Published online 2022 April 20 **Original Article**

Isolation and Eradication of Ovarian CD44+ Cancer Stem Cells via Notch Signaling Pathway Mediated by Ectopic Silence of MAML1

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Received 2021 December 06; **Revised** 2021 December 30; **Accepted** 2022 February 10.

Abstract

Background: Ovarian cancer is the fifth leading cause of cancer-related deaths among women globally. Cancer stem cells (CSCs) are a subpopulation of tumor cells involved in ovarian tumor formation, metastasis, relapse, and chemoresistance. Moreover, the Notch signaling pathway has a pivotal role in CSCs maintenance.

Objectives: This study was designed to isolate CSCs from the A2780 cell line and determine the effectiveness of Mastermind-like transcriptional coactivator 1 (MAML1) inhibition, a key factor of the Notch pathway, in targeted therapy against ovarian CSCs.

Methods: The $CD44^+$ or $CD133^+$ CSCs were isolated from the ovarian A2780 cell line using magnetic cell sorting. The isolated CSCs were also evaluated for stemness markers expression, self-renewal capacity, cell cycle progression, and chemoresistance compared to their negative counterparts. Afterward, MAML1-shRNA was used to inhibit the Notch pathway in $CD44^+$CSCs. The role of MAML1 was also evaluated in the $CD44^+$ CSCs epithelial-mesenchymal transition (EMT) process and migration.

Results: In addition to the high expression of stemness markers, such as Sox2 and Musashi1, ovarian $CD44^+$ or $CD133^+$ CSCs had a high ability for sphere formation, higher percentage in the G1 phase to S phase, and decreased sensitivity to chemotherapy drug compared to CD44- or CD133- cells. Besides, silencing MAML1 significantly reduced the levels of EMT markers and cell migration in CD44+ CSCs, compared to scramble.

Conclusion: Mastermind-like transcriptional coactivator 1 can be considered a pivotal factor in the targeted therapy and eradication of $CD44^+$ CSCs through the inhibition of the Notch signaling pathway in an ovarian cancer patient with a special focus on the ovarian A2780 cell line.

Keywords: CD133, CD44, Drug resistance, Ovarian neoplasm, Neoplastic stem cell, Recurrence

1. Background

Ovarian cancer is a heterogeneous malignancy with high genomic and histopathologic diversities (1). One of the most common type of ovarian cancer is epithelial ovarian cancer (EOC) which consists of different subtypes including serous, endometroid, and mucinous (2). Majority of the epithelial EOC cases present with high-grade serous carcinomas (HGSC) and a 5-year survival rate of 35-40% (3). Of note, conventional and traditional treatments for ovarian cancer have always been associated with tumor recurrence mainly due to the inability of complete removal of a certain type of cancer cells. These cells, known as cancer stem cells (CSCs), have been characterized by regeneration, tumorigenesis and chemoresistance (2). Drug resistance of CSCs is associated with increased drug efflux (4), and DNA repair process (5). These cells can be identified by some intracellular and surface cellular markers (4), including CD133, CD44, and CD54 (6).

CD44 acts as a trans-membrane receptor for the hyaluronic acid (HA) and many extra cellular matrix (ECM) components. It is also a co-receptor of growth factors and cytokines which is involved in various signal transductions. Since the CD44 is a CSC marker involved in self-renewal and tumor progression, it can be used to isolate the CSCs. Besides, CD44+ cells not only have the sphere formation ability in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mic (7), but also are introduced as a diagnostic factor in EOC patients (8). CD133 (prominin-1), an indicator marker of hematopoietic stem and progenitor cells (9), is one of the most common cell surface markers used to isolate CSCs from a variety of malignant cells including glioblastoma, breast, prostate, colon and ovarian cancers (10). The increased expression of CD133 is regarded as a diagnostic biomarker for disease progression (11), such as ovarian cancer identified by Fradina et al.(12). This marker regulates a number of intracellular and extracellular factors, including epigenetic factors, signaling pathways, and microRNA (miRNAs) (13).

Notch pathway has a pivotal role in self-renewal and maintenance of CSCs (14). Notch receptors (Notch 1-4) and ligands (Jagged1-2, Delta1, and

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Delta-like 1-4) interactions between two adjacent cells trigger the gamma secretase activations and Notch signaling machinery (15). Besides, intracellular portion of Notch (ICN) binds to and activates the cellulose synthase-like (CSL) family of DNA-binding transcription factors through CBP/p300 and recruitments of mastermind like proteins (MAML) (16). Finally, MAML1, CSL, and ICN complex activates the transcription of Notch signaling pathway target genes, which is positively involved in cell growth, proliferation, differentiation, and apoptosis of cancerous cells and epithelial-mesenchymal transition (EMT) process (17,18). EMT promotes invasiveness of cancer due to converting carcinoma cells to the high migratory and mobility mesenchymal cells. EMT-associated transcription factors including Slug, Snail and Twist trigger chemo-resistance in breast, ovarian, and nasopharyngeal carcinoma. Besides, zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2) as well as fibronectin are among highly expressed factors during EMT (19). As well, different microRNAs have been verified as powerful regulators of EMT in multiple tumors such as members of the let-7, miR-34, miR-200, and miR-302 families (20).

2. Objectives

In the current study, we hypothesized that CSCs could be a major cause of recurrence, metastasis, and malignancy in ovarian cancer and the effective targeted therapy could be achieved by Notch silencing. Based, we isolated CSCs from the A2780 ovarian cell line via surface markers and then MAML1 silencing (the main component of Notch transcription machinery) was assessed in the eradication of ovarian CD44+ CSCs.

3. Methods

3.1. Isolation of CSCs and sphere formation

The A2780 ovarian cell line was purchased from the Biotechnology Research Center of Bouali Research Institute of Mashhad (Mashhad, Iran). Short tandem repeat (STR) profiling analysis was performed to confirm the validity of this cell line. The A2780 cell line was then cultured in Roswell Park Memorial Institute Medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) (Gibco, UK), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, UK) at 37°C, 95% humidity and 5% CO2. Then, single-cell suspensions prepared by 0.25% trypsin+1 mM ethylenediaminetetraacetic acid (EDTA) were subjected to flow cytometry analysis to evaluate the expression levels of CD44 or CD133. In this regard, single cells were incubated with fluor chrome-conjugated antibodies (Miltenyi Biotech, Germany) diluted 1: 11 in phosphate-buffered saline (PBS) + 0.5% FBS at 4°C for 10–15 min. Finally, the labeled cells were evaluated by fluorescence activated cell sorting (FACS) Calibur flow cytometer and the results were analyzed by the FlowJo 7.6.2 software (Tree Star, Ashland, OR). To obtain the highest enriched population of CD44+/CD44- or CD133+/CD133- cells, magnetic activated cell sorting (MACS) system (Miltenyi Biotech, Germany) had been modified and optimized via dissolving cell pellet in a combined buffer kit (MACS BSA Stock Solution 1:20 autoMACS Rinsing Solution), the FCR Blocking Reagent and CD44 or CD133 microbeads (Miltenyi biotech, Germany). After incubation the mixture at 4 °C for 20 min in a gentle rotator, the cells were washed with combined buffer kit and proceed according to the kit instructions. To confirm cells isolation accuracy, the expression of CD44 or CD133 biomarker was examined using flow cytometry.

Tumor sphere formation capacity was evaluated to verify the self-regenerative nature of CD44+ or CD133+. Based, CSCs were cultured in low attachment plates containing serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium including 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich), 2% B27 supplement, 5 µg/ml Insulin and 1% penicillin/streptomycin and examined with an inverted microscope (OPTIKA, Italy).

3.2. RNA isolation and Real-Time PCR

Total RNA was obtained with Pars Tous extraction kit (Tehran, Iran) according to the company's instructions. Then, RNA samples were treated using DNaseI (Thermo Fisher Scientific, US) followed by reverse transcription using Easy cDNA Synthesis Kit (ParsTous, Iran). Comparative relative real-time PCR of the selected markers (Table 1) was performed in Light Cycler (Roche, Germany) using SYBR Green (AMPLIQON, Denmark) as indicator dye. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizer. To evaluate the mRNA expression of tested genes, Ct values were measured for each individual sample. The higher mean Ct value shows gene down-regulation and its significant level was evaluated by T-test.

3.3. Chemoresistance assay

The cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with ddifferent amounts of (0.01 to 10 µg/ml) 5FU (Gmbh, Germany). After 48h, cells were incubated with 50 µl of 5 mg/ml of dimethyl thiazolyldiphenyltetrazolium (MTT) dye (Sigma; Dorset) for 4h at 37 C. The absorbance of cells were then measured at 570 nm using Spectrophotometer WPA Biowave II (Wolf-labs, UK).

Table 1. Primer sequences for the real time PCR

Gene name	Sequence	Thermal profile	Size (bp)
CD44s	F: TCCAACACCTCCCAGTATGACA R:GGCAGGTCTGTGACTGATGTACA	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	83
CD44v3	F: GCACTTCAGGAGGTTACATC R: CTGAGGTGTCTGTCTCTTTC	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	181
Sox2	F: AACAGCCCGGACCGCGTCAA R: TCGCAGCCGCTTAGCCTCGT	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	189
Nanog	F: GCAATGGTGTGACGCAGAAGGC R:GCTCCAGGTTGAATTGTTCCAGGTC	95 °C(10 min)[95 °C(30 s)/65 °C(30 s)/72 °C(30 s)]40	137
Oct4	F: CCTGAAGCAGAAGACGATCA R: CCGCAGCTTACACATGTTCT	95 °C(10 min)[95 °C(30 s)/63 °C(30 s)/72 °C(30 s)]40	148
ABCG2	F: TGAGGGTTTGGAACTGTGG R: GATTCTGACGCACACCTGG	95 °C(10 min)[95 °C(30 s)/65 °C(30 s)/72 °C(30 s)]40	155
ALDH1	F: GATCCCCGTGGCGTACTATG R: TGGATCTTGTCAGCCCAACC	95 °C(10 min)[95 °C(30 s)/62 °C(30 s)/72 °C(30 s)]40	202
LGR5	F: CCTTCCAACCTCAGCGTCTT R: AGGGATTGAAGGCTTCGCAA	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	248
MAML1	F: GCGGAACAGGAGAAGCAAC R: GGCACGGCAGCAGAGG	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	125
Notch1	F: ACGCCTACCTCTGCTTCTG R: GCACACTCGTAGCCATCG	95 °C(10 min)[95 °C(30 s)/59 °C(30 s)/72 °C(30 s)]40	127
Msi1	F: GAGACTGACGCGCCCCAGCC R: CGCCTGGTCCATGAAAGTGACG	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	213
Klf4	F: TCTTCTCTTCGTTGACTTTG R: GCCAGCGGTTATTCGG	95 °C(10 min)[95 °C(30 s)/55 °C(30 s)/72 °C(30 s)]40	210
Snail	F: CTAGGCCCTGGCTGCTACAA R: ACATTCGGGAGAAGGTCCGA	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	177
Slug	F: GCCAAACTACAGCGAACTGG R: TGGAATGGAGCAGCGGTAG	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	150
Twist1	F: GGAGTCCGCAGTCTTACGAG R: TCTGGAGGACCTGGTAGAGG	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	201
Fibronectin	F: AGGAAGCCGAGGTTTTAACTG R: AGGACGCTCATAAGTGTCACC	95 °C(10 min)[95 °C(30 s)/61 °C(30 s)/72 °C(30 s)]40	106
Zeb2	F: GGGACAGATCAGCACCAAAT R: CGCAGGTGTTCTTTCAGATG	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	110
HES1	F: CCCAACGCAGTGTCACCTTC R:TACAAAGGCGCAATCCAATATG	95 °C(10 min)[95 °C(30 s)/58 °C(30 s)/72 °C(30 s)]40	304
HEY1	F:ACGGCAGGAGGGAAAGGTTAC R:CTGGGAAGCGTAGTTGTTGAGATG	95 °C(10 min)[95 °C(30 s)/58 °C(30 s)/72 °C(30 s)]40	177
HEY2	F:AGAAAAGGAGAGGGATTATAGAGAAAAGG R: AGCGTGTGCGTCAAAGTAGC	95 °C(10 min)[95 °C(30 s)/59 °C(30 s)/72 °C(30 s)]40	200
GAPDH	F:GGAAGGTGAAGGTCGGAGTCA R:GTCATTGATGGCAACAATATCCACT	95 °C(10 min)[95 °C(30 s)/60 °C(30 s)/72 °C(30 s)]40	108
miRNA200c	F: TAATACTGCCGGGTAATGATGGA R: Universal PCR Reverse Primer	94 °C(3 min)[94 °C(15 s)/60 °C(30 s)/72 °C(45 s)]40	-
miRNA34a	F: TGGCAGTGTCTTAGCTGGTTGT R: Universal PCR Reverse Primer	94 °C(3 min)[94 °C(30 s)/60 °C(30 s)/72 °C(45 s)]40	-
U6 snRNA	F:GTGCTCGCTTCGGCAGCACATAT R: Universal PCR Reverse Primer	94 °C(3 min)[94 °C(30 s)/60 °C(30 s)/72 °C(45 s)]40	-

Table 2. MAML1 shRNA sequences

ShRNA types	Sequences 5'——3'
shRNA1	F: GATCCCCGGCTGGACTACGGCAATACAAATCAAGAGTTTGTATTGCCGTAGTCCAGCTTTTTA R: AGCTTAAAAAGCTGGACTACGGCAATACAAACTCTTGATTTGTATTGCCGTAGTCCAGCCGGG
shRNA2	F: GATCCCCGGAGCCACCGAGTAACTTGAATCAGAATCAAGAGTTCTGATTCAAGTTACTCGGTGGCTTTTTTA R: AGCTTAAAAAAGCCACCGAGTAACTTGAATCAGAACTCTTGATTCTGATTCAAGTTACTCGGTGGCTCCGGG
shRNA3	F: GATCCCCGGATGCCAGACCTCAACCTTATCAAGAGTAAGGTTGAGGTCTGGCATTTTTTA R: AGCTTAAAAAATGCCAGACCTCAACCTTACTCTTGATAAGGTTGAGGTCTGGCATCCGGG
shRNA4	F: GATCCCCGG CATATCGCAAAGCAACCTCATTCAAGAGATGAGGTTGCTTTGCGATATGTTTTTA R: AGCTTAAAAACATATCGCAAAGCAACCTCATCTCTTGAATGAGGTTGCTTTGCGATATGCCGGG

3.4. Cell cycle analysis

For cell cycle analysis, cells were suspended in propidium iodide (PI, 50 µg/ml), Triton ×-100 (0.1%), sodium citrate solution (0.1%), and RNase (100 µg/ml). Following at least 60 min incubation at 4 °C in darkness, cell cycle distribution was examined using a flowcytometry (Franklin Lakes, USA) and analyzed with the Mod Fit LT software (version 4.1).

3.5. Construct and transfection

Four MAML1 shRNA sequences (Table 2) were designed and subcloned into PRNAT-H1-Neoby vector using BamH1 and HindIII enzymes. The overview of mentioned vectors map has been demonstrated in Figure 1. To select the vector with the maximum gene silencing rate, all four designed vectors were separately added with Fusofect

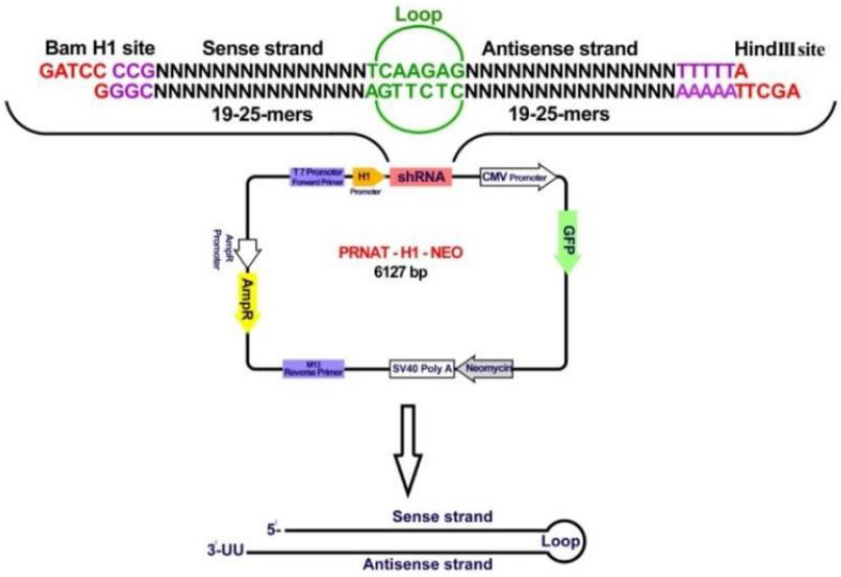

Figure 1. Process of cloning MAMAL1 shRNA into PRNAT-H1-NEO vector

synthetic nanoparticles into the human embryonic kidney (HEK293) cell line as a positive control cell line.

Since the transfection efficiency of ovarian CSCs is low, the HEK293 cell line was chosen to select the best vector carrying MAML1 shRNA with transfection efficiency over 90% as supported by evidence (21). Afterwards, CD44+ A2780 cells (2×10^5 cells/well) were transfected with the selected MAML1-PRNAT-H1 construct using polyethylenimine (PEI)-pro transfection reagent. After 48 h, transfection efficiency was confirmed using fluorescent microscope and Real-Time PCR for MAML1.

3.6. Analysis of EMT markers and miRNAs

In addition to MAML1 and its downstream targets, to measure the expression level of EMT makers, including Hes1, Hey1, Hey2, Snail, Slug, Twist1, Fibronectin, Zeb2, miRNA200c and miRNA34a in transfected cells following ectopic silence of MAMAL1, the relative comparative Real-Time PCR were performed and the results compared to that of scramble. GAPDH was used as internal control gene. All the primer sequences are presented in Table 1.

3.7. Scratch assay

The CD44+CSCs, were incubated in 6-well plates until reaching to the 100% confluent monolayer. After cells transfection, medium was exchanged with fresh medium (2% FBS) followed by cell scratching using p200 pipet tip. The cell-free area was photographed by inverted microscope within a desired time frame and analyzed by MATLAB software.

3.8. Statistical analysis

The statistical analysis was done using two-way ANOVA test. Significance level was set at $P < 0.05$. Each experiment was performed in duplicate. Data analysis was carried out through Graph Pad Prism 8.0 and excel software. Error bars in bar charts were expressed as standard deviation (SD).

4. Results

4.1. Isolation and characterization of CSCs

Since the expression percentage of CD44+ or CD133+ markers in A2780 cell line was 0.9%, and 0.1% (Figure. 2A, B), respectively. This cell line was subjected to the MACS technique to separate CSCs which was resulted in isolation of CD44+ (94.3%) or CD133+ (90.5%) cells. (Figure 2C, D).

To recognize whether CD44+ or CD133+ A2780 cells have sphere-forming capability, the CD44+ or CD133+ cells were cultured in the specific medium. After five days, many CD44+ (Figure. 3A) or CD133+ cells (Figure. 3B) were proliferated and formed spherical colonies in various sizes and asymmetrical shapes. This finding could further indicate the self-regenerating nature of CD44+ or CD133+ cells.

4.2. Analysis of stemness markers

To ensure that CD44+ or CD133+ cells have stemness properties, the mRNA expression levels of several important stemness markers were analyzed by the Real-Time PCR technique. Compared to their negative counterparts, CD44+ or CD133+ cells had a significant ($P<0.05$) over-expression in stemness markers (Figure.4), which approved the high efficient isolation of CD44+ or CD133+ CSCs. It would be mentioned that markers of interest were selected based on their specific expression in each of the CSCs cells.

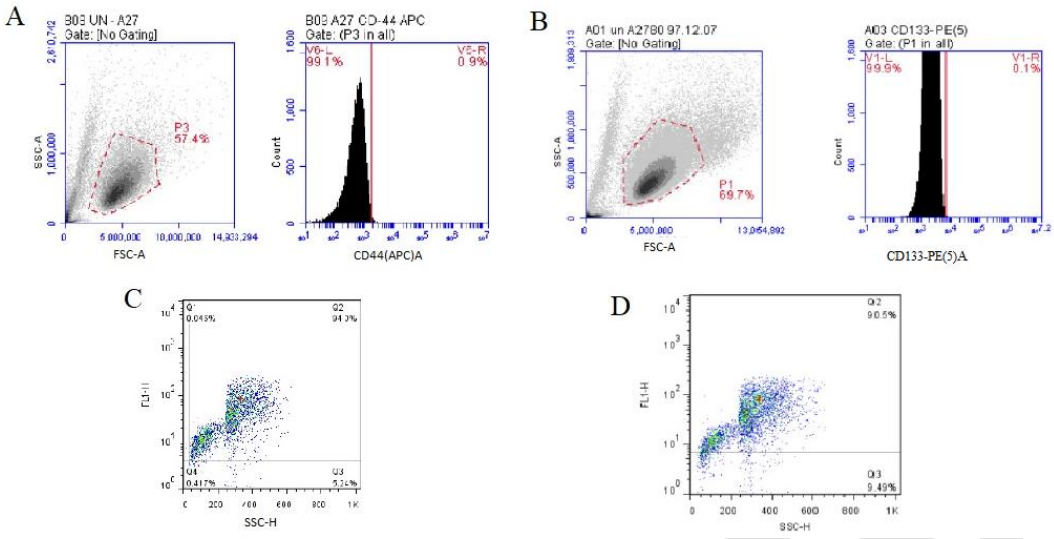

Figure 2. Expression of CD44 or CD133 surface marker in A2780 cell line. Flow cytometry analysis showed that the frequency of CD44+ (A) or CD133+ (B) cells in the A2780 cell line respectively was 0.9%, and 0.1%. Also, the high expression of CD44 (C) or CD133 (D) in isolated cells confirmed their accurate isolation

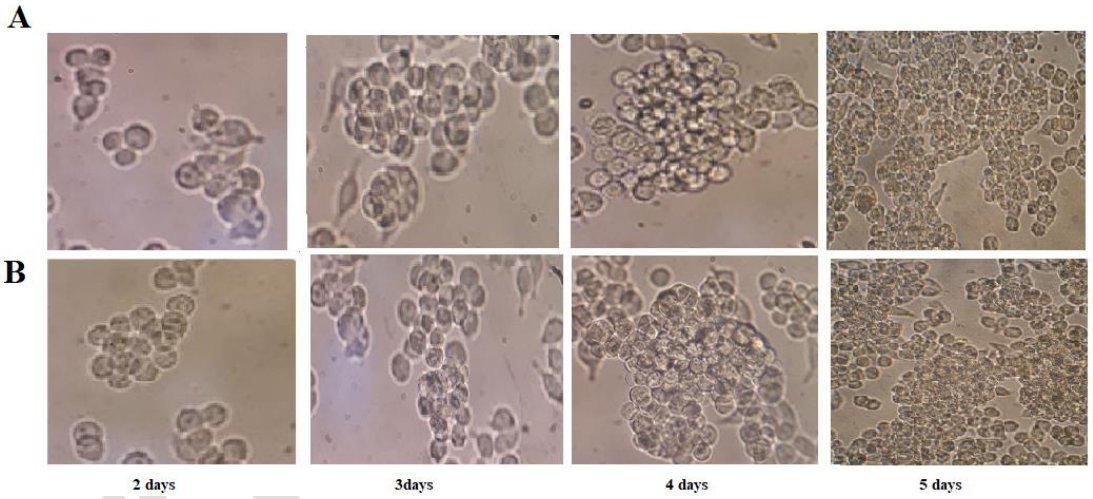

Figure 3. Spheroid colony formation ability in CD44+ (A) or CD133+ (B) cells isolated from the A2780 cell line under specific serum-free condition at the indicated times. Scale bars represent 200 µm

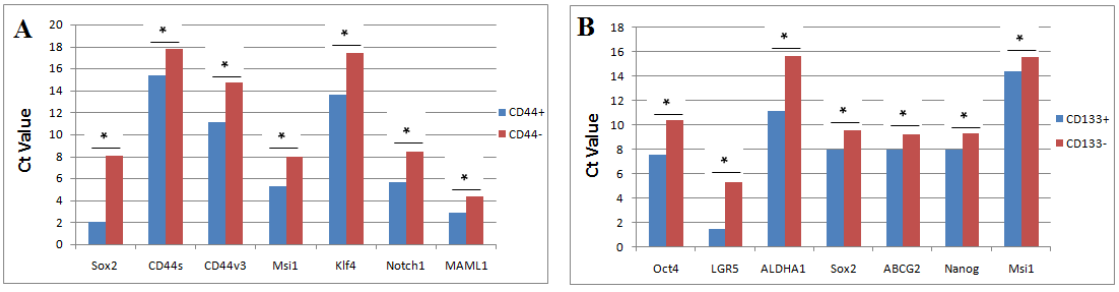

Figure 4. Expression of stem cell markers in CD44+ (A) or CD133+ (B) cells. Compared to their negative counterparts, CD44+ enriched CSCs overexpressed Sox2, CD44s, CD44v3, Msi1, Klf4, Notch1 and MAML1 markers. While, significant increased expression of Sox2, Nanog, Oct4, ABCG2, ALDHA1, LGR5 and Msi1 markers was observed in CD133+ cells compared to their negative counterparts. Data are shown as mean ± SD (* p<0.05)

4.3. Drug resistance

In this regard, CSCs cells were treated serially with diluted concentration of 5FU for 48 h. As it was demonstrated in Figure. 5, CD44+ cells showed remarkable higher viability at 10 µm/L 5FU compared to CD44- cells (P<0.01). While, CD133+ cells showed significant higher viability at 0.1 and 0.01 µm/L 5FU compared to CD133- cells (P<0.01).

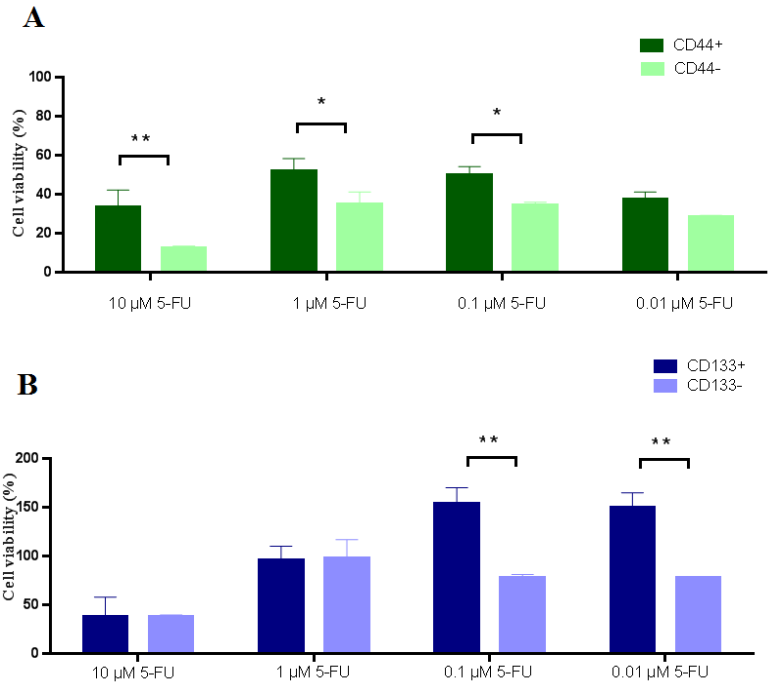

Figure 5. Resistance to 5Fu in CD44+ (A) or CD133+ (B) enriched CSCs. CD44+ cells showed higher viability at 10 µg/ml compared to CD44- cells. Moreover, CD133+ cells indicated higher viability at 0.1 and 0.01 µg/ml compared to CD133- cells. * P value<0.05, ** P value<0.01

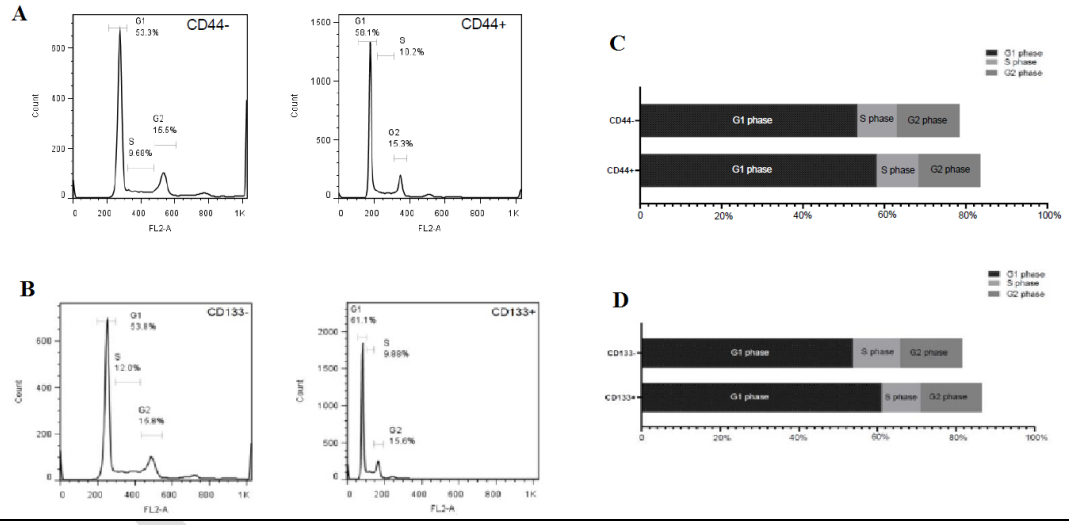

Figure 6. Cell cycle regulation in A2780-isolated CSCs. Histograms represent flow cytometric analysis of cell cycle in CD44- and CD44+ cells (A) or CD133- and CD133+ cells (B). Boxes demonstrate the longer length of CD44+ (C) or CD133+ (D) in G1 phase rather than S one

Therefore, CD44+ or CD133+ cells had higher resistance to chemotherapeutic agents than their negative counterparts.

4.4.Cell cycle regulation

PI flowcytometry was used to compare the percentage of CD44+ or CD133+ cells with their negative counterparts regarding cell cycle regulation. The results evidenced that the main number of CD44+ or CD133+ A2780 cells were accumulated in G1 phase. In contrast, higher percentage of CD44- or CD133- cells were in the S phase, indicating the diverse role of CD44+ or CD133+ in cell cycle regulation compared to their negative counterparts (Figure. 6).

4.5. Transfection and expressional analysis

To evaluate the effects of MAML1, the main co-activator of Notch pathway, on preservation of CSCs, HEK293 cells were transfected with MAML1 silencing vectors with high efficiency (more than 70%). Subsequently, real-time PCR results

demonstrated that the best down-regulation of MAML1 was achieved by shRNA3-MAML1 recombinant vectors.

4.6. Role of MAML1 in biology of CD44+ CSCs

Since CD44+-enriched CSCs have higher expression of MAML1, they were selected for further analysis. Therefore, CD44+ CSCs were transfected with constructed PRNAT-H1-Neo-MAML1 silencing vector (selected based on the best down-regulation of MAML1) using PEI-pro transfection reagent (Figure. 7) leading to noticeable down-regulation of MAML1 followed by significant decreased expression of the Notch target genes (important downstream targets of MAML1), including Hes1, Hey1, and Hey2 compared to scramble (P<0.05) (Figure 8A).

4.7. The role of ectopic silence of MAML1 on EMT markers expression

Following down-regulation of MAML1 in A2780 cells, the cells changed morphologically from epithelial (cobblestone) to mesenchymal (spindle) phenotype (Figure 8B). It seems that the morphological changes following the MAML1 silencing can be associated with significant reduced levels of Snail, Slug, Twist1, Fibronectin, and Zeb2 as well as miR-200c and miR34a in silenced cells compared to scramble (P<0.05) (Figure 8C).

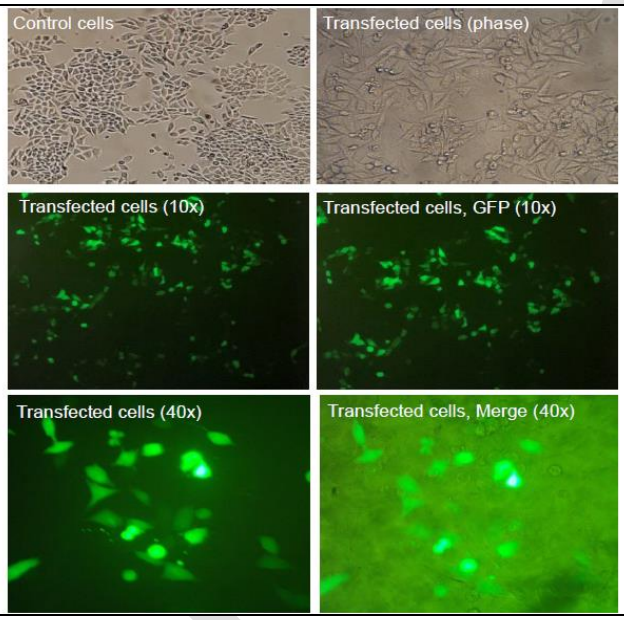

Figure 7. Phase and fluorescence view of the transfected CD44+ CSCs following ectopic silence of MAML1 revealed the high transfection efficiency

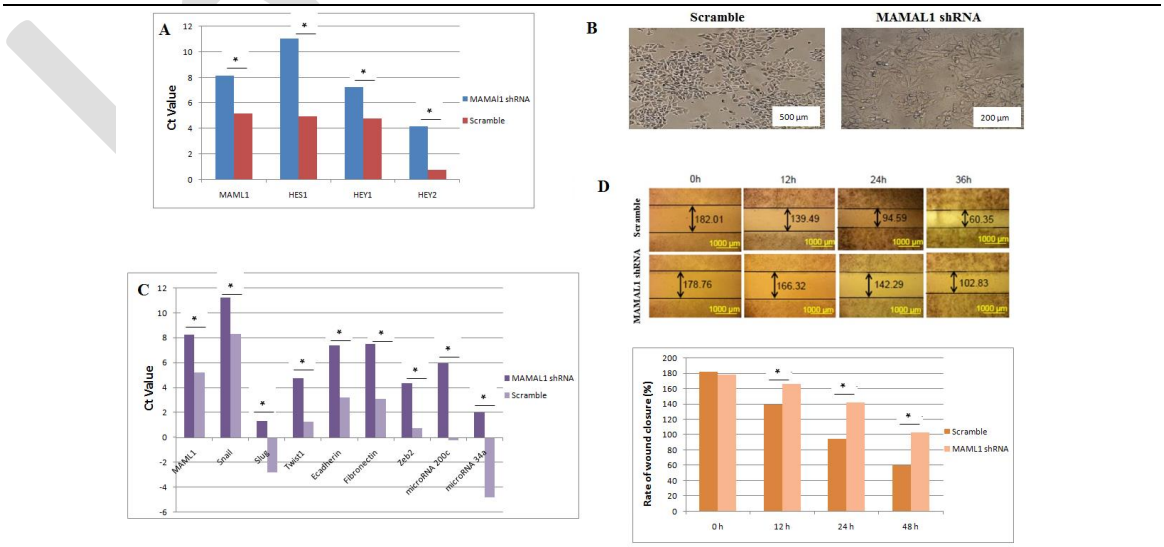

Figure 8. Role of MAML1 in biology of CD44+ CSCs. (A) increased expression of Notch target genes in the level of mRNA in transfected cells compared to scramble. (B) morphological changes in CD44+ cells prior to and 48 h following the MAML1-shRNA transfection. (C) significant enhanced mRNA expression of Snail, Slug, Twist1, Fibronectin, Zeb2, miRNA200c and miRNA34a in A2780 cells 48 h post-transfection compared to scramble. (D) migration of MAMAL1-transfected cells was decreased after ectopic silence of MAML1 during 36 h intervals compared to the scramble. Data are shown as mean ± SD (* p<0.05)

4.8. The role of ectopic silence of MAML1 in ovarian CSCs migration

One of the most significant features of the CSCs is their ability to migrate and cause metastasis via EMT process. To evaluate this process, a scratch assay was implemented to analyze the probable role of the Notch pathway in the cell migration. Our findings provided evidence that the migration of CSCs was significantly reduced following the MAML1 silencing compared to scramble ($P<0.05$) (Figure. 8D).

5. Discussion

Ovarian cancer is the fifth leading cause of death among women worldwide (22). Although, the upgraded treatment approaches have improved the survival rate of the patients in the last decade**,** tumor recurrence and high mortality rate are still frequent due to the lack of complete tumor cells elimination. Because of tumor relapse and drug resistance after standard chemotherapy, it is important to find a novel strategy to overcome this obstacle (23). One of the main factors in resistance of ovarian cancer to chemotherapeutic agents is the presence of CSCs (24). Given the importance of ovarian CSCs in drug resistance and tumor recurrence, their elimination can be considered as an effective therapeutic option (25). Based, targeting CSCs via surface markers has been introduced as a desirable strategy to eradicate these cells. There are lines of evidence shown that CSC surface markers such CD133, dehydrogenase1/2 (ALDH1/2), LGR5, EpCAM, CD44, CD34, CD24, CD117, MyD88 and CDH1 have been used to isolate CSCs from ovarian malignant cell lines (26-35). Amongst, CD44 is an adhesion protein that has been widely defined as a CSC marker in different malignancies such as gastric, colon, esophageal squamous cell carcinoma (ESCC**)**, and breast cancers (36-39). As well, CD133 not only is one of the most common marker for CSCs' isolation in many types of tumor cells, but also has been reported to develop tumorgenesis in glioma cells and human lung cancer (35,40). Importantly, it was evidenced that targeting CD44 and CD133 surface markers in the ovarian cell lines with specific agents reduced tumor progression (41,42).

In the present study, for the first time, CD44+ or CD133+cells were isolated from the ovarian A2780 cell line and their stemness characteristics have been validated via sphere formation, high expression of stemness markers, chemoresistance and cell cycle regulation. Because of higher expression of MAML1 in CD44+ enriched A2780 CSCs, they were selected for further mechanistic analysis. In a comparable study in 2015, it was revealed that sorted CD133+CXCR4+ population could highly express stemness markers such as OCT4, SOX2, KLF4 and NANOG and had the highest potential in sphere formation in OVCAR-3, OVCAR-4 and OVCAR-5 cells (43).

Another study has reported that CD133+ cells display higher resistance to platinum-based therapy, the first-line drugs being regularly used in ovarian cancer (44). In another study conducted in 2018, CD133+ cells derived more spheres formation, larger tumors, and higher expression of ring finger protein 5, smoothened, frizzled class receptor, POU class 5 homeobox1, NANOG and c-MYC compared to CD133− cells (45). Accordingly, a higher percentage of CD133+, and CD44+ CSCs isolated from colorectal cancer patients was established in the G0/G1 phase (46). Therefore, it seems that the CD44 and CD133 could be introduced as key markers for ovarian CSCs. In detail, in the current study we used surface markers to isolate CSCs as a more specific method compared to other low specific techniques such as intracellular marker of ALDH (47), or application of side population (SP) cells with high expression of ATP-binding cassette (ABC) transporters (48).

Among different methods to eliminate ovarian CSCs, it can refer to targeting the self-renewal signaling pathways such as WNT, Notch, and SHH (10). NOTCH pathway operates via both canonical and non-canonical pathways respectively involved in biology and maintenance of CSCs and normal SCs (49). Therefore, it is important to target the Notch pathway only in CSCs without any undesirable impacts on normal SCs. Since MAML1, a main transcription co-activator of Notch signaling pathway, only interferes with the canonical pathway, upregulates HES1,5,7/HEY1, two genes keeping the features of SCs and inhibiting the CSCs differentiation, and take parts in the ESCC progression and metastasis (50). We selected it to target NOTCH pathway in A2780 CSCs. In this regard, our findings pinpointed that silence of MAML1 in CD44+ A2780 CSCs resulted in significant downregulation of the Notch target genes, EMT markers, and migratory potential of CSCs. Similarly, it was shown that there was a significant correlation between MAML1 expression and 5FU resistance in CD44+ ESCC CSCs (29). Consistently, correlation between MAML1 and Twist as an EMT marker was also confirmed in our previous study (51). Also, we showed that MAML1 regulates EMT markers expression through Notch pathway in breast cancer (52). Furthermore, therapeutic targeting of the main players of TGF-β, Wnt, Notch, TNF-α, NF-κB, RTK signaling pathways involved in EMT process could affect growth of CSCs (53). Moreover, we have found that suppression of MAML1 also reduced the expression of miR-34a and miR-200c. The role of miRNAs and their various clusters, including the miR-200c, has been revealed in targeting genes and key pathways in CSC maintenance and survival, such as the BMI1 autogenesis gene, apoptosis, Notch and EMT pathways (54).

6. Conclusion

Given the importance of CSCs in treatment failure and enhanced tumor recurrence, precise eradication

of these cells could be an effective treatment for different malignancies such as ovarian cancer. Since this therapeutic approach is particularly challenging, finding the main factor involved in chemo-therapeutic resistance is of great value**.** The results of the present study showed that targeting canonical Notch pathway in CD44+ ovarian CSCs can be introduced as a novel targeted therapy to eliminate CSCs evidenced by decreased EMT and migration.

Acknowledgments

We would like to thank the Department of Human Genetics of Bu-Ali Research Institute (Mashhad, Iran) and the Department of Biology of Shahid Chamran University of Ahvaz (Ahvaz, Iran**).** The study leading to these results received funding from Mashhad University of Medical Sciences under Grant Agreement No. 951565 and Shahid Chamran University of Ahvaz under Grant Agreement No. SCU.SB99.78

Footnotes

Conflicts of Interest: Authors declare no conflict of interest.

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