

## Epigenetic Modulations Induction Using DSCR1 Ectopic Expression in Breast Cancer Cells

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**Abstract:** Today, prognosis, diagnosis and treatment of cancers are progressing with non-invasive methods, including investigation and modification of the DNA methylation profile in cancer cells. One of the effective factors in regulating gene expression in mammals is DNA methylation. Methylation alterations of genes by external factors can change the expression of genes and inhibit the cancer. In the present study, we investigated the effect of Down syndrome critical region 1 gene (*DSCR1*) ectopic expression on the methylation status of the *BCL-XL*, *ITGA6*, *TCF3*, *RASSF1A*, *DOK7*, *VIM* and *CXCR4* genes in breast cancer cell lines. The effect of *DSCR1* ectopic expression on cell viability in MCF7, MDA-MB-468, MDA-MB-231 and MCF10A cell lines was evaluated using MTT assay after the cells treated by lentivirus vectors harboring *DSCR1* for 72 hours. Methylation status of *BCL-XL*, *ITGA6*, *TCF3*, *RASSF1A*, *DOK7*, *VIM* and *CXCR4* genes in breast cancer cell lines was assessed by Restriction Enzyme PCR (REP) method. Also, methylation changes of these genes in breast cancer cell lines after treatment by lentivirus vectors harboring *DSCR1* for 7 days were analyzed by REP method. To confirm the effect of *DSCR1* on methylation of genes, Real-time PCR was performed. The MTT assay results indicated that *DSCR1* ectopic expression reduced cell viability in all three human breast cancer cell lines. Our results showed that *DSCR1* ectopic expression after 6 days reversed the hypomethylation status of the *BCL-XL*, *ITGA6*, *TCF3*, *VIM* and *CXCR4* genes and hypermethylation of *RASSF1A* and *DOK7* genes. The expression levels of *BCL-XL*, *ITGA6*, *TCF3*, *VIM* and *CXCR4* mRNA significantly reduced ( $P<0.05$ ) and the expression levels of *RASSF1A* and *DOK7* mRNA significantly increased ( $P<0.05$ ). Our findings reveal for the first time the impact of *DSCR1* ectopic expression on the methylation status of breast cancer cells and identify a novel agent for epigenetic therapy.

**Keywords:** Breast cancer, methylation, *DSCR1*, REP PCR.

### 1 Introduction

Cancer is a multi-factorial disease caused by genetic and epigenetic factors include DNA methylation [Hanahan and Weinberg (2011); Ravindra, Tiwari, Sharma et al. (2009)]. Methylation of CpG sites is an epigenetic regulator of gene expression [Vaissière, Sawan and Herceg (2008)]. General changes in the level of methylation are seen as a distinct feature in tumor cells [Spruck, Rideout and Jones (1993)]. DNA hypomethylation of

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oncogenes and hypermethylation of tumor suppressor genes are associated with cancer in numerous studies [Jones and Buckley (1990)]. Today, DNA methylation has been widely considered in the treatment of cancer since epigenetic changes are more likely to be reversed than genetic changes. One of the ways to make epigenetic changes in cancer cells is gene therapy [Stoff-Khalili, Dall and Curiel (2006)].

Change in the DNA sequence or epigenetic programming of a gene may be altered gene function [Thakore, Black, Hilton et al. (2016)]. Epigenetic drugs reverse the aberrant gene expression profiles associated with various diseases such as cancer. Several epigenetic drugs targeting DNA methylation enzymes have been tested in clinical trials [Forrest and Khalil (2017)]. Gene ectopic over expression using gene therapy, gene expression profile can be altered through genetic and epigenetic changes in cancer cells. Gene therapy is the leading way to treat cancer in many investigational models. This method is based upon the recognition of tumor suppressor or suicide proteins encoding genes and transferring them into tumor cells by means of a genetic vector [Zhang, Zhi, Lu et al. (2016)]. Viruses are mostly chosen as vehicles for gene therapy because they have evolved very effective mechanisms of gene transfer and expression [Tarun, Wasila, Aby et al. (2009)]. Lentiviral vectors (LVs) can deliver large cDNAs to dividing and nondividing target cells [Minami, Miura, Aird et al. (2006)]. These vectors, such as those based on human immunodeficiency virus 1 (HIV-1), transduce a variety of cell types [Kafri, van Praag, Gage et al. (2000)].

Angiogenesis has vital role to tumor progression therefore it can be a significant target for anticancer treatment [Ring and Blair (2000)]. Vascular endothelial growth factor-A (VEGFA; also referred to as VEGF) is one of the critical factors for solid tumor growth through stimulating of angiogenesis [Miller, Trigo, Wheeler et al. (2005)]. It is suggested that the reduction occurrence in many cancer types in Down syndrome patients can be due to the high expression of some genes on chromosome 21 such as Down syndrome critical region-1 (DSCR1) encoding a protein which can suppress (VEGF)-mediated angiogenic signaling via the calcineurin pathway [Jain (2005)]. The DSCR1 (also known as RCAN1) gene consists of seven exons, alternatively transcribed or spliced of exons 1-4 produces different mRNA isoforms [Baek, Zaslavsky, Lynch et al. (2009)]. Calcineurin-NFAT signaling activates expression of RCAN1-4 isoform, in response to different stimuli such as VEGF [Davies, Ermak, Rothermel et al. (2007); Hesser, Liang, Camenisch et al. (2004); Minami, Horiuchi, Miura et al. (2004)]. The calcineurin and nuclear factor of activated T cells (NFAT) is the principal mediator of VEGF signaling in endothelial cells pathway [Davies, Ermak, Rothermel et al. (2007); Hesser, Liang, Camenisch et al. (2004); Yang, Rothermel, Vega et al. (2000)]. VEGF activation on endothelial cells cause to intracellular calcium increasing and the calcium regulated Ser/Thr phosphatase calcineurin activation. Calcineurin dephosphorylates the NFAT transcription factors family and in consequence permitting their nuclear entry and induction of DSCR1 as its endogenous inhibitor. A negative feedback loop as a result of Upregulation of DSCR1 inhibits calcineurin activity [Hesser, Liang, Camenisch et al. (2004); Minami, Horiuchi, Miura et al. (2004); Yang, Rothermel, Vega et al. (2000); Minami, Jiang, Schadler et al. (2013)].

The aim of this study was to evaluate the effect of DSCR1 ectopic expression on methylation status and expression level of some key oncogenes (such as BCL-XL, ITGA6, TCF3, VIM and CXCR4) and tumor suppressor genes (such as RASSF1A and DOK7) in MCF7, MDA-MB-231, MDA-MB-468 breast cancer cells.

The Vimentin gene encodes an intermediate filament protein is considered to have a pivotal role in epithelial-to mesenchymal transition (EMT). In breast cancer, Vimentin expression is upregulated during EMT, and is highly expressed in the prognostically poor basal-like subtype of breast tumors [Katz, Dubois-Marshall, Sims et al. (2011); Challa and Stefanovic (2011); Ivaska (2011); Chen, Han, Skoletsky et al. (2005)].

The CXCR4 chemokine together with its ligand, CXCL12, are involved in the mechanism of breast cancer metastasis. CXCL12 binding to CXCR4 initiates various downstream signaling pathways such as increase in intracellular calcium, gene transcription, chemotaxis, cell survival, and proliferation [Muller, Homey, Soto et al. (2001)].

BCL-XL is critical antiapoptotic factor that is encoded by BCL2L1 gene. Cancer cells prevent apoptosis by overexpression of anti-apoptotic proteins such as BCL-XL protein. BCL-XL induced epithelial-mesenchymal transition (EMT), cell migration and metastasis [Niture and Jaiswal (2014); Choi, Chen, Tang et al. (2016)].

TCF3 is a member of the TCF family with various isoforms and overexpression of this gene has been detected in different cancers such as breast cancer. TCF3 implicated in epithelial to mesenchymal transition, tumor aggressiveness, E-cadherin repression, pluripotency and self-renewal [Li, Cai, Wang et al. (2014); Sánchez, Liu, de Barrios et al. (2012)].

ITGA6 overexpression has been shown in cancer stem cells which have mesenchymal features (cell adhesion, migration, and invasion) and breast cancer tissue that is associated with a poor prognosis and reduced survival rates [Kacsinta, Rubenstein, Sroka et al. (2014); Hu, Zhou, Zhao et al. (2016)].

The downstream of tyrosine kinase (DOK) family of adaptor proteins consists of 7 members. DOK7 promotes trans autophosphorylation and activation of muscle specific kinase (MuSK). Hypermethylation of the DOK7 gene was identified in primary breast cancer tissues and cell lines [Heyn, Carmona, Gomez et al. (2012)].

RASSF1A is a member of the RASSF family of tumor suppressors that expressed in all epithelial cells and inactivated in breast cancer tumors by epigenetic silencing including promoter hypermethylation. RASSF1A promote apoptosis and restrict the cell cycle [van der Weyden and Adams (2007)].

In this paper we studied epigenetic modulations induction effect of DSCR1 ectopic expression in breast cancer cells for the first time to investigate this new approach as a new strategy for human cancer therapy.

## **2 Material and methods**

### **2.1 Samples**

The human breast cancer cell lines (MCF7, MDA-MB-468 and MDA-MB-231) and normal breast cell line (MCF10A) were purchased from National Center of Genetic and Biological Reserves (Tehran, Iran). Breast cancer cells were cultured using Dulbecco's

Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO). MCF10A were cultured in DMEM/F12 supplemented with FBS, EGF and insulin.

### **2.2 Construction of the lenti viruses harboring DSCR1 (LV DSCR<sup>+</sup>)**

The LV DSCR1<sup>+</sup> virus particles were constructed in transfected human embryonic kidney (HEK 293T) cells. HEK 293T cells were transfected with plox-MD2 (pmd2G) plasmid which contained G protein of the vesicular stomatitis virus (InvivoGen), pLV-HELP expression vector encoding the non-integrate packaging proteins Gag- Pol- Rev- Tat (InvivoGen), psPAX, integrated packaging plasmid (InvivoGen) and finally transfer vector EX-S0552-LV-105-B harboring DSCR1 gene (GeneCopia). The culture medium was discarded 14 hours after transfection and fresh culture medium was added to the culture flask. 24 hours after changing the medium, lenti virus particles were released into culture medium. Lenti viruses were collected and concentrated by MILLIPORE falcons to be kept in -70°C.

### **2.3 Ectopic expression of DSCR1 gene in HEK293T**

HEK 293T cells were infected with LV DSCR1<sup>+</sup>. Total RNA was extracted 24 hours after infection (RNX plus, Fermentas, EU) and cDNA synthesis was performed using cDNA synthesis kit and Oligo-(dT) primers (Fermentas, EU). Real time-PCR was performed in a 15 µl reaction volume by designed primers for DSCR1 gene (Tab. 1).

**Table 1:** Primer sequences for RE PCR and real time PCR

Gene	Size (bp)	Strand	Sequence (5'→3')	Annealing temperature
BCL-XL	191	F	CGTCCCTCACTGAAACCTTG	60
		R	ACACAGGAATTGCGAAGCTC	60
ITGA6	110	F	GAGAACAACGGGCTCATTCA	60
		R	TCCCGAGTGTCCAAGTTGA	60
TCF3	193	F	GCCTGAGTTCTGTCCAAAGTC	60
		R	CTGTGCGCTTAGTCCATGAC	60
RASSF1A	160	F	GCAAGTTCACCTGCCACTAC	60
		R	CATCCTCGCCCTTCCCATAC	60
DSCR1	140	F	AACAAGTGGAAGATGCGAC	60
		R	ATCACTCTCACATACATGGAC	60
βactin	161	F	GAGACCTTCAACACCCAGCC	60
		R	AGACGCAGGATGGCATGGG	60
DOK7	110	F	TGGAAGAGTAGGTGGCTGGT	60
		R	TTCCACAAGCACAGCTCAAC	60
VIM	197	F	CTGAAGTAACGGGACCATGC	60
		R	AGAGTGGCAGAGGACTGGAC	60

CXCR4	120	F	GGCCTCAGTGTCTCTACTG	60
		R	GGCTGCGCTCTAAGTTCAA	60

### **2.4 MTT Assay to determine effective concentrations of NDV and LV-DSCR1<sup>+</sup>**

MTT assay was performed to determine the effective concentrations for LV-DSCR1<sup>+</sup> concentrations in breast cancer and normal breast cell lines. All cell lines were seeded in four separate 96-well microtiter plates (7000 cell/well) in three repeat wells and incubated for 24 hours. LV DSCR1<sup>+</sup> were added to breast cancer cells with different amounts (0- 1- 2- 3- 4- 5- 6-7  $\mu$ l). LV DSCR1<sup>+</sup> were added to normal breast cells with different amounts (0- 1- 3- 6- 12- 24- 48  $\mu$ l) for 72 hours. Then 20  $\mu$ l of MTT solution was added to growth medium of all plates. The supernatants were carefully aspirated after 4-6 hours and 100  $\mu$ l dimethyl sulfoxide (DMSO) was added for dissolving formazan crystals. The microplate Reader (Bio-Rad, USA) was used to determine the plate's absorbance values at 580 nm. The plates were shaken gently and optical density (OD) was measured at 580 nm to determine the relative cell viability.

### **2.5 Transduction of breast cancer and normal breast cells with LV DSCR1<sup>+</sup>**

Breast cancer and normal breast cells ( $2 \times 10^5$  cells/mL) were cultured in a 25 cm<sup>2</sup> plastic culture flask in appropriate medium and 10% FBS for 24 hours. Concentrated LV DSCR1<sup>+</sup> was added to cultures and incubated for 12 hours. The viral transduction was repeated twice to obtain better DSCR1 expression in cells.

### **2.6 DNA isolation**

DNA was extracted from cell population of breast cancer and normal breast cells before and after LV DSCR1<sup>+</sup> transduction by Roche DNA extraction Kit (Roche Diagnostics, Germany). DNA concentrations are measured by Nano Drop<sup>TM</sup> spectrophotometer at a wavelength of 280/260 nm.

### **2.7 Obtain the appropriate CpG islands in genes promoter and primer design**

Gene promoter sequences obtained from a transcriptional regulatory element database (TRED). Methylated regions in the promoter sequences were determined from EMBOSS CpGplot databas. The region that the percentage of methylation was close to  $\geq 60\%$  was selected from all methylated regions in promoter. Restriction endonuclease recognition sites were studied in selected methylated position using NEBcutter analysis tool and a methylated sensitive restriction enzyme that its recognition site was located in this area was selected. Then primer sequences were designed on both sides of selected restriction enzyme recognition site. All the primers used for the analysis of methylation status were designed using primer 3. Primers used to amplify BCL-XL, ITGA6, TCF3, VIM, CXCR4, RASSF1A and DOK7 genes with REP method were listed in the Tab. 1.

### **2.8 Restriction endonuclease quantitative PCR (RE-PCR)**

Detection of hyper or hypo methylated CpG islands in the promoter region of the candidate gene including BCL-XL, RASSF1A, TCF3, VIM, CXCR4, DOK7 and ITGA6 were carried out by restriction endonuclease quantitative PCR method (RE-PCR). Isolated DNA samples were digested using methylation-sensitive restriction endonuclease enzymes (RE-enzymes) (TaKaRa, Japan) such as SacII, SmaI and NaeI. For each enzyme, methyl groups block the cleavage site and digestion was suppressed. The ratio between unmethylated and methylated promoters in different samples was analyzed using MS-PCR in the LightCycler system (Rotor-GeneQ, Qiagen). Unmethylated (hypomethylated) DNA samples were digested using RE-enzymes and PCR products were not detected. Digested methylated (hypermethylated) DNA samples were amplified by PCR and products were detected.

### **2.9 Digestion of DNA samples**

40 ng of each DNA sample was digested using specific RE-enzyme at 37°C for overnight.

### **2.10 RE-PCR**

PCR amplification was done on digested and undigested DNA samples before and after LV DSCR1<sup>+</sup> transduction that performed in a lightcycler<sup>TM</sup> system (Rotor-GeneQ, Qiagen). PCR (35 cycles of denaturation for 60 seconds at 95°C, annealing for 40 seconds at 60°C, and extension for 45 seconds at 72°C) was performed using specific primers (Tab. 1).

### **2.11 Real time PCR**

All real time PCR reactions were performed in a lightcycler<sup>TM</sup> system (Corbett Real-Time Thermal) using specific primers and SYBR Green Master mix (Bioneer, Daejeon, Korea) following these conditions: 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 60°C for 30 seconds.

Methylation index={Ct value of treated DNA}-{Ct value of untreated DNA}.

### **2.12 Statistical analysis**

Percent of promoter methylation change in cancer and normal samples were analyzed using gel analyzer software (GelAnalyzer 2010a). The Real time RT-PCR data analyzed with LinReg software which estimates the efficiency and Crossing Threshold (CT) for each reaction. The SPSS version 21.0 software (Chicago, SPSS Inc) was used for statistical analysis. Differences in promoter methylation of candidate genes between digested and undigested samples before and after LV DSCR1<sup>+</sup> transduction were analyzed by *T*-test. *p* value <0.05 was accepted as a statistically significant.

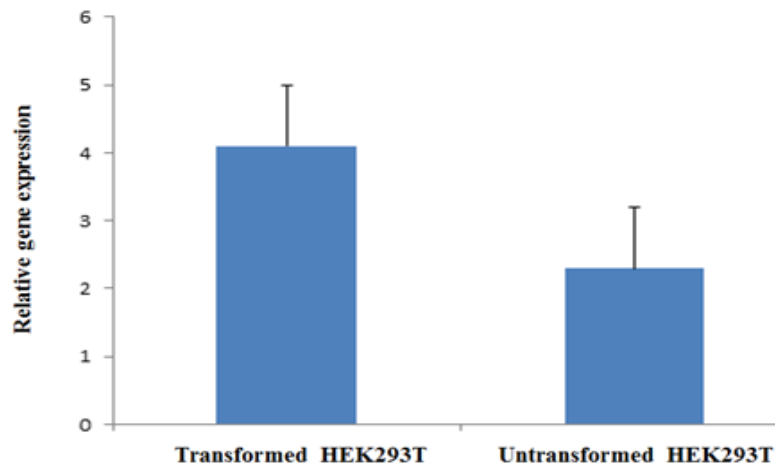
### **3 Results**

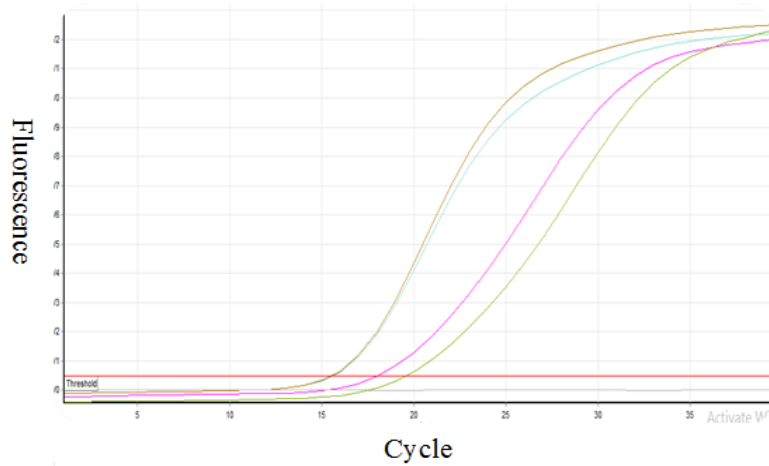
#### **3.1 Production of LV DSCR1<sup>+</sup> in HEK 293 T cells**

The actual LV DSCR1<sup>+</sup> vector was amplified and packaged in HEK 293 T cells. These cells were co-transfected by three plasmids. The sign of viral production was an early color change in the medium from red to yellow, compared to non-transfected HEK 293 T cells. DSCR1 mRNA expressions were detected by quantitative real time RT-PCR in HEK293 T cells. Expression of DSCR1 was confirmed by amplification of 140 bp fragments from HEK 293<sup>DSCR1<sup>+</sup></sup>, compared to no amplification from control HEK 293.

#### **3.2 Expression analysis of DSCR1 in HEK 293**

Virus particles LV DSCR1 was added medium of HEK293 culture. After 24 hours, total RNA was extracted and cDNA was synthesized. DSCR1 expression was quantified by real-time PCR in transformed and untransformed cultures compared to B-actin housekeeping gene expressions. Ectopic expression of DSCR1 was detected in transformed HEK293 compared to untransformed culture (Fig. 1).



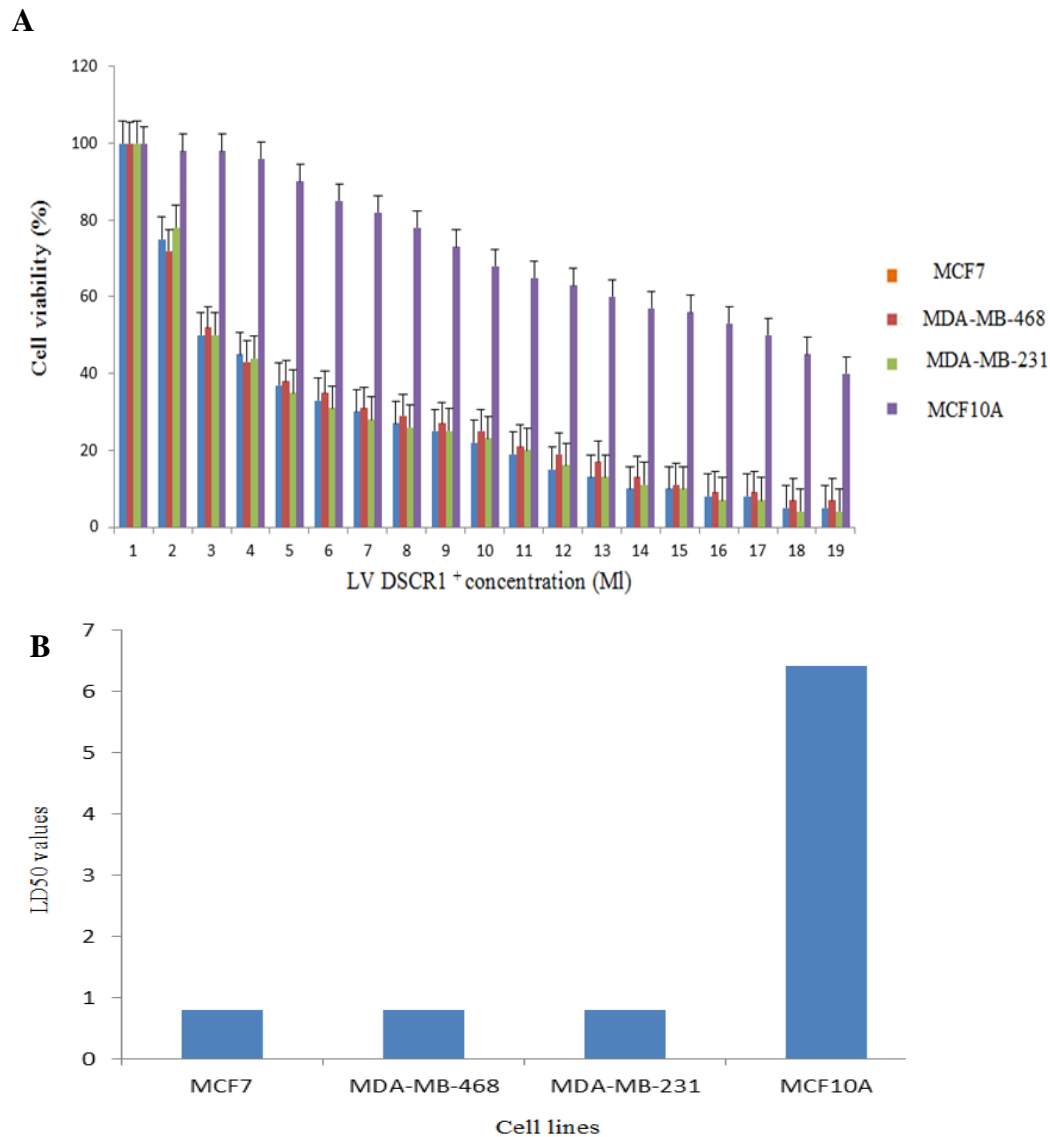


**Figure 1:** DSCR1 gene expression in transformed HEK293 T cells in comparison to the untransformed HEK293 T cells through application of the real time RT-PCR. B-Actin gene amplification was tested in transformed and untransformed HEK293 T cells as an internal real time RT-PCR positive control. Ectopic expression of DSCR1 was detected in transformed HEK293 compared to untransformed culture

### 3.3 Cytotoxicity of LV DSCR1<sup>+</sup>

The effective and lethal doses for LV DSCR1<sup>+</sup> were determined on breast cancer cell lines (MDA-MB-468, MDA-MB-231, and MCF7) and normal breast cell line (MCF10A) using MTT assay. The optimum volume of LV DSCR1<sup>+</sup> that required for killing 50% of cells (IC<sub>50</sub> value) was determined in four cell lines. As shown in Fig. 2, cells viability decreased with increasing concentrations of LV DSCR1<sup>+</sup> after 72 hours. LD50 values were 0.8  $\mu$ l/7000 breast cancer cell lines and 6.4  $\mu$ l/7000 normal breast cells lines respectively.



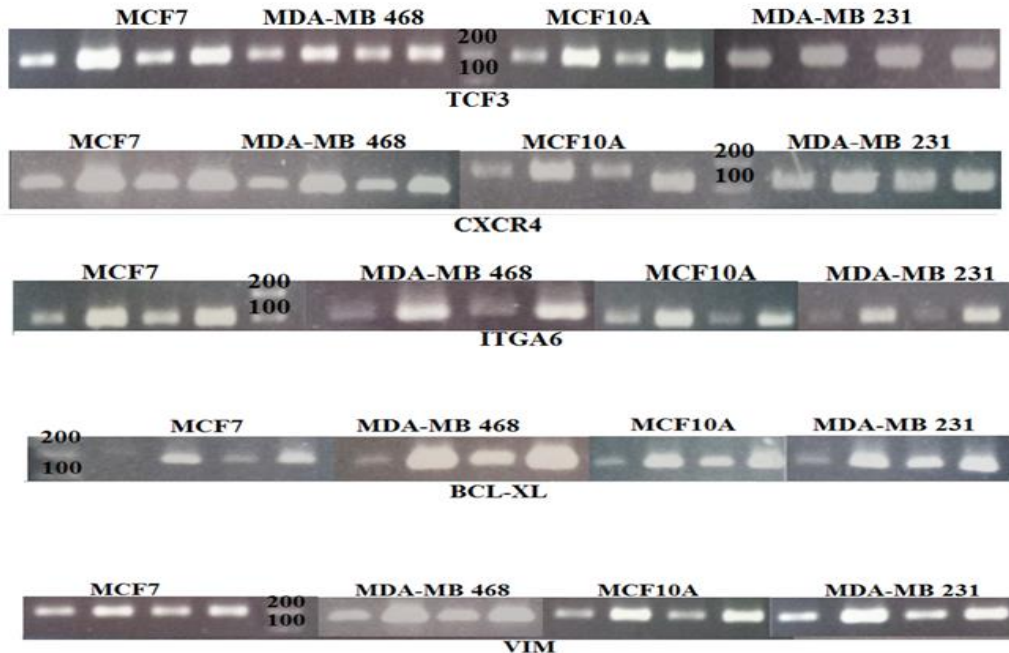


**Figure 2:** LV DSCR1<sup>+</sup> reduced cell viability in breast cancer and normal breast cells. A) Cells were treated with different volume of LV DSCR1<sup>+</sup> for 72 hours before they were subjected to a MTT assay. The viability of untreated cells was considered 100%. The experiments were performed in triplicate. Cells viability decreased with increasing concentrations of LV DSCR1<sup>+</sup> after 72 hours. B) LD50 values were 0.8 μl/7000 breast cancer cell lines and 6.4 μl/7000 normal breast cells lines respectively

### 3.4 Methylation status of the oncogenes in breast cancer and normal breast cell lines

The methylation status of oncogenes including VIM, CXCR4, BCL-XL, ITGA6 and TCF3 in breast cancer cell lines (MCF7, MDA-MB-468 and MDA-MB-231) and normal breast cell line (MCF10A) detected using REP technique (Fig. 3). The MDA-MB-231 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the VIM

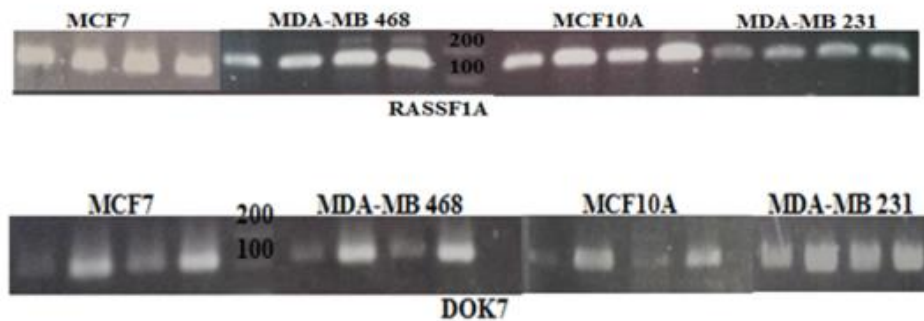
CpG islands. In contrast, MCF7 cell line had strongly methylated promoter (Fig. 3). Furthermore, CXCR4 promoter in MDA-MB-468 and MCF7 cell lines were partially methylated and in MDA-MB-231 were strongly methylated (Fig. 3). The MCF7 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the TCF3 CpG islands. In contrast, MDA-MB-231 cell line had strongly methylated promoter (Fig. 3). The MCF7, MDA-MB-468 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the BCL-XL and ITGA6 CpG islands (Fig. 3).



**Figure 3:** Methylation status of the oncogenes in breast cancer and normal breast cell lines using REP method. 1.5% agarose gel electrophoresis was used for oncogenes PCR products in breast cancer and normal breast cells. The results show that whenever digested DNA has less PCR products than incomplete DNA, the region under study is hypomethylated in the promoter of these oncogenes. The MDA-MB-231 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the VIM CpG islands. In contrast, MCF7 cell line had strongly methylated promoter. Furthermore, CXCR4 promoter in MDA-MB-468 and MCF7 cell lines were partially methylated and in MDA-MB-231 were strongly methylated. The MCF7 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the TCF3 CpG islands. In contrast, MDA-MB-231 cell line had strongly methylated promoter. The MCF7, MDA-MB-468 and MDA-MB-468 breast tumor cell lines showed a partially methylation in the BCL-XL and ITGA6 CpG islands

### 3.5 Methylation status of the tumor suppressor genes in breast cancer and normal breast cell lines

The methylation status of tumor suppressor genes including RASSF1A and DOK7 in breast cancer cell lines (MCF7, MDA-MB-468 and MDA-MB-231) and normal breast cell line (MCF10A) detected using REP technique (Fig. 4). The MDA-MB-231, MCF7 and MDA-MB-468 breast tumor cell lines had strongly methylated promoter in the RASSF1A CpG islands (Fig. 4). Furthermore, DOK7 promoter in MDA-MB-468 and MCF7 cell lines were partially methylated and in MDA-MB-231 were strongly methylated (Fig. 4).



**Figure 4:** Methylation status of the tumor suppressor genes in breast cancer and normal breast cell lines using REP method. 1.5% agarose gel electrophoresis was used for oncogenes PCR products in breast cancer and normal breast cells. The results show that whenever digested DNA has less PCR products than incomplete DNA, the region under study is hypomethylated in the promoter of these oncogenes. The MDA-MB-231, MCF7 and MDA-MB-468 breast tumor cell lines had strongly methylated promoter in the RASSF1A CpG islands. Furthermore, DOK7 promoter in MDA-MB-468 and MCF7 cell lines were partially methylated and in MDA-MB-231 were strongly methylated

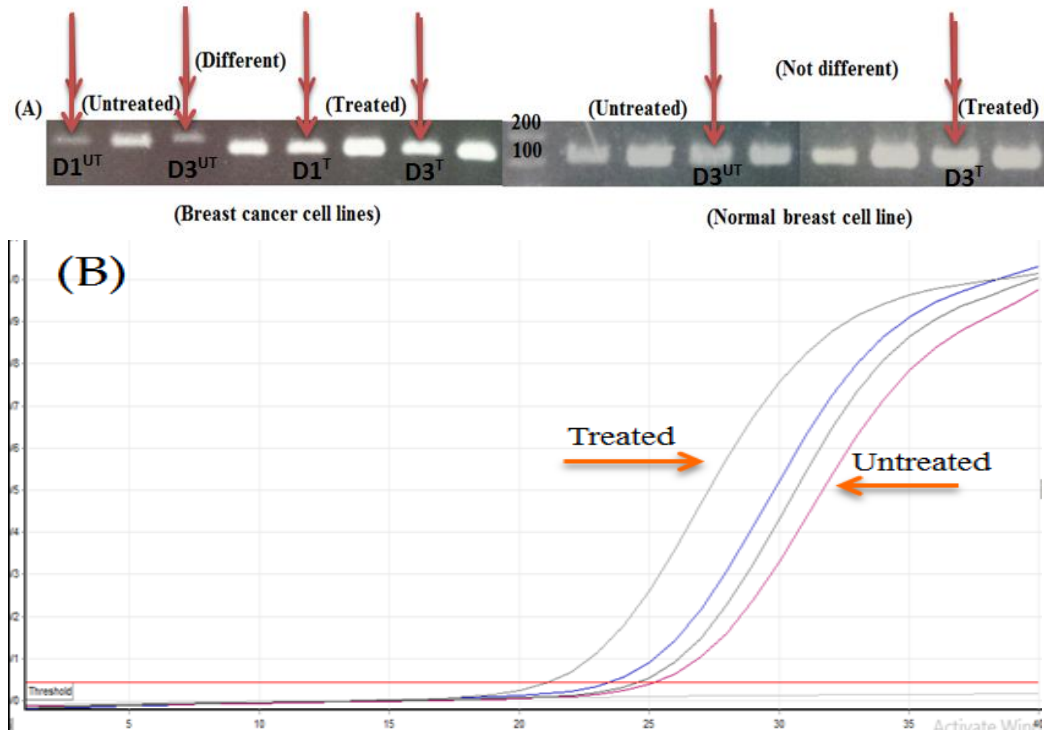
### 3.6 Hypomethylation status of oncogenes reversed in breast cancer cell lines by DSCR1 ectopic expression

0.8  $\mu$ l of LV DSCR1<sup>+</sup> per 7000 breast cancer cells and 6.4  $\mu$ l of LV DSCR1<sup>+</sup> per 7000 normal breast cells were used to induce epigenetic modulation of oncogenes and tumor suppressor genes in cells. Seven days after lenti viruse transduction, the appearance of PCR bands of oncogenes (BCL-XL, ITGA6, TCF3, VIM and CXCR4) in digested samples in breast cancer cells became more intense (Fig. 5(A)). Furthermore, the appearance of PCR bands of oncogenes in digested samples in normal breast cells did not change after lenti viruse transduction (Fig. 5(A)).

### 3.7 Real time PCR was performed to confirm the effect of DSCR1 ectopic expression on methylation of oncogenes

Relative gene expressions were evaluated by the  $\Delta$ Ct method. The gene dosage ratios ( $2^{-\Delta\Delta Ct}$ ) for oncogenes in breast cancer cell lines were calculated as 0.44 ( $P < 0.05$ ) after

DSCR1 ectopic expression and 0.23 ( $P < 0.05$ ) before lenti viruses transduction (Fig. 5(B)). The results showed that the relative amount of PCR product of oncogenes was increased after treating with LV DSCR1<sup>+</sup> after 7 days. Furthermore, DSCR ectopic expression reversed hypomethylation status of tumor suppressor genes in breast cancer cell lines after 7 days.



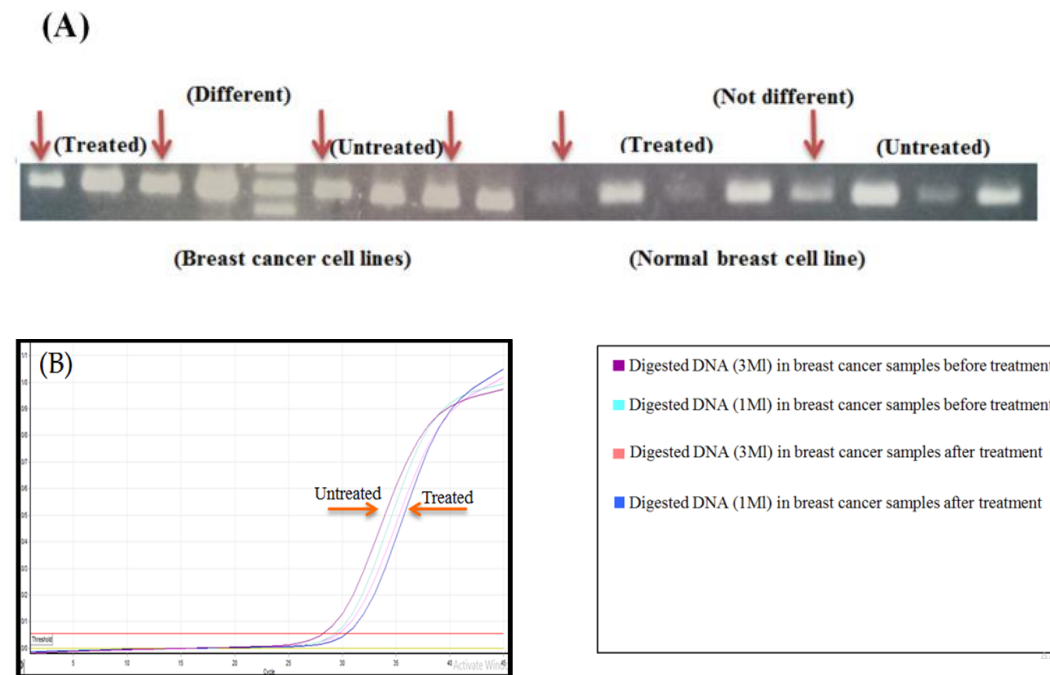
**Figure 5:** Hypomethylation status of oncogenes reversed in breast cancer cell lines by DSCR1 ectopic expression. A) 1.5% agarose gel electrophoresis was used for oncogenes PCR products in treated and untreated breast cancer and normal breast cell lines. The density of the D1<sup>UT</sup> band with D1<sup>T</sup> and D3<sup>UT</sup> is compared with D3<sup>T</sup>. Comparing the bands can be concluded that the appearance of PCR bands of oncogenes (BCL-XL, ITGA6, TCF3, VIM and CXCR4) in digested samples shown with the arrow in breast cancer cells became more intense after lenti virus transduction. The appearance of PCR bands of oncogenes in digested samples in normal breast cells did not change after lenti virus transduction. B) Based on the real time PCR results, the CT amount of treated samples are less than CT of the untreated samples. These results confirmed the effect of DSCR1 ectopic expression on reverse the hypomethylation status of oncogenes in breast cancer cells. (D1<sup>UT</sup>: PCR product of 1 microliter of un-treated DNA that digested using restriction enzyme. D3<sup>UT</sup>: PCR product of 3 microliter of un-treated DNA that digested using restriction enzyme. D1<sup>T</sup>: PCR product of 1 microliter of treated DNA that digested using restriction enzyme. D3<sup>T</sup>: PCR product of 3 microliter of treated DNA that digested using restriction enzyme)

### 3.8 Hypermethylation status of tumor suppressor genes reversed in breast cancer cell lines by DSCR1 ectopic expression

Seven days after lenti virus transduction, the appearance of PCR bands of tumor suppressor genes (RASSF1A and DOK7) in digested samples in breast cancer cells became weaker (Fig. 6(A)). Furthermore, the appearance of PCR bands of tumor suppressor genes in digested samples in normal breast cells did not change after lenti virus transduction (Fig. 6(A)).

### 3.9 Real time PCR was performed to confirm the effect of DSCR1 ectopic expression on methylation of tumor suppressor genes

The gene dosage ratios ( $2^{-\Delta\Delta C_t}$ ) for tumor suppressor genes in breast cancer cell lines were calculated as 0.22 ( $P < 0.05$ ) after DSCR1 ectopic expression and 0.39 ( $P < 0.05$ ) before lenti viruses transduction (Fig. 6(B)). The results showed that the relative amount of PCR product of tumor suppressor genes was decreased after treating with LV DSCR1<sup>+</sup> after 7 days. DSCR1 ectopic expression reversed hypermethylation status of tumor suppressor genes in breast cancer cell lines after 7 days.



**Figure 6:** Hypermethylation status of tumor suppressor genes reversed in breast cancer cell lines by DSCR1 ectopic expression. **A)** Seven days after lenti virus transduction, the appearance of PCR bands of tumor suppressor genes (RASSF1A and DOK7) in digested samples shown with the arrow in breast cancer cells became weaker. The appearance of PCR bands of tumor suppressor genes in digested samples in normal breast cells did not change after lenti virus transduction. **B)** Based on the real time PCR results, the CT amount of treated samples are more than CT of the untreated samples. These results

confirmed the effect of DSCR1 ectopic expression on reverse the hypermethylation status of tumor suppressor genes in breast cancer cells

#### **4 Discussion**

In this approach for the first time we investigated that DACR1 ectopic expression could significantly induce the methylation alterations in breast cancer cell lines. In current study LV DSCR1<sup>+</sup> were used to transduction of breast cancer and normal breast cell lines for intensify their methylation alterations induction properties. Based on our results, we detected that the MDA-MB-231 and MDA-MB-468 cell lines were partially methylated in VIM promoter whereas MCF7 cells were almost completely methylated. Furthermore, CXCR4 and TCF3 promoter in MDA-MB-468 and MCF7 cell lines was partially methylated and MDA-MB-231 was almost completely methylated. Also, the MCF7, MDA-MB-231 and MDA-MB-468 cell lines were partially methylated in BCL-XL and ITGA6 promoters. In addition, MCF7, MDA-MB-468 and MDA-MB-231 were almost completely methylated in RASSF1A and DOK7 promoters. The results showed that treatment of breast cancer cells with 0.8µl of LV DSCR1<sup>+</sup> per 7000 breast cancer cells partially reversed the hypomethylation status of the oncogenes (VIM, TCF3, BCL-XL, ITGA6 and CXCR4). In addition, treatment of breast cancer cells with 0.8 µl of LV DSCR1<sup>+</sup> per 7000 breast cancer cells partially reversed the hypermethylation status of the tumor suppressor genes (DOK7 and RASSF1A).

Hypermethylation of tumor suppressor genes as well as hypomethylation of oncogenes are properties of tumor cells [Jovanovic, Rønneberg, Tost et al. (2010)]. As DNA methylation is a reversible process, DNA methylation inhibitors are used as anticancer agents. DNA methylation inhibitors would demethylate and increase expression of tumor suppressor genes. However, these inhibitors might lead to activation of oncogenes in addition to activating tumor suppressor genes, which might result in increased metastasis [Ateeq, Unterberger, Szyf et al. (2008); Yu, Zeng, Xiong et al. (2010)]. Because of the DSCR1 ectopic expression causes hypermethylation of oncogenes BCL-XL, ITGA6, TCF3, VIM and CXCR4 and hypomethylation of tumor suppressor genes DOK7 and RASSF1A, this study prompts the idea of using DNA demethylating agents in combination with LV DSCR1<sup>+</sup> for cancer treatment.

Baek et al. showed that a transgenic copy of DSCR1 is sufficient to confer significant suppression of tumor growth in mice by dampening VEGF- calcineurin signaling [Jain (2005)]. Minami et al demonstrated that overexpression of DSCR1 inhibited lung metastases in mouse models [Yang, Rothermel, Vega et al. (2000).].

Treatment of breast cancer has been commonly performed by frequently tumor surgery, chemotherapy or radiotherapy. Some problems such as difficult surgery and chemo-resistance limit the cancer treatment. Considering the lethality, limited efficacy, and toxicities of the commonly used treatments strategies, there is large demand for the use of effective and combined therapy to suppress such a complex and noxious disease [Ravindra, Tiwari, Sharma et al. (2009)]. Gene therapy is an approach is applying for the destruction of tumor cells. In this investigation DSCR1 gene were used as a methylation changes induction factor for cancer inhibition.

Lentiviral vectors can deliver transgenes to a wide variety of dividing and nondividing cells and maintain stable long-term transgene expression [An, Wersto, Metzger et al. (2000)]. However, integration of a provirus into host chromatin has induced some adverse issues, such as insertional mutagenesis and induction of malignancy in human and mouse models [Hamaguchi, Woods, Panagopoulos et al. (2000); Li, Düllmann, Schiedlmeier et al. (2002)]. Development of lentiviral vectors to deliver transgenes has provided adequate safety and efficiency in clinical applications due to modifications in the packaging cassette that have limited the potential risk for insertional mutagenesis and replication competent of lentiviruses [Cockrell and Kafri (2007)].

Although the anti-cancer properties of DSCR1 have been proven, no studies have been done to date on the effect of DSCR1 ectopic expression on the change in the methylation status of oncogenes and tumor suppressor genes. In this study, for the first time, induction of methylation changes of oncogenes (BCL-XL, ITGA6, TCF3, VIM and CXCR4) and tumor suppressor gene (DOK7 and RASSF1A) using DSCR1 ectopic expression was studied.

## 5 Conclusion

Based on the results, we can conclude that DSCR1 ectopic expression could inhibit breast cancer through reversal of hypomethylation status of oncogenes and hypomethylation status of tumor suppressor genes. Evidence that we present in this investigation demonstrated that LV-DSCR1<sup>+</sup> can be efficient methylation alteration drug for breast cancer treatment.

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