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Evaluation of the efficacy of MST-312, as a telomerase inhibitor, in the treatment of patients with multiple myeloma after stem cell transplantation

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ABSTRACT

High-dose chemotherapy and stem cell transplantation are the best treatment options in patients with multiple myeloma. Numerous medicines have been studied as a maintenance treatment after transplantation. Still, the use of medications that, in addition to their maintenance properties, eliminate or delay relapse of the disease has always been researchers' purpose. Therefore, this study was performed to evaluate the efficacy of MST-312 after stem cell transplantation in patients with multiple myeloma. For this purpose, 73 patients with multiple myeloma after stem cell transplantation were studied. Thirty-five patients were in the case group, and 37 patients were in the control group. The case group was treated with 100 mg/day MST-312. Stem cell survival was evaluated in the two groups. Also, the expression of TNFα and IL-6 genes were evaluated by the Real-time PCR technique. The results showed no significant difference between the two groups in terms of stem cell survival in the first year (P=0.72) and second years of treatment (P=0.66). But there was a significant difference between the two groups regarding progression-free survival (PFS) in the first year (P=0.041) and the second year (P=0.029). These results indicate that MST-312 inhibits the progress of the disease by inhibiting the telomerase activity of myeloma cells. Genetic evaluations also showed that IL-6 and TNF-α genes were significantly reduced in the case group. Therefore, it could be suggested that MST-312 has a selective inhibitory effect on myeloma cell growth and can be indicated as a suitable candidate for treating multiple myeloma.

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Introduction

Multiple Myeloma (MM) is the second most common hematologic malignancy, accounting for 10-15% of blood diseases (1). Typically, plasma cells are the last B-cell lymphocytes involved in producing antibodies. Still, their uncontrolled proliferation in multiple myeloma leads to bone pain, infection, anemia, bone fractures, and elevated blood calcium levels (2). The average survival in this disease before starting chemotherapy is about seven months. One of the leading causes of cell malignancy is disruption of the NF-κB signaling pathway (3).

NF-κB transcription factor plays an essential role in the survival and proliferation of different B lymphocyte tumors, especially MM (4). Interleukin-6 (IL-6) is the primary growth factor for malignant plasma cells, promoting tumor cell survival and resistance to apoptosis in MM cells (5, 6). This cytokine interacts with other factors involved in the pathogenesis of MM, such as tumor suppressor genes and oncogenes (7). Also, a region for NF-κB transcription factor binding is observed in the promoter of the interleukin-6 gene (8). On the other hand, NF-κB leads to the production of various inflammatory mediators, including TNF-α (9).

Tumor necrosis factor (TNFα) is an inflammatory cytokine involved in cellular proliferation, apoptosis, and survival messages (3). This message mediates survival through NF-κB activity. Studies have shown that TNFα increases cell proliferation by activating the NF-κB pathway in myeloma cells (10, 11). Increased TNFα levels in patients with MM are also associated with disease severity. On the other hand, TNFα leads to NF-κB activation and a 5-fold increase in interleukin-6 (12).

Telomerase is another factor involved in cellular signaling pathways (an enzyme with two catalytic subunits, TERT and two ribonucleotide subunits, and increases telomere length at the end of chromosomes. Telomerase is an enzyme expressed in 90-80% of

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cancers; in the normal state, only stem and sex cells have active telomerase (13). Tumor cells with high telomerase activity show increased proliferation, resistance to treatment, invasion, and decreased apoptosis (14). Recent studies, however, have revealed the non-telomeric role of telomerase. Activation of telomerase and NF-κB play a crucial role in many cancers. Abnormal expression of telomerase leads to increased expression levels of NF-κB and its target genes (15).

Studies have shown that the regulation of telomerase-mediated gene expression is specific for interleukin-6 and TNFα (15-17). Therefore, simultaneous inhibition of telomerase and NF-κB seems to be a promising treatment strategy in malignancies (17). Because current treatments for this disease have many side effects and despite these drugs, the MM is still considered an incurable malignancy, so there is a need to recognize newer drugs (18). Among these, various methods have been proposed to inhibit multiple factors involved in cellular signaling, including inhibition of telomerase activity (19). Because chemically derived drugs are less compatible with the human body than herbal medicines; therefore, herbal medicines are now more important for research in the field of chemotherapy (3). The results of previous studies indicate the anti-cancer effects of catechin compounds (20, 21). These studies have shown that epigallocatechin gallate (EGCG), the primary catechin in green tea, potently and directly inhibits telomerase (21). MST-312 (a derivative of epigallocatechin) is more effective in inhibiting telomerase than epigallocatechin's main compound. These compounds have revived hope for cancer treatment among scientists. Surprisingly, it also has no side effects on normal blood cells (mononuclear cells) (22).

This study aimed to investigate the effect of telomerase inhibitor MST-312 on the expression of inflammatory cytokines that are effective in the pathogenesis of multiple myeloma in patients with this disease after stem cell transplantation.

Materials and methods

Patients

This open-label study was performed on patients with multiple myeloma who underwent autologous stem cell transplantation and was referred to the stem cell transplant clinic. We obtained conscious consent from all patients before entering the study. Patients were randomly divided into the case (receiving MST-312) and control groups. In the case group, MST-312 was started at a daily dose of 100 mg. Patients who underwent autologous stem cell transplantation and had acceptable bone marrow recovery after transplantation were included in the study. If patients at the time of enrollment had a history of venous thrombosis, neutropenia, motor neuropathy, or ischemic events and were randomly assigned to the case group, they were excluded from the case group and entered the control group. Also, if any mentioned complications happened during the follow-up period, the patients were removed from the case group and followed up in the control group. Finally, we determined the overall and progression-free survival rates in each case and control group. The drug's effectiveness was checked out by comparing two groups in terms of OS (probability of survival from transplant to death due to any cause) and DFS (likelihood of disease-free survival from transplant to recurrence or death for any reason).

A total of 73 patients were considered for inclusion in the study. Thirty-five patients were in the MST-312 group and 38 patients were in the control group. Of the 35 patients in the MST-312 group, 23 patients (66%) continued treatment, and 12 patients (44%) were transferred to the control group due to drug intolerance or other reasons. Out of 38 patients in the control group, 36 patients (95%) remained in the control group, and two patients (5%) were transferred to the MST-312 group.

Stem cell transplantation

The transplant preparation regimen was used according to the center protocol, including melphalan 200 mg/m^2 of the body surface. Co-trimoxazole and acyclovir were used in all patients for prophylaxis of PCP, VZV, and CMV infections. In the first ten days after transplantation and five days before transplantation, allopurinol 200-300 mg/day was administered to prevent hyperuricemia due to the destruction of hematopoietic tissue. In case of fever and neutropenia, intravenous antibiotics imipenem, vancomycin, and amphotericin B were used. In case of fever and signs of CMV activation, which was confirmed by PP65 test more than 3/50,000,

intravenous acyclovir at a dose of 10 mg/kg was used in 2 doses for 14 to 19 days. The presence of acute GVHD and 1/50,000 PP65 was also an indication for preemptive treatment of CMV infection.

All patients were in the isolation room during admission to the transplant ward and used routine blood and platelet transfusion regimens as needed. Neutrophil and platelet transplantation time, they had ANC greater than $0.5x10^9$/l and PLT greater than $20x10^9$/l for three consecutive days so that the patient does not require a platelet transfusion.

Genetic evaluations

At the end of the experiment, 5ml of peripheral blood was received from both groups. The RNA in their blood was extracted by a HigherPurity™ Total RNA Extraction Kit (Canvax Biotech, USA). The Revert Aid First Strand Complementary DNA (cDNA) kit (Fermentase, USA) was used to synthesize the DNA. 20µl per reaction containing 9µl of nuclease-free water, 1µl of random hexamer primer, 4µl of Reaction Buffer 5X, 2µl of mixed dNTP (10mM), 1µl of RiboLock RNase Inhibitor (20U/µl), 1µl Revert MuLV (200U/µl) was added to 2µl of total RNA (1µg per reaction). For cDNA synthesis, each 20µl reaction was placed in a thermocycler with the following temperature program:

It was incubated for five minutes at 25°C followed by 60 minutes at 42°C. The reaction was terminated by heating it at 70°C for 5 min. Real-time PCR reaction using 7.5µl of Mastermix Green 2X (Ampliqon), 1.5µl of cDNA, 1µl of each forward and reverse primer (10pmol), and 4µl of water. Temperature cycles include an initial activation phase at 95°C for 15 minutes and then with 40 temperature cycles. Each cycle has a denaturation phase at 95°C for 15 seconds and an annealing/elongation phase at 60°C for 60 seconds. The reaction was performed in ABI Biosystem. Melting curve analysis was performed to confirm the specificity of the products, and the GAPDH gene was used as a housekeeping gene to normalize the results of gene expression. The relative expression of target genes was calculated by the comparative ΔΔCT method according to formula $2^{-\Delta\Delta CT}$, and the expression of each gene was repeated three times. The Primer design for GAPDH, TNFα, and IL-6 were performed according to the sequence obtained from the Ensemble database by Oligo V.7.0 software. BLAST server was used to ensure the specificity of the primer connection (Table 1).

Table 1. The forward and reverse sequences of the studied gene primers

Gene		Primer Sequence
IL-6	Forward	5′- AGCCAGAGCTGTGCAGATGA -3′
	Reverse	5′- CTGCAGCCACTGGTTCTGTG -3′
TNFα	Forward	5′- GCTGCACTTTGGAGTGATCGG -3′
	Reverse	5′- TGGGCTACAGGCTTGTCACT -3′
GADPH	Forward	5′- GAAGGTGAAGGTCGGAGTC -3′
	Reverse	5′- GAAGATGGTGATGGGATTTC -3′

Statistical analysis

Data analysis was performed based on protocol and intention to treat/ITT and cases where there was no difference between groups. The results of the "intention to treat / ITT" analysis were reported. We used the frequency and percentage to describe the qualitative measures, and the median and amplitude of the changes were used for the small measurements. Platelets were estimated. A comparison of disease-free survival in the two groups was reported using log-rank test statistics.

Molecular section data were also analyzed by SPSS ver. 21 software and Student's Two-tailed's test. $P<0.05$ was considered as a significant level.

Results and discussion

The mean age in the MST-312 group was 51.37 years (minimum 32.25 and maximum 68.11 years) with 14 males and 21 females. The mean age of the control group was 53.75 years (minimum 34.12 and maximum 68.41 years), of which 17 were male, and 21 were female. The amount of cells injected during transplantation in the two groups is shown in Table 2. The mean WBC engraftment time in the MST-312 group was ten days, and the control group was 11 days ($p = 0.41$) and the mean PLT engraftment time in the MST-312 group was 13 days, and the control group was 14 days ($p = 0.08$). The mean onset of MST-312 after transplantation was 1.5 months. (Minimum 0.5 and maximum 7.8 months) and the average duration of use of MST-312 after transplantation was 13.2 months (minimum two and maximum 22.3 months).

Stem cell survival rate in the MST-312 group was 94.2% and in the control group was 97.1% in the first year ($P = 0.72$). In the second year, the cell survival

rate was 82.1% in MST-312 groups and 90.2% in the control group (P = 0.66).

Table 2. The amount of injected cells in the two groups

Cell Index	Control Group (n=38)	MST-312 Group
	Average (minimum-maximum)	Average (minimum-maximum)
WBC (×10^8)	12.65 (4.9-26.8)	11.55 (5.4-27)
MNC (×10^8)	8.1 (2.4-23)	7.7 (4.2-17.8)
CD34+ (×10^6)	1.5 (0.3-7)	1.5 (0.2-6.3)

In the first year, the progression-free survival (PFS) rate was 90.7% in the MST-312 group and 81.2% in the control group (P = 0.041). In the second year, the survival rate without disease progression (PFS) was 74.9% in the MST-312 group and 53.7% in the control group (P = 0.029).

The results of the molecular section showed that the telomerase inhibitor MST-312 reduced the expression of inflammatory genes, IL-6 and TNF-α (Fig 1).

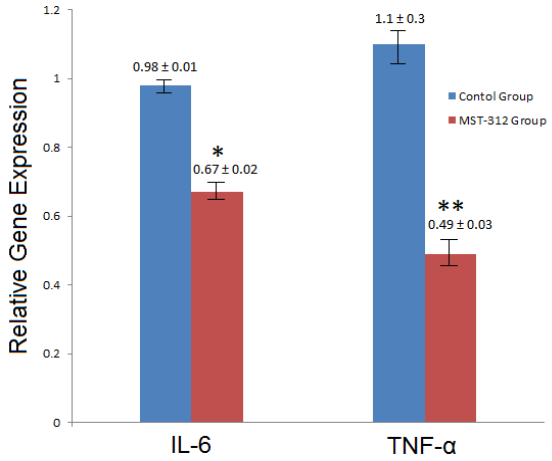

Figure 1. The effect of MST-312 on the expression of inflammatory genes IL-6 and TNF-α

The incidence of multiple myeloma is 4 per 100,000, which is almost the same worldwide. The incidence is slightly higher in men than in women (23). Despite the development of various treatments and drugs, high-dose chemotherapy and stem cell transplantation (allogeneic and autologous) is still the best treatment choice in patients (24). Numerous studies have demonstrated increased overall survival and disease-free survival interventions in these treatments. However, stem cell transplantation (ST) is not a definitive treatment for multiple myeloma, and the disease will usually relapse after the transplant (25). Therefore, treatment requires methods that can increase the lifespan of these transplanted stem cells (5). One of these methods can be targeted therapy (26).

Targeted therapy in cancer means treatment with agents and substances that prevent the growth and spread of cancer by interfering with specific molecules and messaging pathways. The development of targeted therapy requires accurate knowledge of drug targets and molecules involved in the carcinogenic process (26). Multiple myeloma, like other cancers, is a candidate for targeted therapy and therapeutic development in this field due to the involvement of specific tumorigenic molecules. Today, advances have been made in understanding the messenger pathways in multiple myeloma and in knowing the key molecules involved in the pathogenesis of the disease. However, it is still an incurable disease, and therefore new therapies are needed (27).

Also, due to the high role of telomerase activity and its relationship with NF-κB transcription factor in multiple myeloma as one of the primary and critical molecules involved in the pathogenesis of this malignancy, scientists have proposed and tested various strategies to inhibit telomerase (18). Inhibition of NF-κB may also damage normal cells, but telomerase has little activity in somatic cells. Therefore, targeting it will cause the most minor damage (3). In previous research, various compounds have been introduced as telomerase inhibitors. Meanwhile, epigallocatechin gallate (EGCG), the primary catechin of green tea, effectively inhibits telomerase. Various derivatives of this substance (MST-199, MST-295, and MST-312) are available, among which MST-312 inhibits telomerase more effectively (28).

The previous study in brain tumors has shown that MST-312 has two different effects depending on exposure to the drug (29). The short-term acute effect following short-term exposure (72h) to MST-312 leads to DNA damage, cell cycle arrest at G2/M, and increased cell death. This effect is independent of telomere shortening and is performed by separating the telomerase complex from DNA, identifying the telomere, and breaking the double-stranded DNA by the ATM pathway. Prolonged treatment (more than a month and a half) leads to a significant shortening of telomere length. MST-312, like other telomerase

inhibitors, has acute effects on short-term treatment independent of telomere shortening (30).

This effect suggests irregular roles of telomerase or, in other words, independent of telomere length reduction. Recent studies have suggested these roles, including cell cycle regulation, gene expression, and telomerase regulation of the NF-κB signaling pathway (3). In this regard, given that transcription factor target genes, NF-κB, including IL-6 and TNF-α play an essential role in the growth and survival of myeloma cells (4), hence, we investigated these two genes in the present study.

This study showed no significant difference between the two groups in terms of stem cell survival in the first year (P = 0.72) and second (P = 0.66). But there was a significant difference between the two groups regarding progression-free survival (PFS) in the first year (P = 0.041) and the second (P = 0.029). These results indicate that MST-312 inhibits the progress of the disease by inhibiting the telomerase activity of myeloma cells. Genetic evaluations also showed that IL-6 and TNF-α genes were significantly reduced in the drug-receiving group. Therefore, it could be suggested that MST-312 has a selective inhibitory effect on myeloma cell growth and can be indicated as a suitable candidate for treating multiple myeloma.

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