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The effect of isolated-exosomes from human umbilical cord Wharton jelly stem cells on expression of driver genes in ovarian cancer cell line

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Abstract

Background: Ovarian cancer is a prevalent cancer among Iranian and global women, with high death rates due to early detection and lack of early diagnostic techniques. The spindle-like microcephaly-associated protein (ASPM) and Minichromosome maintenance complex component (MCM7) genes are crucial for tumor genesis, invasion, and metastasis, and abnormal expression can lead to tumor emergence. It has been showed that exosome from Wharton jelly stem cells effectively inhibit cancer cell growth in patients. So, the purpose of this study is to investigate the effect of exosomes isolated from Wharton's jelly mesenchymal stem cells (WJ-MCSs) on the expression of ASPM and MCM7 genes in the ovarian cancer OVCAR-3 cell line

Method and Material: After the primary culture of WJ-MCSs, culture medium was prepared, and exosome was extracted using the exosome kit. The SEM microscope and western blot technique were used to check the validity of the extracted exosome. The OVCAR-3 cell line was cultured and treated with different concentration of exosomes. The MTT test and wound healing assay were used to investigate the inhibitory effects of the Wharton jelly stem cell exosome (WJ-MSCs-Exo) on OVCAR-3 cancer cells. After mRNA extraction, the expression of the ASPM and MCM7 genes was evaluated using the qPCR method in the WJ-MSCs-Exo-treated OVCAR-3 cell.

Results: Exosome extraction was confirmed by western blotting of CD-81, CD63, and CD-9 and SEM technique. The MTT and wound healing assay tests showed that the WJ-MSCs-Exo significantly reduced the migration and decreased the viability of OVCAR-3 cells, respectively. Moreover, the mRNA expression of ASPM and MCM7 was considerably elevated in OVCAR-3 cells treatment with WJ-MSCs-Exo ($p< 0.05$)

Conclusion: Overall, WJ-MSCs-Exo could prevent significantly cell growth and migration of ovarian cancer OVCAR-3 cell line in a concentration-dependent manner. This findigs could be associated by modulating the expression of genes increased expression of ASPM and MCM7 genes.

Key words: WJ-MCS; Exosome; ASPM; MCM7; Ovarian cancer

1. Introduction

Ovarian cancer is the fifth leading cause of cancer-related death in women and the probability of death from ovarian cancer increases with age. However, this disease can occur at any age. One of the signs of ovarian cancer is genomic instability. Although ovarian cancer is classified into several clinical subtypes, each subtype exhibits significant genetic and progressive variability, and among the most common types of ovarian cancer is ovarian epithelial cancer[1, 2].

Progress in the application of stem cells has significantly improved the treatment of ovarian cancer[3]. Mesenchymal stem cells (MSCs) can regenerate and transform into various types of cells[4, 5]. However, MSCs obtained from Wharton's jelly are more primitive in terms of development and show characteristics between adult and embryonic stem cells. The distinctive characteristics of Wharton's jelly mesenchymal stem cells (WJ-MSCs) have made them intriguing options in cell therapy and regenerative medicine. WJ-MSCs have the potentiality to cure tissue injury due to their strong capability for adhesion and inducing angiogenesis[6, 7].

The therapeutic potential of MSCs in cancer is still controversial. While some studies indicate that MSCs may contribute to the suppressive effects of MSCs on cancer cells[8]. Investigation has revealed that the inhibitory effect of intraperitoneal administration of MSCs on non-Hodgkin's lymphoma[9]. Moreover, human umbilical cord mesenchymal stem cells (hUCMSCs) significantly reduced metastatic breast cancer cells (MDA-MB-231) growth following intravenous injection in contrast to the control cohort, with evidence suggesting these effects are mediated via exosomes[10, 11]. It has been suggested the effects of WJ-MSCs are mediated via extracellular vesicles (EVs) known as exosomes. Exosomes, a small subtype of EVs (40 to 160 nm), are detectable in bodily fluids and influence cell physiological activities through paracrine mechanisms. They can initiate epigenetic modifications, contribute to genetic reprogramming, and transmit inhibitory or stimulatory signals. Depending on their cell of origin, exosomes exhibit similar traits and functionalities as the parental cell. Specifically, exosomes originating from MSC demonstrate identical functions to the own source cell like MSC[12, 13].

The ability to find genes that distinguish ovarian cancer classes into molecular subtypes has been made possible by advancements in omics technology. Additionally, studies in this area have found genes linked to ovarian cancer that are either frequently mutated in the disease or have a hereditary form. This has produced a vast amount of data regarding drug resistance, metastasis, development, and progression, pushing the limits of precision medicine and improving patient care[14, 15]. Studies have linked abnormal spindle-like microcephaly-associated protein (ASPM) to ovarian cancer through bioinformatics analysis. ASPM is involved in mitosis and DNA damage repair, but their expression is deregulated at the RNA level in ovarian epithelial cancer[16, 17]. Studies have determined that ASPM is correlated with clinical data provided from primary cultures of patients with ovarian cancer[18]. Minichromosome maintenance complex component (MCM7), a gene involved in DNA replication and synthesis, was found to be over-expressed in high-grade serous carcinomas compared to serous borderline tumors of the ovary[19].

Given the importance of driver genes in exosome research and the introduction of exosomes as a new method in cancer treatment and drug delivery, we intend to investigate the effect of WJ-MSCs-derived exosome (WJ-MSCs-Exo) on the expression of two selected driver genes, ASPM and MCM7, in the ovarian cancer cell line, OVCAR-3.

2. Methods and Materials

Culturing of Wharton's jelly mesenchymal stem cells (WJ-MSCs)

Human umbilical cord was obtained from a full-term infant born through cesarean section according to the Ethics Committee of Tabriz Azad University. The cord was chopped into 2-cm pieces after being twice cleaned in PBS solution. Each portion was explanted in the T25 flasks after the vessels were taken out and the Wharton's jelly was divided into 4 to 5 mm. DMEM media were introduced to each flask and the T25 flasks were incubated with 5% CO2 at 37 °C. The cells were subcultured once they had achieved 80% confluence.

Exosome isolation

For obtaining exosome, the WJ-MSCs (passage 3 or 4) were cultured until 80 % confluence in media supplemented 10 % FBS. The cells were washed with PBS, and incubated with serum-free media. After 24 hours, WJ-MSCs-Exo was isolated from the supernatant of the cells by exosome isolation kit (Ana Cell Teb, Iran). In the first step, centrifugation was performed at 3000 rpm for 10 minutes to remove derbies and suspended cells. Then, the sample and reagent A (3, 3′-diaminobenzidine (DAB: 20X) heated to 37°C) were mixed in a ratio of 1:5 and incubated at 4°C for 12 hours. At the end of the incubating period, the solution was centrifuged at 3000 rpm for 40 minutes at 4°C. In the next step, the supernatant was discarded and the white pellet (exosomes) was dissolved in 200µl reagent B (H2O2: 20X). The isolated exosome was stored at 4°C for several days and at -20°C or -80°C for a long time. In order to confirm WJ-MSCs-Exo, western blotting of CD markers including CD81, CD63, and CD9 was performed.

Protein quantitation

Protein concentration of the isolate exosome was quantified using a Bradford method according to the instructions. The fractions were quantified against a bovine serum albumin standard to determine protein concentration

Scanning electron microscopy (SEM)

For microscopic observation of the isolated exosome, a small amount of exosome was fixed in 100-200µL of 2.5% glutaraldehyde solution and then washed with PBS buffer. The exosome sample was dehydrated by ethanol and dry surface covered with a thin layer of gold. Finally, the morphology and size of exosomes were evaluated and observed by scanning electron microscope (Olympus BX50).

OVCAR-3 Cell line culture

The human ovarian epithelial carcinoma cell line, OVCAR-3, was obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI1640 media without phenol red was used in cell culture. The media was supplemented with 20% heat-inactivated FBS, 50 IU/ml of penicillin, and 50µg/ml of streptomycin. Every two days, a new media was used. In a 37°C incubator, cells were cultured in 75 cm^2 tissue culture flasks using a humidified combination of 95% air and 5% CO2.

MTT assay

OVCAR-3 Cell (10,000 cells/well) cells were harvested in a 96-well plate. After 24 h, the culture medium was replaced with WJ-MSCs-Exo (Dose: 2, 4, 8, 16, 24, 32 and 48 µg/ml) for 24, 48, and 72 h. Then, MTT solution (0.5mg/mL) added to all of the wells, followed by incubation of the plate at 37 °C for 4 h. When the supernatants removed, 100µLof DMSO added to all of the wells, and the absorbance at 570 nm was measured by a microplate reader (BioRad, CA).

Migration assay

OVCAR-3 Cells were seeded into six-well plates. After reaching 90% confluence, wounds were created in each well with a sterile 1 ml micropipette tip. The floating cells were washed twice with PBS. The OVCAR-3 Cells received different treatments. Images of each scratch were observed at 0, 24, 48, and 72 h with an optical microscope (Olympus, Japan). The percentage of wound closure was assessed using ImageJ (version 1.52a; Media Cybernetics, USA)

Real-time quantitative PCR

Total RNA isolation was performed using RNAX-Plus solution kit (Cinnagen, Tehran, Iran) according to the manufacturer's protocol and Then, RNA samples reverse-transcribed to cDNA using a Parstous first strand synthesis kit (Tehran, Iran). The purity and the quantity of the RNA and cDNA were determined by spectrophotometer at optical densities of 260 nm and 280 nm, respectively. Real-time was performed on each cDNA sample and quantified on a LightCycler®96 real-time PCR detection system (Roche Molecular Systems, Inc) using an SYBR Green PCR Master Mix (Yekta Tajhiz, Tehran, Iran). The following shows the sequence of primers used for the Q-PCR: ASPM forward 5′-GCTGCCATTATTATTCAGAAGCATTGT-3′, ASPM reverse 5′-GCACTGCAGTTAGTTT TCTGTATCTT-3′; MCM7 forward 5′-GATGCCACCTATACTTCTGCCG-3′, MCM7 reverse 5′-GCTCTTTGACATCTCCATTAGCC-3′; GAPDH forward 5′-GAAGGTCGGAGTCAACG GAT-3′, GAPDH reverse 5′-CTTCCCGTTCTCAGCCATGT-3′.

Statistical analysis

Results from at least three independent experiments are reported as the means ± standard deviation (SD). Statistical analyses were performed using SPSS v21. A value of $P<0.05$ was considered to be statistically significant

3. Results

Characteristics of WJ-MSCs-Exo

Scanning electron microscopy (SEM) and western blotting were used to characterize the WJ-MSCs-Exo. As presented in Figure 1A, the results obtained from the SEM show the extracted exosomes from the condition medium of WJ-MSCs have various sizes. The size of all vesicles is in the range of 30 to 150 nm and most of them are less than 150 nm, so according to the size reported for the exosome, the findings show the accuracy of separating exosomes from condition medium. Furthermore, the exosome biomarker proteins (CD81, CD63, and CD9) were detected by western blotting. The results showed that CD81, CD63, and CD9 were highly expressed in the suspension extracted (Fig 1B)

WJ-MSCs-derived exosome inhibits tumor growth in vitro

As shown in Figure 2, no significant changes in viability percentage of OVCAR-3 cells were observed within 48 h of incubation with 48µg/ml dose of WJ-MSCs-Exo in comparison to the 24 h incubation. The results of the MTT test in OVCAR-3 cells treated with WJ-MSCs-Exo show a decrease in cell survival depending on time and drug dose. According to the obtained results, a significant reduction survival rate of OVCAR-3 cells was observed within 72 h incubation with 48% dose of WJ-MSCs-Exo ($P < 0.05$), which is considered the IC50 in this study (Fig 2).

WJ-MSCs-Exo impede cell migration

To investigate the effect of WJ-MSCs-Exo on wound healing in vitro, we performed a wound healing assay. The wound healing assay or scratch test by comparing the prepared images

illustrated that WJ-MSCs-Exo significantly inhibits the wound healing promotion in ovarian OVCAR-3 cancer cell line as compared to the control group (Fig 3).

Real-time PCR

WJ-MSCs-Exo at a dose of 48µg/ml significantly increased the expression of ASPM and MCM7 genes. WJ-MSCs-Exo significantly increased ASPM gene expression in ovarian cancer cells (OVCAR-3 cell line) by 1.86 times compared to the control group, with $p < 0.0001$. Moreover, MCM7 gene expression increased 2.04 times in the treatment group compared to the control group ($p < 0.0001$), indicating significant changes (Fig 4).

Discussion

WJ-MSCs from human umbilical cord tissue have potential for treating chronic diseases due to their systemic and local efficacy through the secretion of diverse active factors. Furthermore, WJ-MSCs have been studied extensively and presented as an anti-cancer treatment in clinical condition[20, 21]. The ongoing study confirmed the antitumor role of WJ-MSCs, It has been showed recently that WJ-MSCs inhibit the growth and proliferation of prostate cancer cell[22]. hendijanni et al., confirmed anti-leukemic effects of WJ-MSCs by induction of apoptosis and reduction in viability of HL-60 and K562[23]. WJ-MSCs can also deliver antitumor factors to primary tumors; this characteristic makes them an excellent means for delivering antitumor factors to the tumor location and making them ideal for cancer treatment. However, there are also limitations in this area include immune system challenges, GVHD risk, and cell transplantation restrictions[20]. To prevent such problems, the use of exosomes in the treatment of diseases can be considered a novel way to overcome the limitations of MSCs[24, 25]. However, the role of WJ-MSCs-Exo on molecular signaling pathway in ovarian cancers is not yet clear and is still unknown. Therefore, in this study, we investigated the anticancer effect of WJ-MSCs-Exo on changes in the expression of two selected driver genes; ASPM and MCM7 genes in ovarian cancer.

Cancer is caused by changes in regulatory genes, which are crucial for cell survival, differentiation and proliferation, under the influence of a complex interplay of environmental and genetic factors[26]. In other words, cancer development occurs through a series of complex interactions between multiple environmental and genetic factors[27]. In recent times, the utilization of gene profiling and gene arrays has been employed for the purpose of identifying genes that exhibit different expression patterns. Studies have linked the changes in the expression of ASPM and MCM7 genes to various cancers, including ovarian cancer. Thus, considering the importance of ASPM and MCM7 genes in the development of ovarian cancer, investigating and evaluating the effects of exosme on the function of ASPM and MCM7 genes may contribute to the proposal of them as crucial and reliable molecular markers in the treatment of ovarian cancer [18, 28, 29].

Aberrant Wnt/β-catenin signaling is also closely related to the high incidence of various human cancers[30]. Wnt signaling pathway has been found to be positively regulated by ASPM, and abnormalities in ASPM gene expression have also been linked to the development of various cancers[31]. Studies have demonstrated the Wnt/β-catenin signaling is aberrantly activated in about 40% of endometrial cancers[32] and individuals with endometrial cancer have higher levels of ASPM expression[33, 34]. Moreover, according to studies, patients with ovarian cancer who have a high MCM7 labeling index are at lower risk of dying as a whole. MCM7 therefore serves as an effective marker for outcome and disease prediction [28, 35]. MCM7 plays a role in the proliferation of tumor cells, colony formation and migration of cancer cells through inhibiting the AKT1/mTOR signaling pathway[36]. According to studies, altering the expression levels of the ASPM and MCM7 genes could be

a beneficial therapy option for people with ovarian cancer. Many studies investigated the changes in the expression of ASPM and MCM7 genes in various cancers cells progression. The product of this gene plays a key role in the Wnt/β-catenin signaling pathway in the development and differentiation of cells and is closely related to tumorigenesis, invasion and metastasis[16, 37]. Our finding showed that the expression level of ASPM and MCM7 genes were significantly increased up to1.86 and 2.04 fold, respectively, in response to WJ-MSCs-Exo treatment in OVCAR-3 cancer cells compared to the control group. In relation to ovarian cancer, the studies conducted in 2011 by ota and his colleagues; showed that the expression level of MCM7 gene in ovarian cancer increases compared to normal tissue [28]. In 2020, a study conducted by Jing-Wei Zhou showed; ASPM gene expression also increases in ovarian tumor tissue and acts as a clinical prognosis[34]. Moreover, the results of this study indicated that WJ-MSCs-Exo exposure inhibited cell viability in OVCAR-3 cells, as evidenced by MTT results at concentrations of 48µg/ml and 72 µg/ml. Furthermore, the migration assay tests showed that OVCAR-3 cells treated with WJ-MSCs-Exo have a considerably lower rate of cell migration than the controls.

Conclusion

WJ-MSCs have been proven to mitigate the growth of malignancies of both hematological and non-hematopoietic origin in vitro. In our work, WJ-MSCs-Exo therapy significantly reduced the viability and migration of OVCAR3. Our findings also shed light on how WJ-MSCs restrict the proliferation of OVCAR-3 cells in vitro by altering the expression of ASPM and MCM7 genes, which act as drivers genes.

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