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Telomerase inhibitor MST-312 induces apoptosis of multiple myeloma cells and down-regulation of anti-apoptotic, proliferative and inflammatory genes

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ABSTRACT

Aims: The telomerase-based therapy of cancer has received a great deal of attention due to the fact that it is expressed in almost all of the cancer cells while it is inactivated in most of the normal somatic cells. Current investigation was aimed to examine the effects of namely telomerase inhibitor, the MST-312, as a chemically modified derivative of epigallocatechin gallate (EGCG), on human multiple myeloma cell line U-266.

Main methods: U-266 cells were cultured and then treated by MST-312. The viability of cultured cells was measured by both trypan blue staining and MTT assay techniques. To examine the apoptosis, annexin-V/7-AAD staining using flow cytometry method was employed. To analysis the expression of Bax, Bcl-2, c-Myc, hTERT, IL-6 and TNF-α genes, the quantitative real-time PCR was employed.

Key findings: We observed the short-term dose-dependent cytotoxic and apoptotic effect of MST-312 against U-266 myeloma cells. Gene expression analysis indicated that the MST-312-based apoptosis was associated with up-regulation of pro-apoptotic gene (Bax) as well as down-regulation of anti-apoptotic (Bcl-2), proliferative (c-Myc, hTERT) and inflammatory (IL-6, TNF-α) genes.

Significance: These findings suggest that telomerase-based therapy using MST-312 may represent a novel promising strategy for treatment of multiple myeloma.

1. Introduction

The malignancy of multiple myeloma (MM) is described as an incurable disease which is caused by proliferation of malignant plasma cells within bone marrow (BM), leading to production of nonfunctional intact immunoglobulins [1,2]. Neoplastic growth of these cells leads to several complications including bone pain, fractures, anemia, infections, and etc. [3]. MM constitutes of approximately 1% of all cancers and about 10–15% of all hematological malignancies worldwide [2]. In order to clarify the exact pathogenesis of MM, more recent investigations have analyzed parameters which were involved in regulation of the growth and survival of myeloma cells [1]. Within these factors, nuclear factor-κB (NF-κB) as a transcription factor plays a key part in the processes of survival and proliferation of myeloma cells [4]. The NF-κB pathway is almost deregulated in all of human cancers and thus is able to affect the main aspects of cancer, including survival, proliferation, angiogenesis, apoptosis resistance, metastasis, and inflammation via up-regulation of the genes which are involved in these processes [5–7]. Over 150 genes are transcriptionally regulated by activated NF-κB, and most of proteins that are encoded by NF-κB-targeted genes incorporate in host immune responses [4]. NF-κB promotes the synthesis of inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) and these in turn control the growth and survival of myeloma cells [1,8]. IL-6 is secreted in both autocrine and paracrine fashions from several cell types, including bone marrow stromal cells, osteoblasts and myeloma cells in myeloma patients. IL-6 serves not only as a growth factor, but also as a survival factor in MM by inhibition of apoptosis of myeloma cells. Interacting with several factors involved in MM pathogenesis including adhesion molecules, tumor suppressor genes, and oncogenes, IL-6 is considered as a pivotal factor in MM progression [1,9]. TNF-α is also a potent pro-inflammatory cytokine which plays important roles in control of cell cycle, proliferation, apoptosis and survival signals [8,10]. It has been revealed that elevated levels of TNF-α in MM patients are correlated with aggressive disease [11].

Considering the essential role of NF-κB and its target genes in

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development and progression of MM, identification of the underlying molecular mechanisms for the constitutive NF-κB activation is highly crucial for logical development of molecular-based therapies.

Telomerase is a proficient reverse transcriptase enzyme which catalyzes the process of synthesizing of the telomeric DNA at the end of chromosomes [12]. The most specialized telomerase component is telomerase reverse transcriptase (TERT) catalytic subunit, in addition to telomerase RNA (TR) template subunit [13]. Despite cancer cells, most somatic cells undergo a finite number of divisions which is directly proportional to the length of their telomeres [14,15]. Suppression of the activity of telomerase occurs during embryonic differentiation; however, it remains active in some cell types including germ cells and stem cells that are highly proliferative. The reactivation of telomerase is observed in 80–90% of cancers, proposing that its activation serves as a critical phase in human carcinogenicity [14,16]. Several lines of evidences have highlighted the non-telomeric (non-canonical) functions for telomerase that are not dependent on its ordinary activity in telomere maintenance [12,17,18]. These activities vary from control of cell cycle pathway, regulation of gene expression [18–21], and inhibition of apoptosis [22–24], to modulation of cell signaling pathways including NF-κB [25,26]. Some other previous studies showed that telomerase is able to regulate NF-κB-dependent transcription directly. Interestingly, these data indicated that the NF-κB regulation which is mediated by telomerase is exclusive to a subset of NF-κB promoters including IL-6 and TNF-α [25,27].

Given the attention that telomerase in combination with NF-κB is hyperactivated in a broad range of cancers, targeting either of them is considered as a promising therapeutic strategy in cancers. Considering the non-canonical activity of telomerase in controlling NF-κB and its targeted genes involved in MM pathogenesis including IL-6 and TNF-α [25], MM may be regarded as a promising candidate for modeling of therapeutic interventions by telomerase inhibitors. To explore the efficiency of telomerase inhibition in MM, the myeloma cell line U-266 was treated by MST-312 as a telomerase inhibitor.

MST-312 is a chemically modified derivative of green tea epigallocatechin gallate (EGCG). In comparison with EGCG, MST-312 has some great advantages including more chemical stability, lower effective dose in induction of telomere shortening, and lower drug-resistance [28]. Even though, foregoing studies have reported that MST-312 has inhibited telomerase in cancer cells [28–30], its mode of action has yet to be fully established.

In the current study, we have hypothesized that MST-312 is able to induce the cytotoxicity and apoptosis of myeloma cells and this may enable the compound to serve as a useful strategy for treatment of MM.

2. Material and methods

2.1. Cell culture and MST-312 treatment

Myeloma U-266 cells (obtained from cell bank of Pasteur Institute, Tehran, Iran) were cultured in suspension form in RPMI 1640 medium which was supplemented by 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere contained 5% CO2 at 37 °C. All of cell culture medium components were purchased from Gibco Life Technologies, USA. MST-312 (5 mg lyophilized powder, 380.35 g/mol; Sigma-Aldrich, USA) was dissolved in 1 mL dimethyl sulfoxide (DMSO; Merck, Germany) and the concentration of 13.14 mM was obtained as a stock solution and stored at 4 °C. The working concentration of 100 μM was prepared by diluting the stock solution in complete medium. MST-312 treatment of U-266 cells was performed using concentrations of 2, 4 and 8 μM of MST-312 which were obtained by diluting the working solution in complete medium using the following formula: $M_1V_1 = M_2V_2$.

2.2. Measurement of cell viability by trypan blue exclusion assay

To investigate the effect of MST-312 on the viability of U-266 cells, trypan blue exclusion test was performed. Briefly, the cells were seeded in 12-well plates (2×10^5 cells/well) and treated with 2, 4 and 8 μM of MST-312. Following treatment and incubation for 24, 48, and 72 h, the cell suspension samples were well mixed up with 0.4% trypan blue solution (Sigma-Aldrich, USA) in 1:1 ratio. The cells were counted using a Neubauer hemocytometer under a light microscope after 1–2 min incubation at room temperature. The viable/live cells were clear and non-viable/dead cells were blue. The viability percentage was calculated using the following formula: (number of live cells / total number of counted cells) × 100.

2.3. Measurement of metabolic activity using MTT assay

The effect of different concentrations of MST-312 on U-266 cells metabolic activity was examined by the MTT colorimetric assay. Briefly, U-266 cells were initially cultured in a 96-well plate (5×10^3 cells/well) and treated with different amounts of MST-312. At 48 h time point, the medium was discarded and the cells were treated by MTT solution (5 mg/mL; Sigma-Aldrich, USA) and incubated at 37 °C for 4 h. Then, 100 μL of DMSO was added to each separate well in order to solubilize the formazan crystals. An ELISA reader was employed for measuring the absorbance at 570 nm. The percentage of metabolically active treated cells was calculated in proportion to the untreated (control) cells (considered as 100% activity).

2.4. Apoptosis assay

Apoptosis analysis was performed to investigate the effect of MST-312 on the apoptosis of U266 cells. Briefly, U-266 cells (2×10^5 cells/well) were seeded in 12-well plates and treated with MST-312. After 48 h, the cells were collected and then rinsed with PBS and stained by FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, USA), as recommended by the manufacturer. Becton–Dickinson FACS was used to quantify the percentage of apoptotic cells. The cells with Annexin V-positive and 7-AAD-negative staining were considered as cells which are entering to the early apoptotic phase while the cells with double positive staining for Annexin V and 7-AAD were regarded as late apoptotic cells.

2.5. RNA extraction and cDNA synthesis

After treatment of U-266 cells with MST-312 for 48 h, total RNA was isolated by TriPure Isolation Reagent (Roche Molecular Biochemical, USA) as recommended by the manufacturer. The quality of extracted RNA was assessed by electrophoresing on the agarose gel and its purity was evaluated by measurement of OD (A260/A280 ratio) using NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). Then, the isolated RNA was reversely transcribed using Fermentas RevertAid First Strand complementary DNA (cDNA) Synthesis kit (Thermo Fisher scientific, USA). The reaction contained 4 μL of 5× Reaction Buffer, 2 μL of dNTP Mix (10 mM), 1 μL of Random Hexamer Primer, 1 μL of RevertAid M-MuLV Reverse Transcriptase (200 U/μL), 1 μL of RiboLock RNase Inhibitor (20 U/μL), and 9 μL of nuclease-free water, in addition to 2 μL of the extracted RNA (1 μg per reaction) in a total volume of 20 μL. The following temperature/time profile was employed for reverse transcription: 25 °C for 5 min, 42 °C for 60 min, and the reaction was finally stopped by heat-inactivating at 70 °C for 5 min.

2.6. Quantitative real-time PCR

The reaction of quantitative real-time PCR was performed in a total volume of 15 μL, containing 7.5 μL of RealQ Plus 2× Master Mix Green (Ampliqon, DK), 1 μL of each of forward and reverse primers (10 pmol),

Table 1
Primer sequences used for quantitative real-time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226
Bax	AGGATCGAGCAGGGCGAATG	TCAGCTTCTTGGTGGACGCA	84
Bcl-2	ATCGCCCTGTGGATGACTGAG	CAGCCAGGAGAAATCAAACAGAGG	129
hTERT	ATGCGACAGTTCGTGGCTCA	ATCCCCTGGCACTGGACGTA	176
c-Myc	GTCCTCGGATTCTCTGCTCTC	CAACATCGATTTCTTCCTCATCTTC	140
IL-6	AGCCAGAGCTGTGCAGATGA	CTGCAGCCACTGGTTCTGTG	145
TNF-α	GCTGCACTTTGGAGTGATCGG	TGGGCTACAGGCTTGTCACT	123

1.5 μL of the cDNA product, and 4 μL of nuclease-free water. Thermal cycling conditions consist of an initial step of activation at 95 °C for 15 min which was continued by 40 cycles designated as a denaturation step at 95 °C for 15 s and an annealing/elongation step at 60 °C for 60 s. These reactions were occurred in the Rotor-Gene Q Real-time PCR System (Qiagen, USA). To verify the specificity of the analyzed genes products, a melting curve was produced. Alterations in the gene expression level (either up- or down-regulation) were measured relative to the control using the $2^{-\Delta\Delta CT}$ formula (GAPDH was employed as a house keeping gene). The sequences of used primers for the quantitative real-time PCR are presented in Table 1.

2.7. Statistical analysis

The two-tailed Student's *t*-test was employed to examine whether the differences between the studied variables were significant. The differences were statistically considered significant if the P values were < 0.05. Data were statistically analyzed by SPSS 18.0 (SPSS, Inc., USA).

3. Results

3.1. MST-312 decreased both cell viability and metabolic activity of U-266 cells

The effect of different concentrations of MST-312 on the viability of U-266 myeloma cells was examined using trypan blue dye exclusion test. The viability of U-266 cells was substantially decreased in a dose-dependent and time-dependent manner, in response to exposure to MST-312 (Fig. 1a). Over 40% reduction in the viability of treated cells was observed when cells treated with 8 μM concentration of MST-312 for 48 h. This showed that MST-312 exerts a direct short-term cytotoxic effect on U-266 cells. Cell viability was severely decreased following longer exposures with MST-312, therefore, short-term exposure of 48 h was used for further investigations. MTT colorimetric assay was used for determining the effect of MST-312 on metabolic activity of U-266 cells. As shown in Fig. 1b, the cellular metabolic activity was considerably decreased in U-266 cells following treatment with MST-312. This cytotoxic effect of MST-312 followed a dose-dependent pattern, with approximately 25, 46, and 62% reduction in metabolic activity of U-266 cells following 48 h of treatment with 2, 4, and 8 μM of MST-312, respectively. These findings suggested the short-term concentration-dependent cytotoxic effect of MST-312 against U-266 myeloma cells.

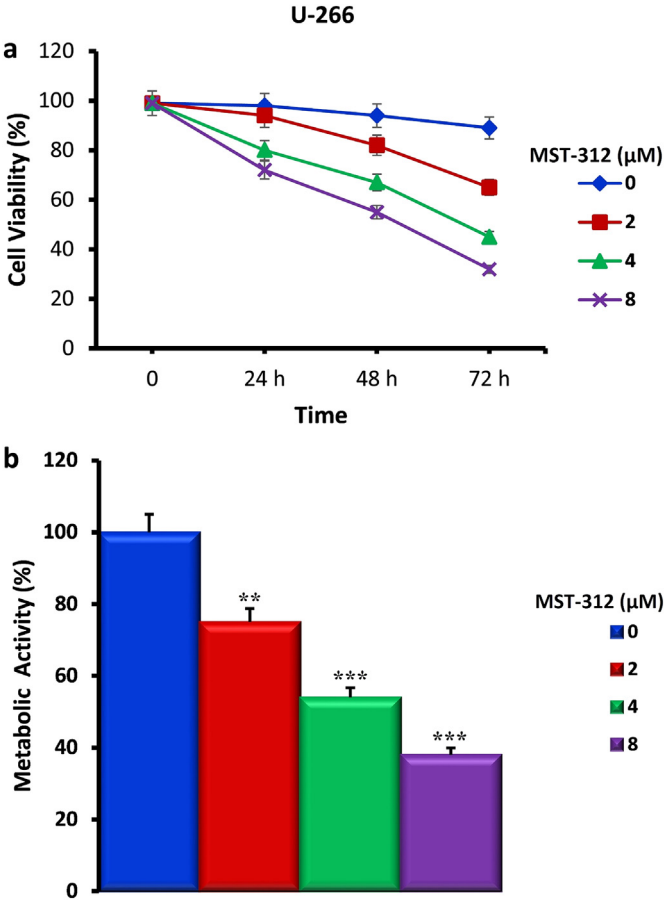

Fig. 1. MST-312 decreased viability and metabolic activity of myeloma cells. (a) U-266 cells were treated with different concentrations of MST-312, and cell viability was evaluated by trypan blue exclusion assay following various times of exposure (mean ± SE, n = 3). After short-term treatment with MST-312, the viability of U-266 cells was substantially reduced in a dose-dependent manner. (b) Metabolic activity of U-266 cells was evaluated by MTT assay after treatment with different concentrations of MST-312 for 48 h (mean ± S.E., n = 3). Following treatment with MST-312, the metabolic activity of U-266 cells was significantly decreased in a dose-dependent manner (**P < 0.01, ***P < 0.001, relative to cells untreated by MST-312).

3.2. MST-312 promoted apoptosis of U-266 cells

To evaluate the effect of MST-312 on cellular death, the apoptosis of U-266 cells was assessed using FITC Annexin V Apoptosis Detection Kit II. As presented in Fig. 2, the short-term incubation of U-266 cells in presence of MST-312 substantially increased the percentage of Annexin V and Annexin V/7-AAD double-positive cells compared to the untreated cells, exhibiting an acute apoptotic effect for MST-312 against U-266 myeloma cells. This apoptotic effect of MST-312 was dose-dependent, by which approximately 25% (total percentage of Annexin V and Annexin V/7-AAD double-positive cells) apoptosis induction was observed in U-266 cells following short-term (48 h) treatment with 2 μM concentration of the compound. This concentration was employed for further experiments.

3.3. MST-312-induced apoptosis was associated with Bax up-regulation and Bcl-2 down-regulation

To further assess the apoptotic effect of MST-312 on U-266 myeloma cells, the expression of apoptosis-related genes was evaluated in the present study. To approach this, U-266 cells were exposed to 2 μM of

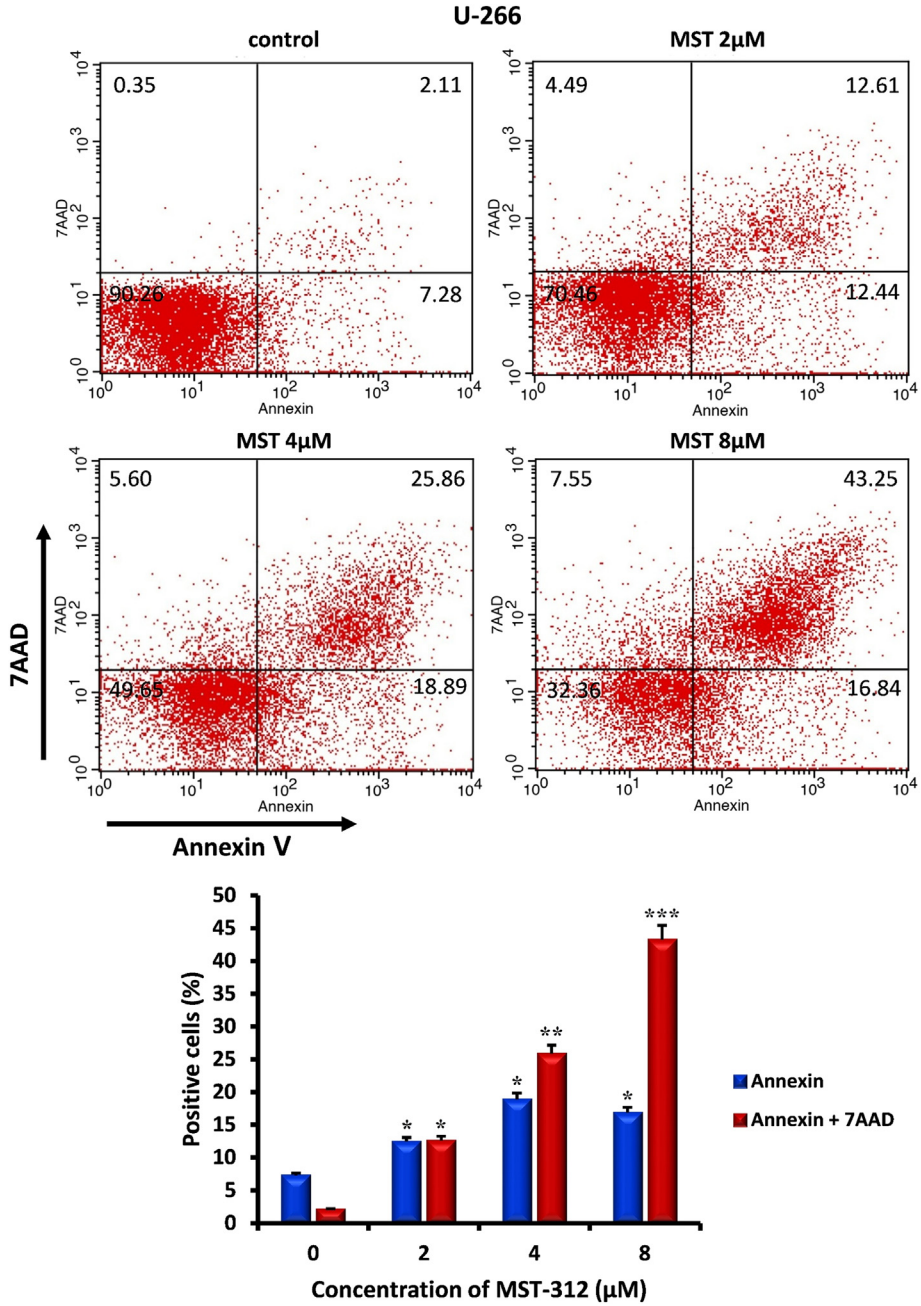

Fig. 2. Acute apoptosis was induced by MST-312 in myeloma cells. U-266 cells were treated with various concentrations of MST-312 for 48 h and subsequently assessed for Annexin V and Annexin V/7-AAD uptake using flow cytometry. One representative experiment of three independent assays is presented (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001, relative to cells untreated by MST-312).

MST-312, and harvested after 48 h of exposure. Bax and Bcl-2 gene expressions were examined by quantitative real-time PCR method. The expression of Bcl-2 as an anti-apoptotic gene was significantly down-regulated while the expression of Bax as a pro-apoptotic gene was up-regulated in U-266 cells that were treated with MST-312, compared to untreated cells (Fig. 3). These findings revealed that MST-312-induced apoptosis of U-266 cells was correlated with simultaneous down-regulation of Bcl-2 and up-regulation of Bax.

3.4. The growth inhibitory effect of MST-312 was correlated with down-regulation of c-Myc and hTERT

Regulation of telomerase mainly occurs at the level of hTERT transcription [31]. The evidences have indicated that different transcription factors exist within the promoter of hTERT that are involved in regulation of its expression [32]. Of the transcription factors, c-Myc is a potent regulator in transcription and expression of hTERT gene [33]. Furthermore, activated c-Myc contributes to the pathophysiology of multiple myeloma and its activity increases alongside with development of the disease [34,35]. To better understand the background leading molecular mechanism of the growth inhibitory effect of MST-312 on U-266 myeloma cells, we investigated the transcriptional alterations of key proliferative genes including c-Myc and hTERT. We observed a significant decrease in the expression of c-Myc and hTERT genes in response to treatment of U-266 myeloma cells with 2 μM of MST-312 for 48 h (Fig. 4). Hence, down-regulation of c-Myc and hTERT by MST-312 and the resulting reduced telomerase activity may altogether enhance the telomerase inhibitory effect induced by MST-312.

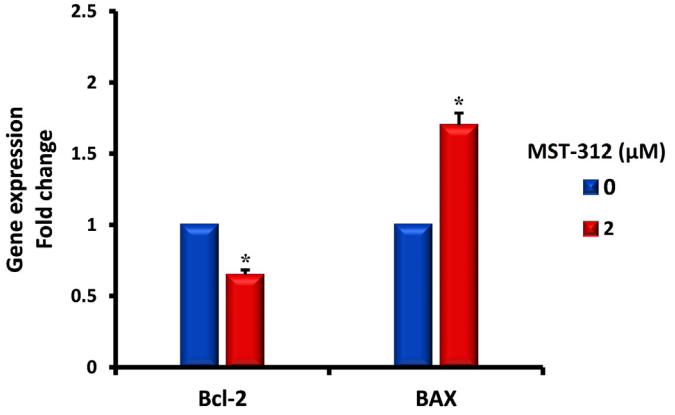

Fig. 3. MST-312 up-regulated the pro-apoptotic gene Bax and down-regulated the anti-apoptotic gene Bcl-2. U-266 cells were treated with 2 μM of MST-312 for 48 h. Then, RNA extraction was performed and cDNA was synthesized. The results of SYBR-Green real-time PCR assay using specific primers are presented. All values were normalized against GAPDH (n = 3, *P < 0.05, **P < 0.01, relative to cells untreated by MST-312).

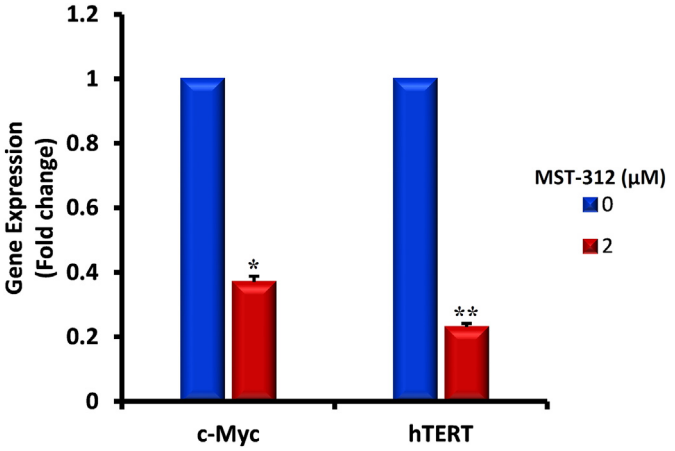

Fig. 4. MST-312 suppressed the expression of c-Myc and hTERT genes. Following treatment of U-266 cells by 2 μM of MST-312 for 48 h, RNA isolation was performed and cDNA was synthesized. The results of SYBR Green-based real-time PCR assay are presented. Normalization of All values was done against GAPDH (n = 3, *P < 0.05, **P < 0.01, relative to cells untreated by MST-312).

3.5. Expression of IL-6 and TNF-α genes was down-regulated by MST-312

Regarding the essential role of inflammatory mediators in the development and progression of MM [1,8], we therefore examined whether MST-312 has affected the expression of some inflammatory genes. For this purpose, U-266 cells were harvested after 48 h of treatment with 2 μM of MST-312, and IL-6 and TNF-α gene expressions were evaluated using quantitative real-time PCR. We observed a significant decrease in the expression of both IL-6 and TNF-α genes in U-266 cells treated with MST-312, compared to untreated cells (Fig. 5). These findings showed that the growth inhibitory effect of MST-312 in U-266 cells was associated with down-regulation of the inflammatory genes, including IL-6 and TNF-α, which are involved in myeloma pathogenesis.

4. Discussion

Lately, Great attention has been paid to telomerase-targeted treatment of cancer. This is due to the fact that telomerase is expressed in almost all of the cancer cells while it is inactivated in most of the

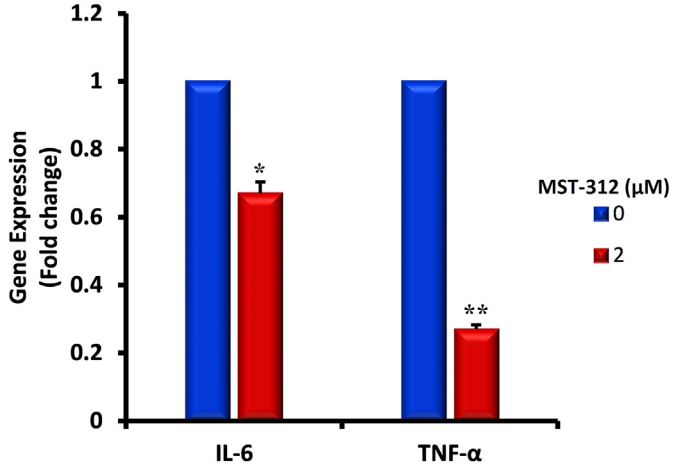

Fig. 5. MST-312 down-regulated IL-6 and TNF-α inflammatory genes. After 48 h treatment of U-266 cells with 2 μM of MST-312, total RNA was isolated and cDNA was synthesized. The results of SYBR-Green real-time PCR assay using specific primers are presented. All values were normalized against GAPDH (n = 3, *P < 0.05, **P < 0.01, relative to cells untreated by MST-312).

normal somatic cells [36]. Various telomerase inhibitors, including nucleoside, non-nucleoside components, and siRNAs, have been designed, so far [37]. MST-312 has been found to serve as a telomerase inhibitor, in addition to being a valuable candidate for telomerase-based cancer therapy [28]. As reported in previous studies, MST-312 possesses two various effects depending on the exposure time. Short-term (72 h) MST-312 treatment leads to an acute (short-term) telomere erosion-independent effect, causing DNA damage, ATM-dependent G2/M cell cycle arrest, in addition to decreasing cell viability. The long-term effect of MST-312 is achieved after a duration of > 1.5 months of exposure, leading to a remarkable telomere shortening [29,30,38,39]. In our previous studies, we have indicated that MST-312 exerts a short-term dose-dependent cytotoxic effect against acute promyelocytic leukemia (APL) and pre-B acute lymphoblastic leukemia (ALL) cells [40,41]. Furthermore, we evidenced that MST-312 exhibited neither cytotoxic nor apoptotic effects against normal human peripheral blood mononuclear cells (PBMCs), which relatively proposes that MST-312 has selectively tumor-growth inhibitory effects [40]. In line with previous reports, the present study also revealed that MST-312 has a deceasing short-term effect on cell viability and metabolic activity of U-266 myeloma cells (Fig. 1). Additionally, the present results demonstrated that U-266 cells underwent apoptosis after short-term exposure with MST-312 (Fig. 2). Similarly, previous studies showed that other types of telomerase inhibitors, including anti-sense oligonucleotides [42,43] or G-quadruplex compounds [44] have short-term cytotoxic effects on tumor cells which are not dependent on critical telomere attrition. The acute effects of inhibiting telomerase are not related to telomere erosion, suggesting that telomerase might have other functions as well as maintenance of telomere. Several studies have highlighted the non-telomeric (non-canonical) activities for telomerase which are not dependent on its conventional (canonical) function that is maintenance of telomere [17,18,45]. Non-canonical functions of telomerase vary from cell cycle regulation, control of gene expression [18–21], and inhibition of apoptosis [22–24] to modulation of intracellular signaling pathways such as NF-κB [25,26]. In the previous study, we reported that MST-312 displayed an anti-NF-κB activity in APL cells and suppressed the genes which are regulated by NF-κB, including the anti-apoptotic genes such as surviving, Bcl-2, and Mcl-1 as well as the proliferative genes such as c-Myc and hTERT [40]. In the current study, it was consistently shown that MST-312 considerably down-regulated the expression of Bcl-2, hTERT, and c-Myc genes in U-266 myeloma cells. Therefore, suppressing the expression of pro-survival genes may possibly explain the acute growth arrest and apoptosis

of myeloma cells in response to MST-312 treatment.

The previous study performed by Ghosh et al. reported that telomerase was able to regulate NF-κB-dependent gene expression directly. Interestingly, this research team has indicated that the NF-κB regulation which is mediated by telomerase is exclusive to a subset of NF-κB promoters including IL-6 and TNF-α. Furthermore, gene expression analysis of AML, ALL or CML primary cells indicated that in > 80% of cases, IL-6 levels were remarkably reduced in response to MST-312 treatment [25]. Notably, Ding and colleagues demonstrated the regulated expression of NF-κB targeted genes by hTERT, regardless of its telomerase activity [27]. Considering the fundamental role of inflammatory mediators in pathogenesis of MM [1,8], we further examined whether MST-312 was able to affect the expression of inflammatory NF-κB target genes (IL-6 and TNF-α) in myeloma cells. In consistency with previous findings, the current study also showed a considerable reduction in the expression of IL-6 and TNF-α genes after treatment of U-266 cells with MST-312. Taken together, these findings propose that inhibition of telomerase activity in MM may probably be associated with effective blockade of the expression of genes serving the processes of development and progression of MM.

In conclusion, the present data may, at least in part, provide evidence that the acute growth arrest and apoptosis induced by MST-312 in U-266 myeloma cells were correlated with up-regulation of the pro-apoptotic gene (Bax) in addition to down-regulation of the anti-apoptotic (Bcl-2), proliferative (c-Myc, hTERT) and inflammatory (IL-6, TNF-α) genes. Thus, the telomerase-based therapy employing MST-312 may probably be regarded as a promising strategy for the treatment of MM.

Conflict of interest

All authors of the present article declared no any conflict of interest.

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