₅₀) in leukemic cells isolated from children diagnosed with B lineage ALL (5). The genetic

basis of resistance to chemotherapy with prednisolone (PRE), VCR, asparaginase (ASP), or daunorubicin (DNR) was determined in other studies (4, 5). In another study, signal transduction genes related to drug resistance were identified in children with ALL as *ex vivo*. The drugs included PRE, VCR, ASP, etoposide (VP16), fludarabine (FLU) (6).

VCR is a vinca alkaloid isolated from leaves of *Catharanthus roseus* (7) and used for the treatment of various cancers (8). VCR influences cells by inhibiting cell division and establishment of microtubules and results in mitotic arrest (9). VCR can induce apoptosis in some leukemic cell lines (7) such as acute T-lymphoblastic CCRF-CEM.f2, pre-B ALL-697

1: Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran.

2: Stem Cell Research Center, Golestan University of Medical Sciences, Gorgan, Iran.

3: Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran.

*Corresponding author: Ali Mostafaie; Tel: +98 9188302044; E-mail: ali_mostafaie@kums.ac.ir.

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and Jurkat. Previous studies showed VCR induced apoptosis by extrinsic and intrinsic pathways in Jurkat cells (10). The most studies investigate the gene profile in VCR-resistant B-ALL cells (4-6). A few studies have explored gene resistant to drugs in T-ALL cells. In a study, gene expression of cells isolated from 7 patients with T-ALL was analyzed. These patients received a four-drug combination of VCR, dexamethasone (DEX), ASP and DNR (3). In another study by Beesley *et al.* sensitive and resistance pathways to VCR and other anti-leukemia drugs were determined using microarray data from T-ALL cell lines derived from pediatric ALL bone marrow specimens. A gene model was made based on resistant genes and used for 3 patients in primary cohort. The patients were treated with 10 drugs and. The evaluation was indicated that T-ALL patients to be sensitive to 6-mercaptopurine. The aim of this study was finding the genes and pathways associated with resistance (11). However, there is not a study that especially evaluates gene expression of pathways associated with cancer drug targets in T-ALL cell line or patients treated with VCR. The aim of this study was to determine a gene expression profile of the T-ALL cell line in response to VCR treatment. So, we evaluated gene expression changes of human cancer drug targets in the T-ALL Jurkat cells to identifying relevant pathways to VCR treatment.

Materials and Methods

Cell culture

The human T-lymphoblastic leukemia cell line (Jurkat, cell line number: C121) was purchased from the cell bank of the Pasteur Institute (Tehran, Iran). The cell line identified in DNA Typing laboratory of the Pasteur Institute (by STR analysis method). Also, the cells were tested for microbial and fungal contaminations, especially mycoplasma contamination, in Microbial Quality Control laboratory of the Pasteur Institute. Cells cultured in RPMI-1640 medium (Gibco, USA, 11875093) with supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA, A25904DG) and penicillin-

streptomycin (Gibco, USA, 15140122) and incubated at 37 °C in a 5% CO₂ atmosphere with 95% humidity. When the cells were obtained from cell bank, first, were cultured and passaged for one month. After that, the cells were harvested in tubes containing DMSO and frozen and maintained in the nitrogen tank. These cells were cultured and passaged again to perform experiments after about one year.

MTT assay

Cell growth inhibition was measured based on the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT) assay. 6×10^4 cells per well of Jurkat were seeded into a 96-well plate and treated with different concentrations of VCR (0.05, 0.1, 0.5 and 1 µg/ml). After 48- and 72-hours incubation at 37 °C, 20 µl of MTT solution (5 mg/ml in PBS) (Sigma, USA, A101161) was added into each well and cells were incubated at 37 °C for 4 hours. After adding DMSO to wells to dissolve the formazan crystals, plates were read using a plate reader at 570 nm against 630 nm. The experiment was performed three times. The 50% inhibitory concentration (IC₅₀) was determined. Cell growth (%) was calculated as following (12):

$$\text{Cell growth (\%)} = \frac{A_{490\text{nm}}(\text{sample})}{A_{490\text{nm}}(\text{control})} \times 100$$

Isolation of total RNA and cDNA preparation

Jurkat cells (3.5×10^5 cells/ml) were treated with vincristine (in a concentration based on IC₅₀) for 72 h. Total RNA was extracted from the harvested cells using RNX-Plus (Sinaclon, Tehran, Iran, RN7713C). The quality and quantity of RNA were estimated by the Picodrop machine (Picodrop Microliter, USA). Then, RNA treated with RNase-free DNase I, for removing genomic DNA, was converted to cDNA using First Strand cDNA Synthesis kit (Parstous, Iran, A101161; Thermo Fisher Scientific, USA, EN0521) according to manufacturer's instruction.

Gene expression analysis by PCR array

The Human cancer drug target RT² Profiler™ PCR Array (Qiagen, USA, PAHS-507Z) was used to screen 84 gene expression changes of 17 pathways. Real-time PCR was performed according to the manufacturer's instruction. The diluted cDNA was mixed with RT2 SYBR Green/ROX PCR master mix (Qiagen, USA, PAHS-507Z) and RNase-free water with a final volume of 2700 µl. Subsequently, 25 µl of the reaction mixture was placed in to each well of a 96 well plate and qRT-PCR was carried out on ABI 7300 machine (Applied Biosystems, USA) by a three -step cycling program including one cycle in 95 °C for 10 min, 40 cycles in 95 °C for 15 s and, finally, 60 °C for 1 min. Each experiment was carried out in duplicate (n= 2). The $\Delta\Delta C_t$ method was used for PCR array data analysis. The analysis was performed using the software in the web portal of Qiagene company (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) The data normalized

with five housekeeping genes including *B2M*, *HPRT1*, *RPL13A*, *GAPDH*, *ACTB*.

Protein-interaction analysis

We model a protein interaction network to analyze the association between human cancer drug target genes induced with VCR in Jurkat cells. STRING 11.0 software was used to construct protein-protein interaction map of genes.

Statistical analysis

The data are presented as mean±standard error mean (SEM). Graph of MTT assay test was processed with GraphPad prism 6.0.

Results**Cell growth assay**

The proliferation percent of Jurkat cells treated with different doses of VCR was examined during 48 and 72 h using MTS assay. Table 1 shows IC₅₀ values VCR in Jurkat cells.

Table 1. IC₅₀ of VCR in Jurkat cells treated for 48 and 72 h.

Time	IC ₅₀ (µg/ml) 95% Confidence Interval
48 h	0.54 (0.2493 to 1.176)
72 h	0.17 (0.1086 to 0.2640)

Gene expression changes

PCR array analysis was performed to identify molecular targets that are affected by VCR in Jurkat cells. Human cancer drug target array results showed that 66 genes of 84 genes had expression levels higher or less than 1.5 in Jurkat cells treated with VCR (0.5 µg/ml) in

comparison to untreated cells, 50 genes were down-regulated, and 16 genes were up-regulated (Table 2). In the scatter plot, the central line indicates unchanged gene expression, blue and yellow dots were down-regulated and up-regulated genes, respectively (Fig. 1).

Table 2. The results of the PCR array for Jurkat cells treated with VCR. Values less and more than 1.5 show gene down-regulation and up-regulation as compared to non-treated cells (control group).

UniGene ID	Gene Description	Gene symbol	Fold regulation
Apoptosis			
Hs.150749	B-cell CLL/lymphoma 2	BCL2	-
Hs.744872	Baculoviral IAP repeat-containing 5	BIRC5	1.95
PI-3 Kinases & Phosphatases			
Hs.338207	Mechanistic target of rapamycin (serine/threonine kinase)	MTOR	2.49
Hs.736397	Phosphoinositide-3-kinase, class 2, alpha polypeptide	PIK3C2A	-2.78

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Hs.656958	Phosphoinositide-3-kinase, class 3	PIK3C3	-5.35
Hs.553498	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	PIK3CA	-9.09
Growth Factors & Receptors			
Hs.488293	Epidermal growth factor receptor	EGFR	-1.77
Hs.446352	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	1.90
Hs.118681	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	-4.25
Hs.390729	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	ERBB4	-2.13
Hs.11392	C-fos induced growth factor (vascular endothelial growth factor D)	FIGF	-3.43
Hs.594454	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT1	-4.92
Hs.646917	Fms-related tyrosine kinase 4	FLT4	2.45
Hs.160562	Insulin-like growth factor 1 (somatomedin C)	IGF1	1.57
Hs.643120	Insulin-like growth factor 1 receptor	IGF1R	-2.48
Hs.700350	Insulin-like growth factor 2 (somatomedin A)	IGF2	2.91
Hs.479756	Kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	-4.00
Hs.479754	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	-5.17
Hs.74615	Platelet-derived growth factor receptor, alpha polypeptide	PDGFRA	-
Hs.509067	Platelet-derived growth factor receptor, beta polypeptide	PDGFRB	-
Drug Metabolism			
Hs.391464	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	ABCC1	-1.66
Hs.523836	Glutathione S-transferase pi 1	GSTP1	-1.88
Hs.196384	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	-
Hs.435136	Thioredoxin	TXN	-
Hs.690011	Thioredoxin reductase 1	TXNRD1	-5.95
G Protein Signaling			
Hs.247077	Ras homolog gene family, member A	RHOA	-2.13
Hs.502876	Ras homolog gene family, member B	RHOB	8.06
Hormone Receptors			
Hs.744830	Estrogen receptor 1	ESR1	2.09
Hs.660607	Estrogen receptor 2 (ER beta)	ESR2	-
Hs.32405	Progesterone receptor	PGR	-10.68
Heat Shock Proteins			
Hs.525600	Heat shock protein 90kDa alpha (cytosolic), class A member 1	HSP90AA1	-2.69
Hs.192374	Heat shock protein 90kDa beta (Grp94), member 1	HSP90B1	-2.95
Receptor Tyrosine Kinase Signaling			
Hs.525622	V-akt murine thymoma viral oncogene homolog 1	AKT1	-
Hs.631535	V-akt murine thymoma viral oncogene homolog 2	AKT2	-
Hs.444356	Growth factor receptor-bound protein 2	GRB2	-4.68

Cathepsins			
Hs.520898	Cathepsin B	CTSB	-
Hs.654447	Cathepsin D	CTSD	-1.88
Hs.731507	Cathepsin L1	CTSL	-3.51
Hs.181301	Cathepsin S	CTSS	-4.75
Cell cycle			
Hs.732435	Cyclin-dependent kinase 1	CDK1	-2.49
Hs.19192	Cyclin-dependent kinase 2	CDK2	-1.77
Hs.95577	Cyclin-dependent kinase 4	CDK4	-
Hs.647078	Cyclin-dependent kinase 5	CDK5	-1.90
Hs.184298	Cyclin-dependent kinase 7	CDK7	-
Hs.382306	Cyclin-dependent kinase 8	CDK8	-6.60
Hs.150423	Cyclin-dependent kinase 9	CDK9	-3.18
Hs.437705	Cell division cycle 25 homolog A (S. pombe)	CDC25A	-
Hs.484551	Mdm2 p53 binding protein homolog (mouse)	MDM2	-7.60
Hs.715871	Mdm4 p53 binding protein homolog (mouse)	MDM4	-10.43
Hs.492203	Telomerase reverse transcriptase	TERT	1.81
Topoisomerases, Type II			
Hs.156346	Topoisomerase (DNA) II alpha 170kDa	TOP2A	-3.56
Hs.475733	Topoisomerase (DNA) II beta 180kDa	TOP2B	-5.96
Transcription Factors			
Hs.631535	V-akt murine thymoma viral oncogene homolog 2	ATF2	-7.34
Hs.597216	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF1A	-8.01
Hs.521181	Interferon regulatory factor 5	IRF5	-2.37
Hs.618430	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	-
Hs.437460	Tumor protein p53	TP53	-
Protein Kinases			
Hs.250822	Aurora kinase A	AURKA	-6.81
Hs.442658	Aurora kinase B	AURKB	-3.03
Hs.98338	Aurora kinase C	AURKC	-3.60
Hs.592049	Polo-like kinase 1	PLK1	-1.56
Hs.398157	Polo-like kinase 2	PLK2	-8.26
Hs.632415	Polo-like kinase 3	PLK3	-1.96
Hs.172052	Polo-like kinase 4	PLK4	-3.51
Hs.531704	Protein kinase C, alpha	PRKCA	1.75
Hs.460355	Protein kinase C, beta	PRKCB	1.75
Hs.155342	Protein kinase C, delta	PRKCD	-2.01
Hs.580351	Protein kinase C, epsilon	PRKCE	-3.35
RAS Signaling			
Hs.37003	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	HRAS	-
Hs.505033	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	KRAS	-2.18

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Hs.486502	Neuroblastoma RAS viral (v-ras) oncogene homolog	NRAS	-3.05
Histone Deacetylases			
Hs.88556	Histone deacetylase 1	HDAC1	-
Hs.744132	Histone deacetylase 11	HDAC11	-
Hs.3352	Histone deacetylase 2	HDAC2	-9.53
Hs.519632	Histone deacetylase 3	HDAC3	-2.23
Hs.20516	Histone deacetylase 4	HDAC4	1.71
Hs.6764	Histone deacetylase 6	HDAC6	-
Hs.200063	Histone deacetylase 7	HDAC7	-
Hs.310536	Histone deacetylase 8	HDAC8	1.62
Poly ADP-Ribose Polymerases			
Hs.177766	Poly (ADP-ribose) polymerase 1	PARP1	-1.50
Hs.409412	Poly (ADP-ribose) polymerase 2	PARP2	1.50
Hs.744855	Poly (ADP-ribose) polymerase family, member 4	PARP4	-8.14
Hs.370267	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase	TNKS	-2.67
Structural protein			
Hs.158336	Netrin 3	NTN3	12.35

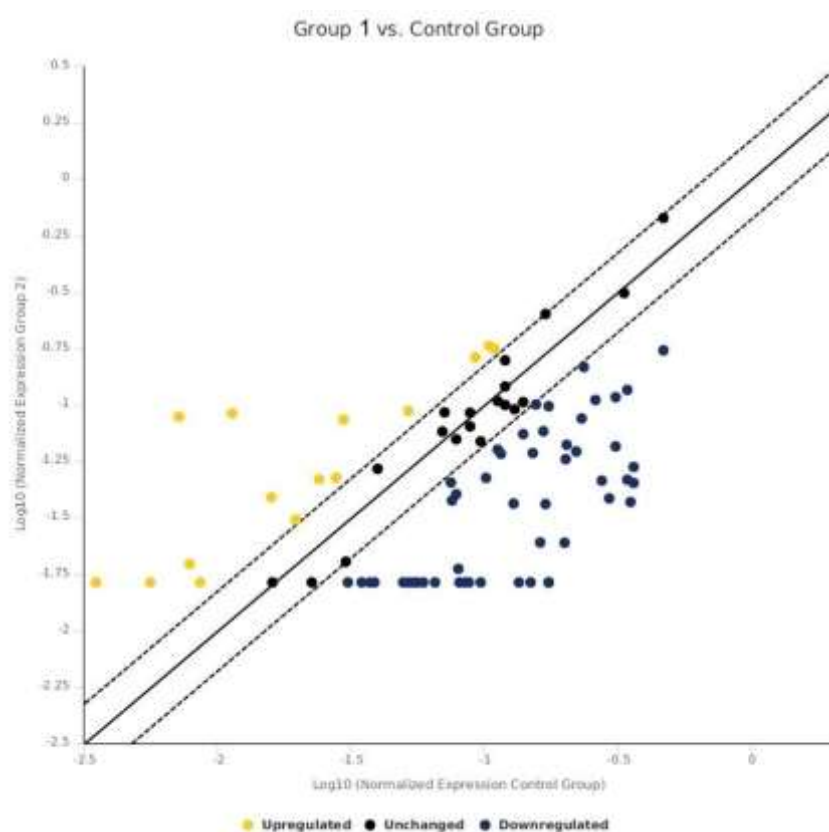


Fig. 1. Effect of VCR on gene expression profiles of Jurkat cells. Eighty-four human cancer drug target genes were evaluated in Jurkat cells treated by VCR in a concentration of 0.5 $\mu\text{g/ml}$. Scatter plot diagram compares the gene expression changes of cells treated by VCR to control cells (untreated cells). The yellow and blue dots show up-regulated and down-regulated genes, respectively and the black dots are un-changed genes.

Discussion

The literature review demonstrates the reports about gene pathways and mechanism of resistance to VCR in ALL cells of patients. However, most of them are B-lineage cells or a combination of B- and T- cells (4-6). Then, the effect of VCR on gene profile of T-ALL cells has not been specifically evaluated until now. Here, we investigated human cancer drug target gene expression changes in Jurkat cells treated by VCR. The mRNA level of 84 genes in seventeen pathways was compared between treated cells and untreated cells (negative control). The pathways included apoptosis, PI3-kinase and phosphatase, growth factors and receptors, drug metabolism, G protein signaling, hormone receptors, heat shock proteins, receptor tyrosine kinase signaling, cathepsins, cell cycle, topoisomerase type II, transcription factors, protein kinases, RAS signaling, histone deacetylases, poly ADP-ribose polymerase, structural protein. The gene expression consideration revealed that VCR induced cell cycle inhibition by decreasing mRNA levels of CDK1, CDK2, CDK5, CDK8 and CDK9. Cyclin-dependent kinases (CDKs) are serine-threonine protein kinases with a direct role in the regulation of the mitosis cell cycle and an indirect role in meiosis, transcription, and DNA repair (13). VCR treatment, also, caused down-regulation of MDM2 and MDM4. MDM2/MDM4 complex decreases the protein level of p53 (a tumor suppressor) (14). TERT was another gene in the cell cycle pathway and was up regulated in this study. TERT is a part of telomerase that catalyzes increasing and stabilizing telomere length. The activity of telomerase results in immortalizing the cells and is critical in carcinogenesis. Previous studies showed telomerase activity was higher in children with acute leukemia in comparison to healthy persons (15). So TERT was one of evaluated genes that resisted against cytotoxic effect of VCR in Jurkat cells.

The expression of CTSD, CTSL and CTSS was decreased under the influence of VCR. The intensity of expression reduction was

higher for CTSL and CTSS genes. Cathepsins (CTS) play an important role in tumor progression. Overexpression of these proteins is correlated with cancers (16).

Apoptosis effects of VCR on Jurkat cells led to down-regulating TOP2A, TOP2B, HSP90AA1, HSP90B1 and transcription factors genes (ATF2, HIF1A). Topoisomerases are necessary for many DNA functions during normal cell growth and, also, are trigger in various physiologic and non-physiologic stresses because of their role in DNA breaking and reconnecting (17). ATF2, HIF1 and HSP90 are anti-apoptotic proteins and overexpressed in cell stress conditions (18-20). As shown in Fig. 2, both of TOP2A and TOP2B have direct interaction with CDK1. HSP90AA1 is a key protein in the gene network activated by VCR and has the most association with other proteins.

The expression level of HDAC2 and HDAC3 in treated cells was 9.53 and 2.23 times lower than untreated cells, respectively. HDAC4 and HDAC8 were up regulated, but they can reduce apoptotic effect of VCR because in their expression level changes are very minor. Histone deacetylases (HDACs) result in chromatin condensation by histone deacetylation and facilitate gene expression. Several studies have shown the anti-leukemic activity of HDAC inhibitors and the potential role of HDACs in treatment of leukemia (21). It was indicated VCR decreased gene transcription in ALL cells, so that the association between HDAC3 and ATF2 (a transcription factor) was shown in Fig. 2.

Down-regulation of the PI3- kinase and phosphatase genes (PIK3C2A, PIK3C3 and PIK3CA) and growth factors and receptor genes (EGFR, ERBB3, ERBB4, FLT1, IGF1R, KDR and KIT) was a response to apoptosis signals of VCR. The member of PIK3C group play several roles in angiogenesis, cell growth and tumor formation (through activity in mTOR signaling pathway) and cell cycle (22, 23). PIK3CA had an interaction with MDM2 in Fig. 2. Bartram et al

described that IGF1R inhibition was effective in decreasing proliferation of T-ALL cells (Jurkat and Molt-4) resistant to VCR and ASP (24). Our results confirmed IGF1R, as a growth receptor, was one of the target genes in Jurkat cells treated by VCR. KIT is a tyrosine kinase receptor and leukemia is the most cancer related to KIT gene mutation (25).

Overexpression of glutathione-s-transferase p1 (GSTP1) is related to resistance to anticancer drugs (26). Expression of ABCC1, also, increases in many human cancer cell lines with multi-drug resistance (27). Then VCR increased the sensitivity of Jurkat cells to drug effect by down-regulating GSTP1 and ABCC1 genes.

According to our results, signaling pathways and hormone receptors were affected in T-ALL cells by VCR treatment. So that, RHOA (G protein signaling), GRB2 (receptor tyrosine kinase signaling) and PGR (a hormone receptor) were downregulated. All the genes in protein kinases pathway were activated by VCR induction that the most of them enhanced cell death. AURKA, AURKB and AURKC are three members of serine/threonine kinase family and regulators of mitosis and meiosis. Overexpression of AURKA and AURKB was detected in solid tumors and ALL and acute myeloid leukemia (AML) (28). In this research, the expression level of PLK genes was decreased in Jurkat cells. PLK1 overexpressed in various cancers and high expression of PLK4 is related to many cases of metastatic cancers. PLK2 is a tumor suppressor but PLK3 has a low expression in many neoplastic tissues, and it seems PLK3 is a pro-apoptotic protein (29). High expression of PRKC was shown in a study by Lei *et al*. They demonstrated overexpression of Rack1 (a receptor of PKC) resulted in raising expression level of PKC α in resistant T-ALL cell lines (Jurkat and CCRF-CEM) to VCR (30). Down-regulation of

PRKCD and PRKCE genes had a positive effect on Jurkat cell apoptotic events in our study.

Decreased mRNA level of KRAS, PARP1 and TNKS genes were other feedbacks of apoptotic events in Jurkat cells due to treatment by VCR.

Some evaluated genes showed resistance to VCR apoptosis induction by increasing expression level, including BIRC5, MTOR, ERBB2, FLT4, IGF1, IGF2, ESR1, NRAS, PARP2. But also, decreasing mRNA level of some genes was resulted in cell survival and resistance to cell death, including TXNRD1, IRF5 and PARP4. From up-regulated genes, ESR1 is the most important resistant gene and interacts with four up-regulated genes of other pathways including TERT, HDAC4, MTOR and IGF1 which had a negative effect on VCR apoptotic process (Fig. 2). ESR1 stimulates the proliferation of cancer cells (31).

In conclusions, in most of evaluated pathways, there were genes with expression changes resulted from VCR apoptotic signals, although some genes were without changes and some genes were resistant to VCR treatment. In this study exactly VCR target genes in T-ALL cells were determined that can be used as biomarkers for ALL therapy and future studies about new anti-leukemic agents. Also, these results can be used to select appropriate drugs to synergize the effect of VCR to improve ALL treatment.

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