The Role of Pomalidomide-Based Epigenetic Effect on DNMT Genes Expression in Myeloma Cell Line

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Abstract- Multiple myeloma (MM) is clonal B-cell malignancy characterized by the progressive proliferation of malignant plasma cells and accumulation of monoclonal immunoglobulin (M-spike) in blood and urine. Pomalidomide is an immunomodulatory agent which has potentially suppressed myeloma cell progression, especially in drug-resistant cases. As epigenetic modifications have an important role in gene regulation and because of the revealing role of DNA-Methyltransferase 1 (DNMT1) overexpression in myeloma pathogenesis, in this study DNMT1, 3a and 3b genes expression of U266 myeloma cell line treated with pomalidomide have been evaluated. In this study after treatment of U266 cells with 1 μM pomalidomide for 48 hours, total RNA extraction and cDNA synthesis was performed. Gene expression of DNMT1, 3a and 3b has been evaluated using real time PCR technique. The result of this study show that pomalidomide can downregulate the expression of DNMT1, 3a, and 3b in 48 hours of treatment as 0.049, 0.058 and 0.055, respectively as comparing with untreated control (P<0.05). Based on these results we conclude that pomalidomide has desired effect on epigenetic modification by downregulation of DNMTs genes expression and has been considered as an effective drug for inhibition of myeloma proliferation.

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Keywords: Multiple myeloma; U266; Pomalidomide; DNA methyltransferase

Introduction

Multiple myeloma is clonal B-cell malignant disorders, characterized by the proliferation of malignant plasma cells in bone marrow, and subsequently the accumulation of monoclonal immunoglobulin in the blood and urine (1,2). The prevalence of the disease in developed countries is estimated to be about 5 per hundred thousand people (3,4). It has been found that the interaction of myeloma malignant cells with the cells in the bone marrow microenvironment plays an important role in the pathogenesis of this disease (1). In fact, there is an interaction between myeloma malignant cells and cells of the bone marrow microenvironment including endothelial cells, mesenchymal stem cells (MSCs), osteoblasts, osteoclasts and cells involved in the immune system, through the secretion of cytokines such as IL-6, IGF-1, IL-1, TNF-α, VEGF, DKK-1, and sFRP. Also, cell-mediated interactions, as well as cell-matrix interactions, result in increasing the expression of CD138 and VLA-4 on myeloma cells and then, stimulate the expression and secretion of growth factors from the bone marrow microenvironment cells (5).

A recent study showed that tumor cells microenvironment have abnormal biology since in tumor cell the biological functions are abnormal. Bone marrow microenvironment is a complex network of the extracellular matrix includes at least four main cell lineage involved in the controlling of bone marrow cell biology named osteoclasts, cells with the mesenchymal origin, lymphoid cells, and endothelial cells. It is shown that osteoclasts and endothelial cells play an important role in supporting the uncontrolled myeloma cell proliferation. In addition to these cells, it has been shown that bone marrow mesenchymal stem cells (BMMSCs) produce high levels of IL-6, and have a prominent role in communicating with uncontrolled proliferation of myeloma cells (6). Also, it is shown that BMMSCs (bone marrow mesenchymal stem cells) have a supporting role in angiogenesis and osteoclastogenesis process (7-10). It

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has been demonstrated that cell biology of BMMSCs in myeloma patients and healthy persons are completely different. In fact, myeloma BMMSCs cells have lower expression levels of CD106 and fibronectin, and higher levels of IL-1β and hyaluronan production compared with normal controls (11). This bi-directional interaction between BMMSCs cells and myeloma cells inhibits the apoptosis rate in myeloma cells via activation of anti-apoptosis signaling pathways. In addition, it has been found that the mechanical connections may cause changes in cellular secretion of cytokines and its growth factor. It indicates that different signaling pathways involved in the interaction of myeloma cells and BMMSCs (12).

It seems that DNA hypomethylation occurred followed by genomic demethylation of repetitive elements. About 55 percent of human genome is composed of repetitive areas and play an important role in regulating gene expression and genomic stability. More than 90 percent of methylated cytosine is located in CpG islands of transposable elements such as Alu, LINE-1, and SAT-α sequences, associated with total genomic DNA methylation content. CpG island methylation is carried out by the enzymatic activity of DNMTs (DNA Methyl Transferases) includes DNMT-1, DNMT-3a, and DNMT-3b. It is known that DNMT class 3 (DNMT-3) plays a role in de novo methylation and DNMT class 1 (DNMT-1) increase methylation level in hemimethylated and fully methylated regions. In recent research, it has been found that epigenetic changes besides the genetic variations play a significant role in myeloma progression (12).

Several studies in recent years have been done with the aim of reducing the rate of tumor progression using monotherapy or multidrug approaches. The use of epigenetic drugs including HDAC (histone deacetylase) inhibitors or DNMT inhibitors plays an important role in controlling the progression of tumor cells. It seems that these drugs act by both cytotoxic function and epigenetic changes and involve the controlling of tumor cells progression. It also is effective in controlling the disease by changing the interaction of myeloma cells with bone marrow microenvironment (13-17). Several studies have been done on the use of immunomodulatory drugs including thalidomide, lenalidomide, and pomalidomide for arresting myeloma cell proliferation. The main mechanism of action of these drugs is through a significant increase in IL-2 and IL-12 receptor levels in order to activate the T-cells and phagocytic system. These drugs also play a role in increasing gene expression of the SOCS-1 gene by reducing its promoter methylation level. However, the exact molecular mechanisms of these drugs remain unclear (14-16).

In this study, we evaluated the effect of pomalidomide on DNMTs genes expression regulation in U266 myeloma cell line.

Materials and Methods

Pharmacological agent and cell culture

Pomalidomide was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and stock solution was prepared in distilled water and subsequently diluted into aqueous media for in vitro studies.

Multiple myeloma (MM) cell line U266 was kindly provided by Stem Cells Technologies, Tehran, Iran. The cell line was cultured in RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA), supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 100 IU/l penicillin and 100 mg/l streptomycin (GIBCO, Grand Island, NY, USA), at 5% CO2 and 37. U266 cells were cultured in a 24-well plate in the culture media with 1 µM concentration of pomalidomide and incubated for 48 hours.

U266 cell viability using MTT assay

U266 cells were cultured in a 96-well plate in the 100 µL culture media with three concentrations of pomalidomide (0.1, 1, and 10 µM). After 24 h, 48 h, and 72 h, 10 µL of MTT solution with a concentration of 5 mg/ml was added to each well and then incubated for 4 h at 37° C. Then, 100 µL of DMSO was added, and the absorbance was analyzed at 570 nm with a reference filter of 620 nm using ELIZA reader. Also, 20 µl MTT solutions were added to each well followed by incubation for 24, 48, and 72 h at 37° C. We selected the concentration of pomalidomide which showed a minimum reduction in cell viability.

RNA extraction, cDNA synthesis and quantification of DNMTs genes expression

Quantitative RT-PCR was performed to evaluate the level of DNMT1, 3a, and 3b gene expression. In brief, differentiated cells were collected at day 14 and immediately prepared for total RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. We used about 1×106 cells of each defined group and extracted total RNA.Then, total extracted RNA was reverse transcribed for the first strand cDNA synthesis using a Fermentas K1622 RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA,

USA). Eventually, quantitative evaluation of gene expression was performed using SYBR Green-based assay Ampliqon III (Ampliqon, Rødovre, Denmark), and an ABI step one RealTime PCR System (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). The thermocycling machine protocol was 95° C for 15 minutes to activate the polymerase enzyme followed by 40 cycles of 95° C for 15 seconds, 60° C for 30 seconds, and 72° C for 30 seconds.

As shown in Table 1, we designed three pairs of forward and reverse primers for DNMT1, 3a and 3b gene amplification, as well as one pair for reference gene (HPRT gene). The results obtained were normalized to HPRT, and relative quantity was determined using comparative CT method. The data were analyzed using the 2–ΔΔCt formula, where ΔΔCt=(Ct post-treatment - Ct pre-treatment) target gene/(Ct post-treatment–Ct pre-treatment) reference gene, as the primer efficiency was between 90-110%. Statistical analysis and graph preparation were performed using Microsoft® Excel SPSS software. The *P* less than 0.05 were considered statistically significant by t-test analysis.

Table 1. Forward and reverse primer sequences designed for gene expression analysis

Gene	Forward Primer	Reverse Primer
DNMT1	ACCTGGCTAAAGTCAAATCC	ATTCACTTCCCGGTTGTAAG
DNMT3a	CCTGTGGGAGCCTCAATGTTA	TTCTTGCAGTTTTGGCACATTC
DNMT3b	GACTCGAAGACGCACAGCTG	CTCGGTCTTTGCCGTTGTTATAG
HPRT	TGGACTAATTATGGACAGGACTGAAC	GCACACAGAGGGCTACAATGTG

Results

The effect of pomalidomide on U266 cell proliferation

U266 cell line was cultured for 24 h, 48 h, and 72 h, and exposed to increasing doses of pomalidomide (0.1, 1, and 10 µM). The resuts of MTT assay demonstrates that pomalidomide significantly reduced cell viability of U266 cells at 48 h with IC50 value 10 µM (Figure 1).

Cells were cultured with three concentration of pomalidomide ranging from 0.1 µM to 10 µM. Pomalidomide significantly suppressed proliferation of cells at 48 h with IC50 values of 10 µM. This data are presented as mean±SD. **P*<0.05.

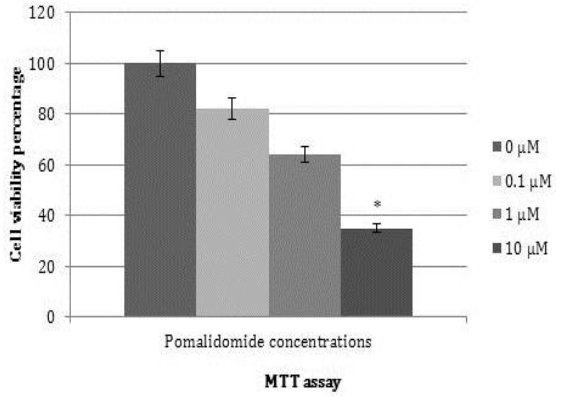

Figure 1. Pomalidomide reduces the viability of U266 cell line in 10 µM concentration

qPCR for DNMT1, 3a, and 3b genes expression level analysis

In this study, in order to assess the effect of pomalidomide on the expression of DNMTs genes, we determined the mRNA levels of three genes (DNMT1, 3a, and 3b) in defined cell culture group using quantitative real time PCR. As shown in Figure 2, U266 cells treated with pomalidomide at 48 h incubation resulted in the significantly decreasing of DNMT1 and 3a and 3b genes expression by 0.049, 0.055 and 0.058 fold, respectively, over the untreated control group (*P*<0.05).

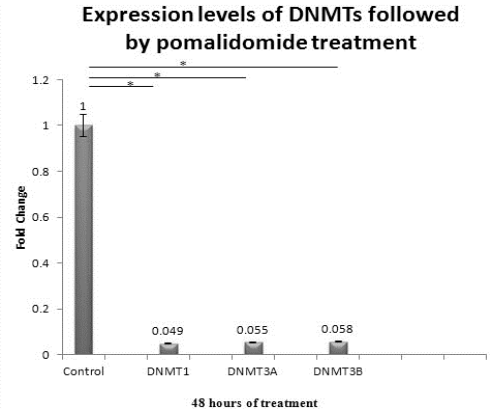

Figure 2. Decreasing gene expression level of DNMT1, DNMT3a and DNMT3b followed by pomalidomide treatment of U266 cells compared to control. The data for each sample represent the mean values of 3 independent experiments (mean±SD) **P*<0.05.

Discussion

In the present study, we evaluated the effect of pomalidomide on DNMTs gene expression profile, as these genes have an important role in *denovo* and maintenance methylation. Our results provide new insights into the efficacy of pomalidomide in decreasing mRNA levels of all three genes DNMT1, 3a and 3b compared to control (P<0.05).

Other studies also illustrated the brilliant role of DNMTs gene overexpression in the pathogenesis of myeloma. The results of Lu QY and their collogues showed that DNMT activity and expression in is higher U266 cells compared to normal control. Also, they showed that the expression and activity of DNMT declined after treatment of U266 cells with phenyl hexyl isothiocyanate (PHI), and cells were predisposed into apoptosis (18). In another study, it has been shown that knockdown of DNMT1 using siRNA in RPMI-8226 myeloma cell line can lead to significant decreasing of cell proliferation, as well as reduction of promoter methylation in tumor suppressor genes including suppressor of cytokine signaling 1 (SOCS1) and p16 (19). A very recent study introduced a novel agent RRx-001 which has nitric oxide-donating and hypoxia-selective epigenetic properties. This agent plays its role in decreasing the viability of MM-cells, either alone or in combination with pomalidomide, bortezomib, and SAHA. Also, RRx-001 have a prominent role in suppression of DNMTs genes expression (20).

Several studies have been focused on the importance of using drugs with epigenetic modification potential. Elucidation of the epigenetic modifications underlying myeloma drug resistance may obviously contribute to the promotion of cancer therapy (21). In a paper, it has been sought that arsenic trioxide (As (2) O (3)) can significantly underexpressed DNMT1, DNMT3A and DNMT3B gene expression level by a dose-dependent manner (22). Another study shows the importance of p53 promoter gene hypermethylation and subsequently declining gene expression level in myeloma progression. Also, IL6 treatment of myeloma cell line KAS6/1 results in increasing the level of DNMT1 gene transcription. Furthermore, IL6 can be establishing the methylation status of p53 promoter and diminishing the level of p53 production. Zebularine as a DNMT inhibitor reverse the methylation modification of p53 and subsequently reduce myeloma cell growth (23).

Based on our results in this paper pomalidomide have a potential effect on DMNT genes inhibition and result in changing the epigenetic modification of myeloma cells. Therefore, DNMTs may be a new therapeutic target in multiple myeloma cells and efficiently inhibit by pomalidomide.

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