

Investigating changes in the post-transcriptional pattern of *VEGF* and *CD146* genes carrying *miR-573* in breast cancer cells treated with tamoxifen

Zkoumání změn posttranskripcního úprav genů *VEGF* a *CD146* nesoucích *miR-573* v buňkách karcinomu prsu ošetřených tamoxifenem

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Summary

Background: Recent developments regarding the contribution of microRNAs (miRNAs) to tumor angiogenesis and the oncogenic effects of miRNAs point to their potential role in breast cancer angiogenesis. Tumor-derived exosomes are considered a rich source of miRNAs that can regulate the function of other cells in the tumor microenvironment, including vascular endothelial cells. This study analyzes the effect of tamoxifen chemotherapy on the expression of a key miRNA, *miR-573*, involved in the angiogenesis of the tumor exosomes and introduces a regulatory link between this miRNA and the *CD146* gene associated with the vascular endothelial growth factor (*VEGF*) messaging pathway. **Materials and methods:** MCF-7 breast cancer cells were purchased and cultured in a complete culture medium. These cells were treated with tamoxifen and then their exosomes were extracted from the culture medium. The RNAs of the exosomes were isolated and the expression of *miR-573*, *VEGF*, and *CD146* genes in the exosomes was investigated using the real-time polymerase chain reaction (PCR) method. **Results:** The results of this study showed that tamoxifen treatment increased the expression of *miR-573* in exosomes derived from MCF-7 cancer cells. The expression of *CD146* and *VEGF* genes in drug-treated cell exosomes had a downward pattern. **Conclusion:** The results of this experiment demonstrated that the treatment of breast cancer cells with tamoxifen reduces the expression of *VEGF* and *CD146* by increasing *miR-573*. Thus, angiogenesis is reduced and, therefore, its anti-tumor effects are applied.

Keywords

breast cancer – tamoxifen – tumor angiogenesis – exosome – miR-573

The authors declare that they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.



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Submitted/Obdrženo: 20. 1. 2023
Accepted/Přijato: 20. 7. 2023

doi: 10.48095/ccko2023447

Souhrn

Východiska: Nejnovější výzkumy týkající se podílu microRNA (miRNA) na nádorové angiogenezi a onkogenních účinků miRNA ukazují na jejich potenciální roli při angiogenezi karcinomu prsu. Exozomy získané z nádoru jsou považovány za bohatý zdroj miRNA, které regulují funkce ostatních buněk v nádorovém mikroprostředí, vč. endoteliálních buněk cév. Tato studie analyzuje účinek chemoterapie tamoxifenem na expresi klíčové miRNA, *miR-573*, která se účastní angiogeneze v exozomech nádoru a působí jako regulační spojka mezi miRNA a genem *CD146*, který je součástí dráhy vaskulárního endoteliálního růstového faktoru (vascular endothelial growth factor – *VEGF*). **Materiál a metody:** Byly zakoupeny buňky karcinomu prsu MCF-7 a kultivovány v kompletním médiu. Tyto buňky byly ošetřeny tamoxifenem a z médií byly následně exrahovány jejich exozomy. Byla izolována RNA exozomů a pomocí metody polymerázové řetězové reakce (PCR) v reálném čase byla vyhodnocena exprese genů *miR-573*, *VEGF* a *CD146* v exozomech. **Výsledky:** Výsledky této studie ukázaly, že ošetření buněk tamoxifenem zvýšilo expresi *miR-573* v exozomech získaných z nádorových buněk MCF-7. Expresi genů *CD146* a *VEGF* v exozomech buněk ošetřených léčivem měla klesající tendenci. **Závěr:** Výsledky tohoto pokusu prokázaly, že ošetření nádorových buněk tamoxifenem snižuje expresi genů *VEGF* and *CD146* zvýšením *miR-573*. Dochází tedy k omezení angiogeneze a tím k protinádorovému působení léčiva.

Klíčová slova

karcinom prsu – tamoxifen – nádorová angiogeneze – exozom – miR-573

Introduction

Breast cancer occurs as a result of the abnormal growth of cells in the breast. That growth pattern stops at a certain stage in benign tumors, whereas the malignancy development continues uncontrollably in malignant tumors that if left untreated, affect and disable all parts of the body [1]. Tamoxifen is a drug used to reduce or stop the effects of the female hormone, estrogen, in the body and is one of the drugs used in breast cancer treatment [2]. MicroRNAs (miRNAs) are a new class of endogenous non-coding RNA molecules that regulate gene expression at the post-transcriptional level by mRNA degradation and/or translation inhibition. There exist more than 2,000 miRNA-encoding genes in the human genome, which make up about one percent of the whole genome [3]. The essential role of miRNA in the processes of carcinogenesis and oncogenesis is obvious. Available evidence suggests that abnormal levels of expression in some specific miRNAs can cause misalignment of cancer cells. MiRNA molecules can play an important role in disease control at the biological stages. These miRNAs can act as oncogenes or tumor suppressor genes. The study of the expression of total genome miRNAs in recent years has shown more detailed information about the intervening role of these small regulatory molecules in human cancers [4].

Exosomes are small vesicles (50–100 nm) that have recently been known as intercellular communication linkers.

Exosomes can transfer lipids, proteins, mRNAs, and miRNAs to the receptor cell when moving to other cells. Cancer cells have been shown to secrete far more exosomes than normal cells, thus transmitting tumor-dependent signaling molecules to surrounding cells [5].

MiRNAs are among the molecules being transferred by exosomes and regulate most of the cell's biological functions. They regulate translation and transcription programs and coordinate pathological as well as physiological processes such as development, cell differentiation, proliferation, apoptosis, and tumor growth [6]. MiRNAs act as small guide molecules in RNA silencing and negatively regulate the expression of various genes, both at the miRNA level and the protein level, by degrading the target miRNA and silencing the translation. One of the recent developments in this field of study is the understanding of their role in oncogenesis. The number of miRNA genes is increasing, and changes in the level of miRNAs play a role in the initiation, progression, and metastasis of various tumors. Biochemical and genetic evidence confirms the role of miRNAs in tumor development. The abnormal expression of miRNAs in cancer cells is a well-known phenomenon, the cause of which is yet unidentified [7]. The exact mechanism by which tumor cell-derived exosomes affect their constituent micro-environmental cells, including endothelial cells, has not yet been determined, and the biological function of exosomal miRNAs in receptor cells is not well

understood. Therefore, this study investigates the effect of tamoxifen chemotherapy drug on the expression of a key miRNA involved in angiogenesis, *miR-573*, in tumor exosomes and introduces a regulatory relationship between this miRNA and *CD146* gene, associated with vascular endothelial growth factor (*VEGF*) messaging pathway.

Materials and methods

Cell preparation and culture

The MCF-7 breast tumor cell line with NCBI CODE 135 and HUVEC cell line were both purchased from the Iranian Genetic Resources Center. Subsequently, the cells were cultured in a full culture medium containing FBS and trypsin (GibCo). The flasks were kept inside an incubator with CO₂ saturation rate 5% and a temperature of 37 °C.

Tamoxifen treatment

Based on the results of various experiments and resource reviews [8], tamoxifen IC₅₀ was a concentration between 54 µM (equivalent to 20 µg/mL) and 68 µM (equivalent to 25 µg/mL). Hence, the concentration of 60 µM (as a concentration close to IC₅₀) was considered for the treatment in this study.

Exosome isolation

Exosomes were isolated from the culture medium of MCF-7 cells. For this purpose, the culture medium was first centrifuged at 3,000 g for 10 minutes to separate the dead cells from the culture medium. Exoquick solution (System

Tab. 1. The sequence of forward and reverse primers used to study gene expression.

Genes	Primers	Primer sequences
VEGF	forward	5-TGCAGATTATGCGGATCAAACC-3
	reverse	5-TGCATTACATTGTTGTGCTGTAG-3
CD146	forward	5-GGGTACCCATTCCCTCAAGT-3
	reverse	5-CAGTCTGGGACGACTGAATG-3
U6snRNA	forward	5-CTCGCTTCGGCAGCACATATACT-3
	reverse	5-ACGCTTCACGAATTGCGTGTC-3
GAPDH	forward	5-ACCCACTCCTCACCTTTGA-3
	reverse	5-CTGTTGCTGTAGCCAAATTGCT-3

Biosciences) was then added to the supernatant. Vortex binds the exoquick solution to exosomes. Then it was incubated for 20 minutes at 4 °C. The centrifugation was afterwards performed at a speed of 1,500 g for 15 minutes. 50 µL PBS was then added to the solution and pipetting was performed. The content was transferred to a microtube and placed on a heater for 5 minutes at 70 °C and then at -20 °C. Exosome isolation was performed from the supernatants of both treated and control cell groups, confirmation of which was done by microscopic examination, digital electron microscope (Digital FESEM, KYKY-EM3200, China), evaluating the size and morphology of the exosomes.

Exosome labeling

PKH26 (PKH26 Red fluorescent – Mini26-1, Sigma) was used to label the exosomes. One mL of the exosome was transferred to a microtube, and 1 mL of diluent C was added to it and gently pipetted. From the diluted paint PKH26, 1 mL was added into the microtube which contained the exosome, and then incubated at room temperature for 5 min. To stop the labeling, 2 mL of diluent C was again added into the microtube and placed at room temperature for 5 more minutes. Then the exoquick solution was added with a ratio of 3 : 1 and kept at 4 °C for 20 minutes. Later, the solution was centrifuged for 20 minutes at 15,000 g. After centrifugation, the top solution was removed and the exosome-containing precipitate was dissolved in 30 µB PBS.

The endothelial cell was then treated with stained exosomes, followed by incubation at room temperature for 12 hours. Due to the presence of miRNAs in exosomes, the exosomal transmission of *miR-206* to fibroblast cells was investigated. In this regard, fibroblast cells were treated by 100 µg/mL tumor exosomes and 50 µg/mL alpha-amanitine. The rate of *miR-206* transferring to fibroblast cells was evaluated at 0, 24, and 48 hours post-treatment. The reason for using alpha-amanitine is to inhibit any transcription activity that can be induced by exosomal treatment in cells.

RNA isolation

RNA isolation from cells and exosomes was performed using the Trizol (Invitrogen) agent. After centrifuging the cells and adding 50 µL of Trizol solution, 15 µL of chloroform was added to it and incubated at room temperature for 2–3 minutes. After centrifugation at 12,000 g, the upper phase was removed and isopropanol was added and then the whole solution was incubated on ice for 10 minutes. The centrifugation was performed another time at 12,000 g for 10 minutes. Subsequently, 1 mL of ethanol 75% was added to the residual precipitate, and centrifugation was performed again at 7,500 g at 4 °C for 5 min. The RNA sample was stored in a freezer at -80 °C. The quantitative analysis of the isolated RNA was done using spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA).

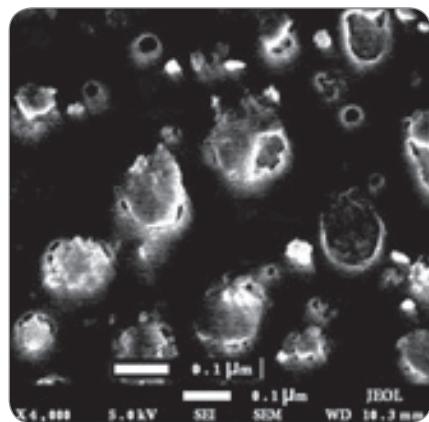


Fig. 1. Exosomes isolated from MCF-7 breast cancer cells' supernatant. As can be seen in the figure, these exosomes are spherical and have a size in the range of 30–150 nm.

Investigating the expression level of CD146, VEGF, and *miR-573*

cDNA was generated using the 1st strand cDNA Synthesis kit (Thermo Fisher Scientific), Oligo dT, and random hexamer primers. In order to perform the real-time PCR reaction, the forward and reverse primers (Tab. 1), cDNA, DEPC water, Mastermix, and CybrGreen dye were all added and the solution was placed in a thermocycler. Data analysis was performed using the threshold cycle comparison method according to the table below. Normalization of changes in miRNA expression level was done in comparison with the endogenous *U6 snRNA* expression level, while the expression level of other genes was compared with *GAPDH*. Data analysis was performed using ABI step one Real-Time PCR Software v2.0.2 (Applied Biosystems, UK), Excel software (Microsoft, 2010), and GraphPad software.

Statistical analysis

Statistical analysis of the obtained data was performed using the SPSS v.20 software, Anova test and Tukey follow-up test. P < 0.05 was considered significantly meaningful in this study.

Results

Exosome isolation confirmation

The size and morphology of the exosomes were evaluated by electron microscopy. The results showed that the

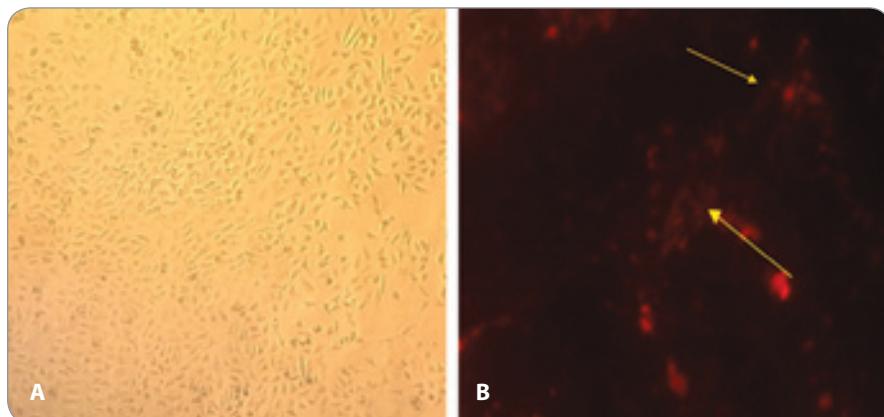
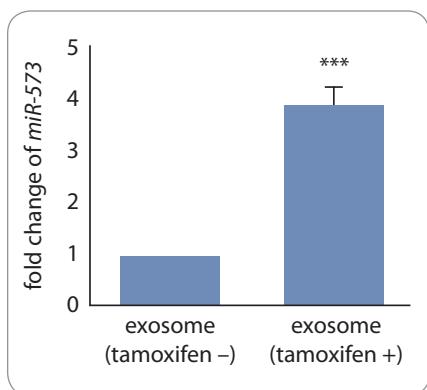


Fig. 2. A) HUVEC endothelial cell line (magnification 90 \times); B) locating PKH26-labeled tumor exosomes in the cytoplasm of endothelial cells.

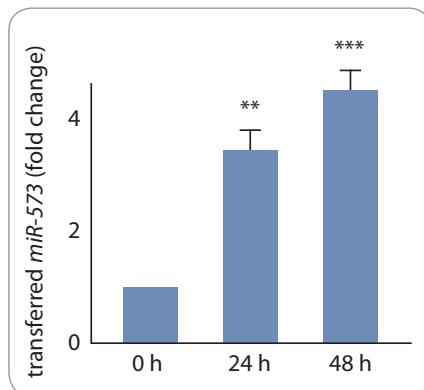


Graph 1. The expression level of miR-573 in MCF-7 cancer cell-derived exosomes treated by tamoxifen compared with the control untreated group. Expression of miR-573 is significantly higher in exosomes derived from tamoxifen-treated cells than the control group.

isolated exosomes had a spherical appearance with a size range of 30–150 nm (Fig. 1).

Measurement of endothelial cells' ability to absorb PKH26 fluorescent-labeled tumor exosomes

In order to determine the ability of endothelial cells to absorb tumor exosomes, HUVEC endothelial cells were treated by PKH26-labeled fluorescent colored exosomes. They were then incubated for 12 hours. Fluorescent microscopic analysis showed that PKH26 fluorescent-labeled exosomes are located in the cytoplasm of endothelial cells (Fig. 2). This indicates the ability of HUVECs to absorb endosomes derived from breast tumor cells.



Graph 2. Exosomal transfer of miR-573 to endothelial cells has a time-dependent manner. Over time, the exosomal transfer has increased significantly.

Examination of miR-573 expression level in exosomes isolated from MCF-7 breast cancer cells

According to Graph 1, the miR-573 expression level in tumor cell-derived exosomes treated with tamoxifen has a higher expression than exosomes derived from the control cell group. This result suggests that tamoxifen treatment may alter the expression profile of miRNAs which have tumor suppressor activities such as miR-573 in tumor cell-derived exosomes. The expression level of miR-573 being treated by tamoxifen has up-regulation.

Transferring miR-573 from MCF-7 breast tumor cell-line to HUVEC endothelial cells

Initially, the exosomal transfer of miR-573 to endothelial cells was investigated due to the presence of miRNAs in the

exosomes. For this reason, HUVEC endothelial cells were first treated by 100 μ g/mL alpha-amanitine, and then the rate of miR-573 transfer to endothelial cells was assessed at 0, 24, and 48 hours post-treatment. The reason lying behind using alpha-amanitine is to inhibit any transcription activity that can be induced by exogenous exosomes in the endothelial cells. As it is seen in Graph 2, the level of miR-573 transcripts in endothelial cells treated by exosomes and alpha-amanitine increased significantly over time.

Effects of breast tumor cells-derived exosomes on the expression level of CD146 and VEGF genes: tumor-derived exosomes that have been left untreated induced the expression level of CD146 and VEGF genes time-dependently. Much to our surprise, in contrast, tamoxifen-treated tumor exosomes reduced CD146 and VEGF expression levels over time. Therefore, given the greater presence of miR-573 in tamoxifen-treated tumor exosomes, it is comprehended that the miR-573 exosomal transfer reduces the expression level of CD146 and VEGF in endothelial cells after 24 and 48 hours significantly and time-dependently. Therefore, it is assumed that the exosomal transfer of miR-573, which is a tumor suppressor, from the mammary tumor cells being treated by tamoxifen may have an inhibitory effect on angiogenesis through decreasing the activity and expression level of CD146 and VEGF pro-angiogenic genes (Graphs 3,4).

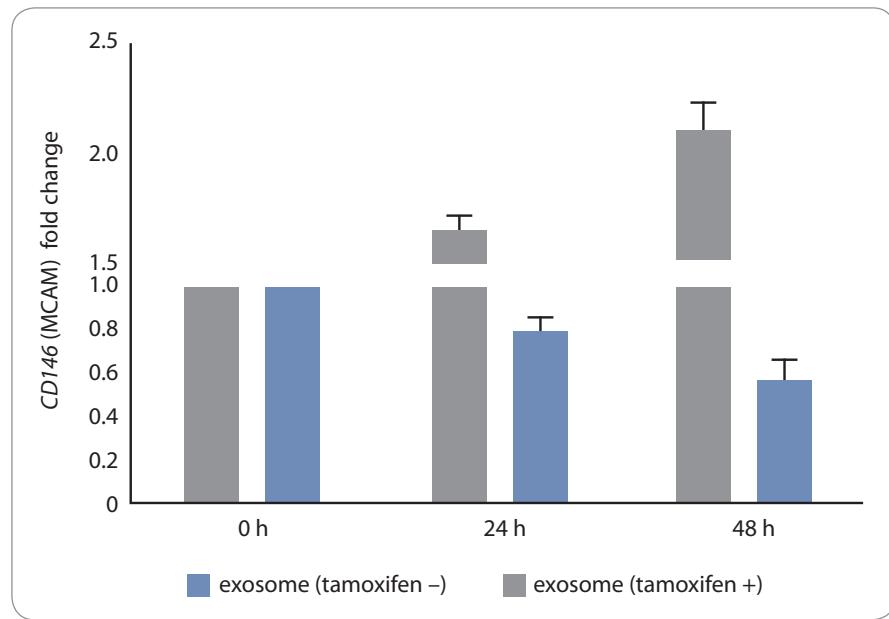
Discussion

Cancer cells seek to activate regulatory pathways for survival and development, as well as to obtain the metabolites and growth factors they need [9]. Since angiogenesis is developed by microenvironmental changes in cancer cells through tumor exosomes, it appears that inhibiting angiogenesis is mediated by the anti-angiogenic chemotherapy drug, tamoxifen, through modulating related miRNAs' expression. Therefore, it is assumed that tumor cell exosomes should be targeted to inhibit angiogenesis [10]. Tumor-derived exosomes are considered as a rich source of miRNAs

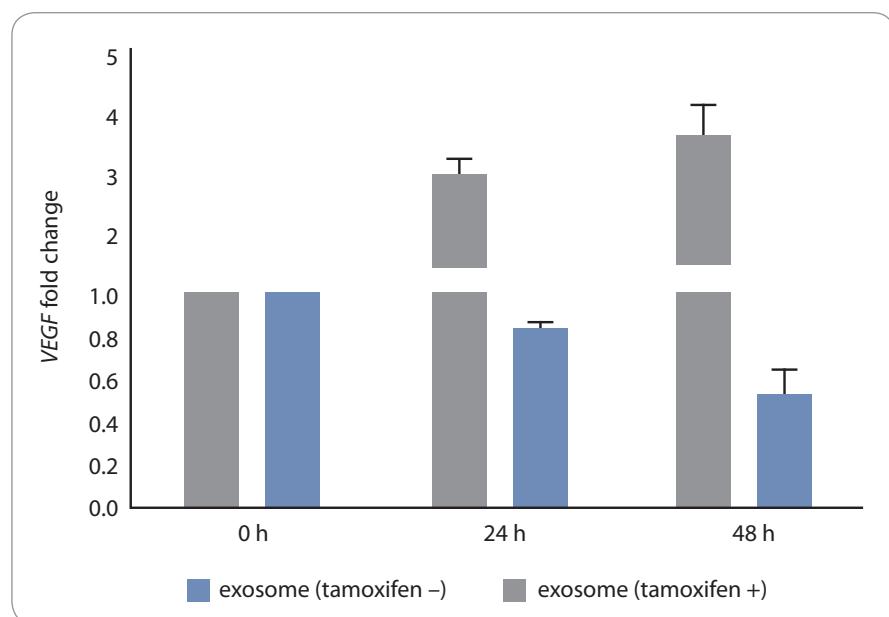
that could regulate the function of other cells in the tumor microenvironment, including vascular endothelial cells. However, the exact mechanisms by which tumor cell-derived exosomes affect their microenvironmental cells, as well as the biological function of exosomal miRNAs in cells, have not been well established [11]. Therefore, this study evaluated the effect of one of the chemotherapy drugs, tamoxifen, on the expression level of a key miRNA involved in angiogenesis, *miR-573*, and tried to introduce a regulatory relationship between this miRNA and *CD146* gene associated with the *VEGF* messaging pathway. The results showed that the expression of *miR-573* in the exosomes derived cells treated by tamoxifen increased significantly and time-dependently. On the other hand, the expression of *CD146* and *VEGF* genes in drug-treated cell exosomes had a downward pattern.

In 2015, Tahakashi et al. investigated the breast tumor-derived miRNAs and introduced these miRNAs as a diagnostic biomarker in the treatment of breast cancer [12]. The result of the presented study, in line with the research of Tahakashi et al., confirmed the role of *miR-573* as a diagnostic biomarker in breast cancer. In 2015, Hanafen et al. concluded that the use of the anti-cancer drug docosahexaenoic acid (DHA) changes the exosomal secretions of breast cancer cells. They isolated MCF7 and MDA-MB-231 breast cancer cell exosomes after treatment with DHA and observed an increase in the secretion of exosomes and the content of miRNAs of DHA-treated exosomes. The expression level of *miRNA-83* in MCF7-treated exosomes had changed. The group concluded that DHA alters the exosomal secretions of breast cancer cells and their miRNA content, thereby inhibiting angiogenesis [13]. Although the chemotherapy drug used in the present study, tamoxifen, is different from that of Hanafen et al., the results are in line with each other in terms of diminishing the expression level of genes targeting the angiogenesis and cancer development.

The *CD146* molecule belongs to the family of immunoglobulins. Recent research suggests that *CD146* is an endo-



Graph 3. inhibitory effect of mammary tumor cell-derived exosomes treated by tamoxifen in comparison with the progressive effect of the control group which has been left untreated on *CD146* gene expression after 24 and 48 hours of treatment (gene expression is normalized by the *GAPDH* gene).



Graph 4. Inhibitory effect of mammary tumor cell-derived exosomes treated by tamoxifen in comparison with the progressive effect of the control group which has been left untreated on *VEGF* gene expression after 24 and 48 hours of treatment (gene expression is normalized by the *GAPDH* gene).

thelial biomarker that promotes angiogenesis. The antibody against *CD146*, called AA98, prevents the migration of endothelial cells and subsequently prohibits angiogenesis and the growth of human tumors, and plays an important

role in cell migration, especially in invasive intermediate trophoblast cells. It has recently been discovered that the *CD146* is a transferring inducer between the epithelium and the mesenchymal tissue, thus expanding the in-

vasion of breast cancer cells. *CD146* is known as a receptor on endothelial cells. Tumor secretions stimulate the dimerization of *CD146* molecules, activation of p38 MAPKs / NF- κ B, and activation of many gene-producing genes including *VEGF*, *MMP-9*, and *IL-8*. AA98, which is the anti-molecular antibody of *CD146*, interferes with this signaling. The association of *CD146* with tyrosine kinase P59, phosphorylase P125, and paxillin promotes the migration of endothelial cells. *CD146* promotes angiogenesis in both laboratory and somatic cell conditions [14].

Increasing the expression level of *CD146* facilitates the endothelial response to the kinase SRC stimulated by *VEGF* or *VEGF*-induced SRC kinase family (SKF), p38 mitogen-activated protein kinase (MAPKs) and NF- κ B activation, and subsequently increases the migration of endothelial cells and tube formation. *CD146* is a co-receptor for *VEGFR-2*; therefore, suppression of *CD146* by *miR-329* impairs signaling and angiogenesis induced by *VEGF* [15].

The expression level of *miR-573* decreases in various tumors and its re-expression in the cell prevents the growth and development of melanoma. Characteristics of rheumatoid arthritis, which is a chronic autoimmune disease, is angiogenesis and cartilage decay in which *miR-573* has a role [16]. *miR-578* and *miR-573* play a role in the process of angiogenesis in breast cancer associated with *BRCA* gene. Laboratory evaluations on HEK293, MCF-7, and SUM149PT cells to investigate the role of *miR-578* and *miR-573* have shown that these miRNAs have been suppressed in breast cancer associated with *BRCA 1/2*. *miR-578* and *miR-573* are associated with the central connection, or focal adhesion, *VEGF*, and the signaling pathway of hypoxia inducible factor-1 (HIF-1). *miR-578* and *miR-573* control angiogenesis in breast cancer associated with *BRCA 1/2*, and do so by regulating *VEGF* and inducing HIF-1 [17]. In line with this research, the present study showed that when the ta-

moxifen-treated exosomes were transferred to the endothelial cell line, the expression of *VEGF* gene had a decreasing pattern.

Conclusion

Based on the results of the present study on the MCF-7 breast cancer cell line, it was found that there was a significant relationship between 60 μ M dose of tamoxifen and MCF-7 cell supernatant exosomes, in a way that the expression level of exosomal *miR-573* showed a significant increase. In this study, the effect of tamoxifen-treated tumor-derived exosomes was evaluated on the HUVEC endothelial cell line to assess the process of angiogenesis and treatment of breast cancer in its early stages. Applying the experiment group to the endothelial cell line reduces the expression of *CD146* gene in endothelial cells significantly over time. The second gene studied in this study was *VEGF*, the result of which was the same as *CD146*. Ultimately, this paper could conclude that the exosomal transfer of *miR-573* to endothelial cells reduces the expression of genes responsible for the angiogenesis process, including *VEGF* and *CD146*, identification of which could mitigate the tumor process.

When breast cancer cells are treated with tamoxifen, the level of *miR-573* which is a tumor suppressor miRNA in the exosomes of these cells, is much higher than in untreated cancer cells. In contrast, when tamoxifen-treated tumor-derived exosomes are transferred to endothelial cells, the expression level of *CD146* and *VEGF* genes declines in a time-dependent manner. This achievement brings us to the idea that the therapeutic effect of tamoxifen on breast cancer lies behind its relationship with the expression level of exosomal miRNAs that regulate the cancer microenvironment.

Acknowledgments

This study was conducted at the cellular and molecular laboratory of Islamic Azad University, thus appreciating the spiritual support of these centers is admitted.

Availability of data and materials

The data used in this study are available from the corresponding author upon request.

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