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Evaluation of stemness genes expression of OCT4, SOX2, Nanog, C-Myc and surface marker of CD133 on myeloma cells

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Abstract

Cancer stem cells that have the ability to proliferation and self-renewal were cause to drug resistance and metastasis in malignancies. In multiple myeloma, these cancer stem cells (MMCSCs) play a very important role in the recurrence of the disease. Detection of these cells can create new ways for treating these patients. In this study, stemness genes and phenotypic markers were investigated as the methods for identifying cancer stem cells in multiple myeloma.Expression of stemness genes of OCT4, SOX2, Nanog, C-Myc and target genes of STAT3 and TCF3 by PCR and Real Time PCR and also expression of surface markers of CD19, CD33, CD133, CD38 and CD56 by flow cytometry in RPMI8226 and U266 were studied.The results of the study showed that myeloma cells were express the stemness genes, and also CD133 surface marker was express in the some of myeloma cells. Identification of myeloma cancer stem cells can be crucial in the diagnosis, treatment and prognosis of multiple myeloma patients. CD133+ myeloma cells that express stemness genes are Multiple Myeloma Cancer Stem Cells (MMCSC) that can be considered as therapeutic targets.

***Keywords*:** Stemness genes; Myeloma cell; Immunophenotyping markers; MMCSC; CD133

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Introduction

Stem cells are primary and pluripotency cells that have the ability of proliferation, self-renewal, generation of diverse mature cell types by differentiation, regeneration of tissues(1). The number of these stem cells in the body is very limited, and most of these stem cells are in the G0 phase(2). The stem cell decision to proliferate or self- renew depends on signals in the cell that are activated in the signal transduction pathways(3).

Among the malignant cells, there is a very small percentage of stem cell-like cells that contribute to the formation, spread, invasion, drug resistance, and recurrence of cancer cells. These cells have been termed "cancer stem cells", which are similar to stem cells in proliferation and self-renewal(4). Cancer stem cells share similar properties with normal stem cells, including phenotype and cellular properties, but what distinguishes these natural stem cells from cancerous stem cells is the unlimited proliferation ability of these cancer cells due to disturbance in regulatory pathways. Although these cancer stem cells are very limited in number, they have a very high clonogenic capacity that is associated with the self-renewal of these cells(5). There are two different reasons for the origin of these

cancerous stem cells. The first reason is the genetic mutation and epigenetic modification that cause the normal stem cells to be transformed into cancerous stem cells, and the second reason is the natural somatic cells of the body that turn into cancerous stem cells due to genetic changes(6).

Notch, Wnt, Hedgehog, Bmi-1 and Hox signaling pathways play a very important role in regulating self-renewal. any alteration or non-regulation of these signaling pathways results in the transformation of normal stem cells or normal somatic cells into malignant cells(7). Maintaining stem cells with their special properties is critical and important. The vital mechanism for keeping stem cells of intracellular signaling pathways is Wnt, MAPK/ERK, Notch, Hedgchog, TGF-β, JAK/STAT, and receptor pathways(8). OCT4, Nanog, and SOX2 transcription factors are the central nucleus of these signaling pathways, which are called by the genes with the same name as stemness genes, and have the main role in regulating the expression of the gene and of the other genes of the signaling pathways(9). Multiple myeloma is the second most common hematologic malignancy caused by the proliferation of clonal plasma cells in bone marrow, and despite the new therapeutic methods due to frequent recurrence and drug resistance, it is still untreatable(10). The presence of cancer stem cells is believed to cause relapse and drug resistance in multiple myeloma and Identification of Multiple Myeloma Cancer Stem Cells (MMCSCs) and cellular and molecular mechanisms in these cells are one of the important topics in this disease(11). In this study, we examined the status of expression of the CD133 marker, and stemness genes in the myeloma cells.

Materials and methods

RPMI8226 and U266 cell lines were obtained from the cell bank of the Hematology Department of TarbiatModarres University, the NT2 cell line was provided by the Department of Biology and Genetics of TarbiatModares University and the human fibroblasts cell line was obtained from the "Bon yakhteh" Research Center. RPMI8226 and U266 cell lines in RPMI1640 complete culture medium contain 2mM L-glutamate, 50 U / ml of penicillin, 50µg / ml Streptomycin and 10% of the fetal bovine serum cultivated in sterile condition with 5% CO2 incubator at 37 ° C. NT2 cell lines and human fibroblasts in a complete DEMM culture medium containing 2mM L-glutamate, 50 U / ml penicillin, 50 µg / ml Streptomycin and 10% of the fetal bovine serum in sterile conditions was cultivated in a 5% CO2 incubator with the temperature of 37 ° C.

RNA extraction and cDNA synthesis:

The cultured myeloma cells were isolated from the culture medium and were washed with PBS buffer. The RNA of the myeloma cells was extracted using the Sinaclon RNX-PLUS kit, based on the kit protocol. The quality and purity of the RNA extracted measured by a photometer. For synthesis of cDNA, the Vivantis kit was used in accordance with the manufacturer's instructions.

Design Primer:

The sequences of OCT4, SOX2, Nanog, c-Myc, STAT3 and TCF genes were obtained from the NCBI site, and using primer-BLAST software, suitable primers for each genes were designed. The precision and specificity of each of the primer was evaluated by Gene Runner software (Table-1).

Genes expression study:

Using real time PCR and according with this protocol 5µl of RealQ Plus 2x Master Mix Green High ROX (Ampliqon) and 0.5µl of cDNA and 0.4µl of Primer and 4.1µl of Nuclease-free water and based on the program : Holding stage temperature at 95ºC for 15min, Cycling stage (95 ºC for 25sec, 60 ºC for 30sec, 72 ºC for 30sec and Number of cycles is 40) and Melt curve (95 ºC for 15sec, 60 ºC for 1min , 95 ºC for 15sec) expression of the OCT4, SOX2, Nanog, c-Myc, STAT3 and TCF genes in RPMI8226 and U266 cell lines was studied. The expression of these genes was also performed by PCR and electrophoresis products along with positive control (NT2 cell line) and negative control (human fibroblast cells) and marker size.

Table-1. Primers list

Genes	Accession number	Primers	Product length
OCT4	NM_001159542.2	Forward: 5'-GGTGCCTGCCCTTCTAGGAATG-3' Revers: 5'-TGCCCCCACCCTTTGTGTTC-3'	137 bp
SOX2	NM_003106.4	Forward: 5'-GACTTCACATGTCCCAGCACT-3' Revers: 5'-CTCTTTTGCACCCCTCCCATT-3'	156 bp
Nanog	NM_001297698.2	Forward: 5'-AATGGTGTGACGCAGGGATG-3' Revers: 5'-TGCACCAGGTCTGAGTGTTC-3'	148 bp
C-Myc	NM_001354870.1	Forward: 5'-CCTACCCTCTCAACGACAGC-3' Revers: 5'-CTTGTTCCTCCTCAGAGTCGC-3'	183 bp
STAT3	NM_003150.4	Forward: 5'-ACGAAGGGTACATCATGGGC-3' Revers: 5'-CTGGATCTGGGTCTTACCGC-3'	164 bp
TCF3	NM_003200.5	Forward: 5'-CCACGGCCTGCAGAGTAAGATAG-3' Revers: 5'-ATGGGGCCGGTGAAACCTG-3'	141 bp
β-actin	NM_001101.5	Forward: 5'-TGAAGATCAAGATCATTGCTCCTC-3' Revers: 5'-AGTCATAGTCCGCCTAGA AGC-3'	168 bp

Measurement of the expression of cell surface markers by flow cytometry:

Conjugated CD19-PE, CD33-FITC, CD38-FITC, CD56-PE, CD133-PE, CD138-PE antibodies were used. After adding 100 µl of PBS to the cells separated from the medium, we add 2 µl conjugate antibody. The cells were placed at 4 ° C for a period of 45 minutes in dark places and were kept away from the light. 500 µl of PBS containing 10% FBS was added to the cells and we measured the cell surface markers by flow cytometry.

Results

Expression of stemness genes in RPMI8226 and U266 myeloma cells:

In evaluation of the expression of OCT4, SOX2, Nanog, and c-MYC stemness genes and STAT3 and TCF3 target genes in RPMI8226 and U266 myeloma cells, β-Actin as internal control, and human fibroblast cells as negative control of stem cell gene expression and NT2 cells as positive expression control Stem cell genes were used by PCR and Real-time PCR.The β-Actin gene has expression in all cells of RPMI8226, U266, Human fibroblast and NT2, and a 168bp band in PCR product was observed. RPMI8226, U266, and NT2 cells express OCT4, SOX2, Nanog, and c-MYC genes, and 111bp, 156bp, 115bp, and 183bp bands are visible in PCR products, but in Human fibroblast cells no bands for this genes were not seen (Fig. 1). The expression of these genes was also examined by Real Time PCR, which expressed in RPMI8226, U266, and NT2 cells and lacked expression in human fibroblasts. The expression of target genes STAT3 and TCF3 are also examined by Real Time PCR.

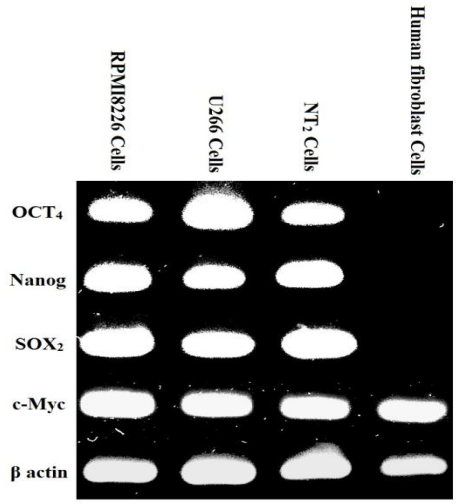

Fig-1. OCT4, SOX2, Nanog, C-Myc, STAT3 and TCF3 genes expression on RPMI8226 and U266 myeloma cells.

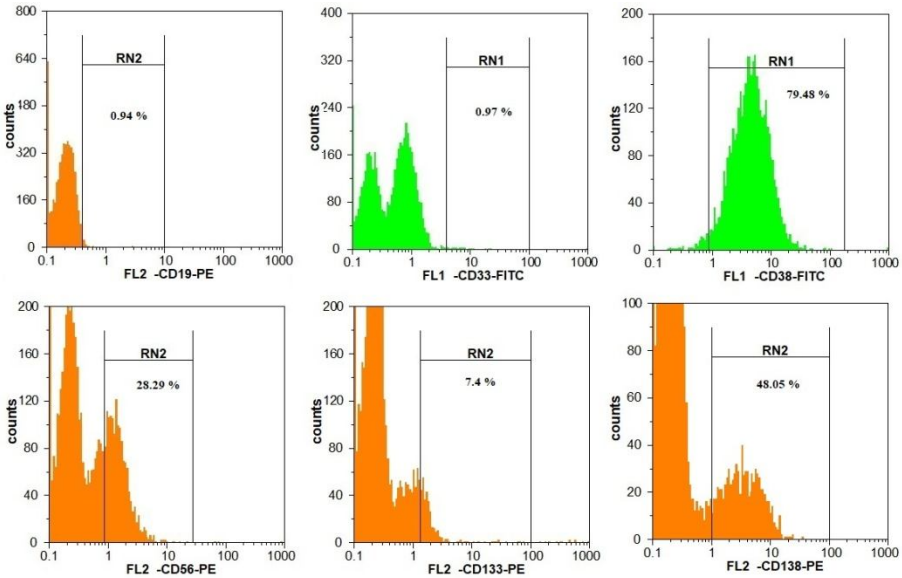

Fig-2. CD19,CD33,CD38,CD56,CD133 and CD138 expression on RPMI8226 myeloma cell line by flowcytometry.

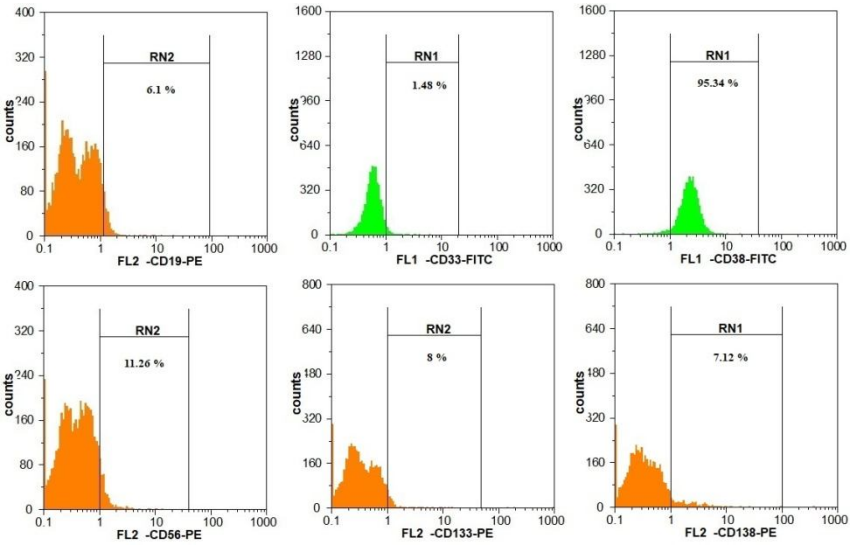

Fig-3. CD19,CD33,CD38,CD56,CD133 and CD138 expression on U266 myeloma cell line by flowcytometry.

CD19, CD33, CD38, CD56, CD133 and CD138 markers expression:

Levels of surface marker expression in the myeloma cells were measured by using flow cytometry (Fig-2 and Fig-3). The results showed that the level of expression of the CD19 surface marker in the RPMI8226 myeloma cells was 1.52% and for the U266 myeloma cells was 3.8%**.** The surface marker of CD33 is 0.57% in RPMI8226 and 2.9% in the U266 myeloma cells**.** Surface marker CD38 with expression of 66.19% in RPMI8226 myeloma cells and 91.8% in U266 myeloma cells of has expression**.** The level of expression of the CD56 surface marker in RPMI8226 myeloma cells is 21.31% and 13.9% in the U266 myeloma cells**.** Surface markers of CD133 and CD138 have expressions of 6.84% and 44.95% in RPMI8226 myeloma cells and 6.61% and 9.47% in the U266 myeloma cells, respectively (Fig. 4**).**

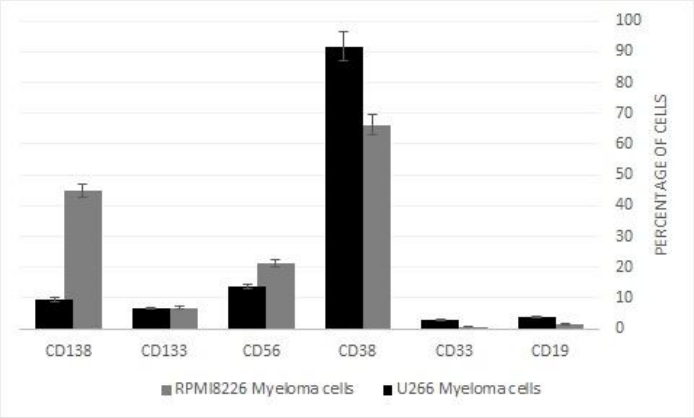

Fig-4. Flow cytometry analysis of RPMI8226 and U266 myeloma cells surface markers. Data are shown as mean ± SD.

Discussion

Multiple myeloma cancer stem cell (MMCSC) is a small, quiescent or cologenic population of the myeloma cells that can cause tumors through interactions with bone marrow Nich(13). These cells have the capacity to self-renew and differentiate into the malignant myeloma cells(14). Self-renewal capacity of the myeloma cells helps maintain the cologenic proliferation of these cells and recurrence of multiple myeloma(15).

It seems that common therapies only eliminate malignant cells and these drugs do not affect on MMCSCs which are resistant cells, and responsible for initiation, drug resistance, metastasis, and malignant recurrence(16). Several markers, such as ALDH1 and CD133, have been studied for CSC detection, but a precise and definitive diagnostic marker for MMCSC has not yet been identified(17).

Stemness genes OCT4, SOX2, Nanog, and c-MYC are expressed naturally in embryonic stem cells(18). These genes play a role in self-renewal, proliferation, differentiation, and maintaining the pluripotency capacity of these cells(19). Stemness genes, especially OCT4 and SOX2, are also expressed in cancerous cells, and the expression of these genes is associated with recurrence of cancer, drug resistance, and poor prognosis in these malignancies(20). The present study showed that stemness genes in RPMI8226 and U266 myeloma cell lines have high expression. Given that the gene of OCT4 is the main gene among stemness genes, it can be argued that its expression leads the expression of other genes, such as SOX2, Nanog. On the other hand, the c-MYC gene has the ability to replicate in all cells that have expressions that. Therefore, this gene is also expressed in the myeloma cells. In embryonic stem cells, OCT4, SOX2, and Nanog genes affect each other, and the OCT4 gene is the first gene that activates stem cell signaling and activates other genes, including SOX2 and Nanog(21). The OCT4 gene plays a major role in generating drug resistance and increasing the invasion of malignant breast cancer through the STAT3 / OCT4 / c-MYC signaling pathway(22). In malignant breast cancer cells, increasing the expression of OCT4 by increasing the expression of the STAT3 gene promotes increased IL-24 secretion, which contributes to the resistance of these cells to radiation therapy(23). In the myeloma cells, the JAK / STAT signaling pathway is active and these cells express the active form of STAT3, which is associated with poor prognosis in multiple myeloma patients. Inhibition of expression of STAT3 gene reduces the proliferation and increases the apoptosis of the myeloma cells (24). Inhibition of the STAT3 and NF-kB transcription factors in the CD138+ myeloma cells reduces adhesion to bone marrow stromal cells, secretion of cytokines, and survival of the myeloma cells(25). TCF3 acts extensively at the genome level on the promoter of genes associated with the self-renewal of embryonic stem cells, neutralizing the effects of the OCT4 and Nanog genes, and its effect

on the OCT4 gene is greater than that of the Nanog gene. TCF3 acts on the promoter of genes associated with the self-renewal of embryonic stem cells and disables OCT4 and Nanog genes. In fact, TCF3 is a self-renewal capacity inhibitor, that lack of TCF3 expression causes an increase in the self-renewal capacity and delay in the differentiation of embryonic stem cells(26). The OCT4 gene binds to the promoter of the Tcl1 gene to activate the signaling pathway for regulating stem cell proliferation(27). One of the pathways for activating OCT4 is the Akt1 signaling pathway, which through the effect of IL-6 on the PI3K / Akt signaling pathway prevents apoptosis in the myeloma cells and proliferation and survival of these cells(28). The OCT4 gene activated the CCND1 gene and it increases cell cycle speed and cell proliferation through shortening the time of the G1 phase and rapidly passing the cells from this phase and entering them into the S phase(29). The OCT4 gene binds directly to the promoter of the CCND1 gene and increases its expression. When the OCT4 gene is inhibited, the expression of the CCND1 gene decreases and causes the cell cycle to be stopped in the G1 phase(30). OCT4 is prevented from phosphorylation of cell cycle pathway proteins, and causes continues mitosis in malignant ovarian cancer cells and increases the proliferation of these cells(31). The myeloma cells express a variable expression of surface markers and vary from one patient to another in expressing these surface markers. Overall, it has been observed that the myeloma cells can markup surface CD138, CD38, CD19, CD56, CD117, CD20, CD27, CD28, and CD33**.** Flow cytometry of patients with multiple myeloma indicates that CD138, CD38, CD56, CD117, CD20 and CD52 surface markers are expressed in which 100% of these patients express CD138 and CD38 markers and 80% of them expressed CD56, but a very small percent of the patients expressed other markers(32). Our results also showed that both the RPMI8226 and U266, express the surface markers of CD38, CD138, and CD56. Normal Plasma Cells express the surface marker CD19, but in the case of multiple myeloma, the CD19 marker is not expressed, or its expression decreased. The expression of CD19 on the myeloma cells will inhibit the proliferation of these cells and reduce the spread of it in the body of the patients(33). About 6.5% to 12% of the patients with multiple myeloma, express a myeloid specific marker of CD33(34). CD133 is one of the most important surface markers for the detection and isolation of stem cells and it has been shown that CD133 plays a role in the proliferation, evolution and biology of malignant cells(35). Our results showed that the RPMI8226 and U266 myeloma cells expressed surface marker CD133. It seems that with respect to the expression of the genes of OCT4, SOX2, Nanog, and c-MYC in the myeloma cells, it is possible to consider the CD133 positive cells of the myeloma cells as multiple myeloma stem cells (MMCSC). The results of this study showed that like many malignancies, the stemness genes of OCT4, SOX2, Nanog, and c-MYC are also expressed in the myeloma cells, and the study of the expression of the target genes of TCF3 and STAT3 in these cells shows that stemness genes in these cells are functional.

Conclusion

Cancer stem cells cause drug resistance, metastasis, and recurrence of cancer. These small numbers of cancer stem cells can survive cancer in patients, and routine treatments cannot kill these cancer stem cells. Multiple myeloma cells are also present in these cancerous stem cells (MMCSC). Identification of myeloma cancer stem cells can be crucial in the diagnosis, treatment and prognosis of multiple myeloma patients. It seems CD133+ myeloma cells that express stemness genes are Multiple Myeloma Cancer Stem Cells (MMCSC) that can be considered as therapeutic targets.

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Conflict of interest

The authors declare that they have no conflicts of interestand do not have any financial relationship with

the Royan institute for Stem Cell Biology and Technology (RI-SCBT).

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