

Use of cellular exosomes as a new carrier in breast cancer gene therapy

Využití buněčných exozomů jako nového nosiče v genové terapii karcinomu prsu

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Summary

Background: Breast cancer is recognized as a major clinical challenge in gynecological diseases worldwide. Exosomes are small vesicles derived from multicellular bodies that are secreted by many cells into the extracellular environment and thus participate in intercellular communication through the transfer of genetic information such as encoded and non-encoded RNAs to target cells. Tumor-derived exosomes are thought to be a rich source of microRNAs (miRs) that can regulate the function of other cancer cells in the tumor microenvironment. However, the exact mechanisms by which tumor cell-derived exosomes affect their neighboring cells, as well as the biological function of exosomal miRs in receptor cells, are not well understood. **Materials and methods:** In this study, after the overexpression of *Mir-205* in breast cancer cells (MDA-MB-231 class), cell-derived exosomes were successfully isolated and characterized by electron microscopy and dynamic light scattering. **Results:** The determination of *Mir-205* expression levels in exosomes secreted from engineered cells confirmed the high expression of this miR in exosomes. It was also found that the treatment of tumor exosomes carrying this miR had an apoptotic induction effect and also had a significant effect on reducing the expression of *Bcl-2* gene transcript in a time-dependent manner in breast cancer cells ($P < 0.001$). **Conclusion:** Overall, this study suggests that exosomal transfer of tumor suppressor miRs to cancer cells could be a suitable platform for nucleic acid transfer to these cells and be highly effective in cancer treatment.

Key words

breast cancer – exosomes – apoptosis – microRNA – gene therapy

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Souhrn

Východiska: Karcinom prsu je celosvětově považován za hlavní klinický problém u gynekologických onemocnění. Exozomy jsou malé váčky, které vznikají v mnohobuněčných organizmech. Jsou vylučovány různými buňkami do extracelulárního prostředí, a tak se účastní mezibuněčné komunikace tím, že přenášejí genetické informace prostřednictvím kódujících a nekódujících RNA do cílových buněk. Exozomy odvozené od nádoru jsou považovány za bohatý zdroj mikroRNA (miR) a mohou regulovat funkci nádorových buněk v mikroprostředí nádoru. Přesné mechanismy, kterými exozomy pocházející z nádorových buněk ovlivňují sousední buňky ani biologická funkce exozomálních miR v receptorových buňkách však nejsou dobře známy. **Materiál a metody:** V této studii byly po nadměrné expresi *MiR-205* v buňkách karcinomu prsu (třída MDA-MB-231) úspěšně izolovány buněčné exozomy a charakterizovány pomocí elektronové mikroskopie a dynamického rozptylu světla. **Výsledky:** Stanovení hladin exprese *MiR-205* v exozomech vylučovaných z upravených buněk potvrdilo vysokou expresi této miR. Bylo také zjištěno, že úprava nádorových exozomů nesoucích tuto miR měla indukční účinek na apoptózu a významný účinek na snížení exprese genového transkriptu *Bcl-2* v buňkách karcinomu prsu v závislosti na čase ($p < 0,001$). **Závěr:** Celkově tato studie naznačuje, že exozomální přenos nádorových supresorových miR do nádorových buněk může být vhodnou platformou pro přenos nukleových kyselin do těchto buněk a může být vysoce účinný při léčbě nádorů.

Klíčová slova

karcinom prsu – exozomy – apoptóza – mikroRNA – genová terapie

Introduction

Exosomes are nano-sized cup-shaped vesicles of the size 40–100 nm, which are released from many cell types into the intercellular space. Pathogens can exploit exosomes to spread their infectivity. The RNA and protein contents of exosomes released from cells into the bloodstream and body fluids are very different in health and disease and can be measured as a diagnostic marker. Exosomes derived from cancer cells have been shown to be rich in tumor marker microRNAs (miRs). More recently, mRNAs and miRs have been identified in the exosome that can be absorbed by neighboring (near or distant) cells and subsequently regulate receptor cells [1]. Therefore, the examination of these miRs in cells can be a criterion for the diagnosis of many diseases.

MiRs have recently been found to be closely linked to various diseases, including cancer. Because of the potential of miRs to target large numbers of mRNAs, these non-coding-RNAs (ncRNAs) are involved in all biological phenomena, including cell cycle regulation, cell growth, apoptosis, cell differentiation, and the stress response [2]. There is growing evidence that miRs play an important role in cancer biology, and recent studies have confirmed the oncogenic and tumor inhibitory role of miRs in cancer cells, and also shown that miRs can be expressed by oncogenes themselves and regulate tumor inhibitory genes. It is possible that the expression of miRs in both *in vivo* and *in vitro*

through the synthesis of pre-miR molecules or antisense oligonucleotides can be regulated, which is a promising prospect for cancer treatment. Evidence suggests that miRs are stable in body fluids, including saliva, urine, milk, and blood [3]. In addition, extracellular miRs for packaging inside exosomes or microvesicles can be loaded into high-density lipoprotein (HDL), or form an AGO2-bonded extracellular protein. The key role of miRs has been identified as regulators of various cellular processes such as evolutionary timing, cell proliferation, cell differentiation, organ development and apoptosis. Abnormal expression of miRs has been reported in many cancers, and there is strong evidence that miRs play a key role as oncogenes or tumor suppressors in the development of many human malignancies [4].

MiR-205 is highly under-expressed in breast tumor cells compared to the normal breast cells. Even *in vivo* analysis reports down regulatory pattern of *MiR-205* in breast cancer cell lines including MCF-7 and MDA-MB-231. Additionally, overexpression of *MiR-205* inhibits cell proliferation and independent growth as well as cell invasion [5]. Moreover, previous studies have proved that *MiR-205* suppresses metastasis. *In vivo* studies have proved the down-regulated condition of *MiR-205* in drug-resistant derivatives. ErbB3 and vascular endothelial growth factor A (VEGF-A) are direct targets for *MiR-205* and this miR applies its suppressive effect via direct interaction with the *MiR-205* bind-

ing site in the 3'-untranslated region (3'-UTR) of ErbB3 and VEGF-A. Hence, these findings propose *MiR-205* as a tumor suppressor in the breast cancer [6]. *MiR-205* enhances the chemo-sensitivity of breast cancer cells to chemotherapy by suppressing VEGF-A and fibroblast growth factor-2 [7], resulting in decreased phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway activity and increased apoptosis upon chemotherapy. As a result, *MiR-205* may be used as a predictive biomarker and a potential therapeutic target in breast cancer treatment [8].

Apoptosis, programmed cell death, happens when cells are not needed. Apoptosis is caused by a family of proteases called caspases. Caspases are made in the form of inactive precursors called procaspase, which are activated by proteolytic digestion in response to symptoms that induce apoptosis [9]. Members of the Bcl-2 family play a key role in regulating changes in mitochondrial membrane permeability. Some proteins in this family, such as Bcl-2, Bcl-XL, and Mcl-1, are inhibitors of apoptosis or anti-apoptosis and maintain cell survival by binding to mitochondrial ducts. In contrast, the binding of pro-apoptotic proteins of the Bcl-2 family such as Bax, Bid, Bak, Bcl-XS to the outer membrane of the mitochondria increases the permeability of this organ and causes the onset of apoptosis [10]. The balance between pre-apoptotic and anti-apoptotic activity of members of the Bcl-2 family is highly determinant of whether a cell

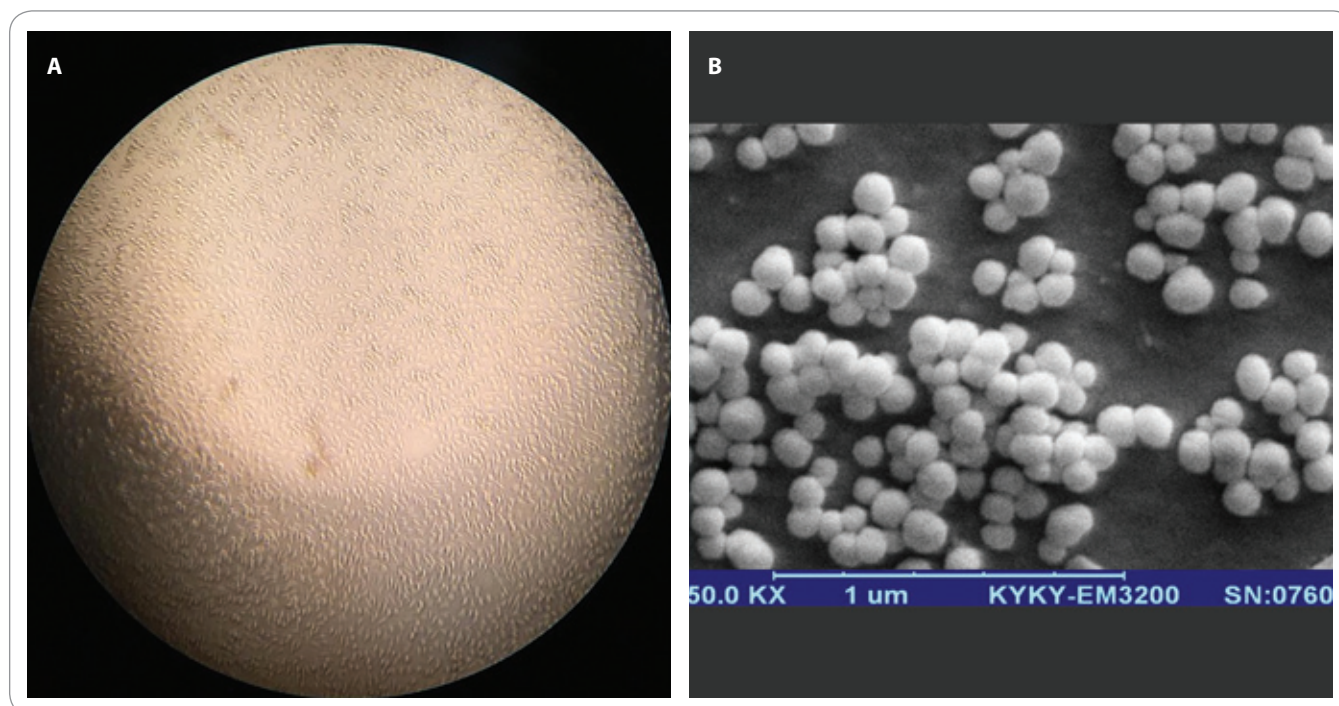


Fig. 1. A) Contrast-phase microscopy image of the morphology of breast tumor cells of MDA-MB-231 cell line after transfusion collected after successive passages of the supernatant medium for exosome extraction. B) Examination of isolated exosomes by electron microscopy.

goes for or survives the apoptosis. Cells induce apoptosis to their adjacent cells via intercellular communication, which happens with the assist of exosomes in microenvironment [11].

Materials and methods

Cell culture

Cell line MDA-MB-231 (cell line derived from invasive breast cancer) was prepared from Pasteur Institute of Iran. These cells were grown in DMEM medium containing 10% of fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, in a humid incubator with 5% CO₂ and 37 °C.

Exosome isolation and purification

Exosomes were prepared from the supernatant of the MDA-MB-231 cell line at passage 3 by differential centrifugation and according to the manufacturer's instructions. In short, the culture media was removed when the cells were developed through up to almost 85% of the plate. Firstly, the supernatants were centrifuged at 3,000×g for 10 min for lessening the amount of residual cells. Then, Exoquick (System Biosciences) solution was added

to the supernatant with a ratio of 5/1. Isolation steps followed as of the manufacturer's instruction, and the last point was to suspend the exosome pellets in 50 μL phosphate buffer solution (PBS) and stored at –20 °C until use.

Exosome Identification

The morphology and size of the purified exosomes were measured by scanning electron microscopy (Digital FESEM, KYKY-EM3200, China). Hence, an aliquot of the abovementioned exosomes was fixed in 2.5% glutaraldehyde on a microscope slide, washed by PBS, and then the ascending amount of ethanol was used for reaching its critical point of dehydration. The slide was then dried on a glass substrate and coated with gold.

Scanning electron microscopy (SEM)

For microscopic observation, a small volume of the purified exosome was fixed with 2.5% glutaraldehyde and washed with PBS. The sample was then dewatered with ethanol on a dry glass surface. The size and morphology of the exosomes were evaluated by electron microscopy (Digital SEM, KYKY-EM3200, China).

Dynamic light scattering (DLS)

40 μL of extracted exosome were dissolved in 300 μL PBS. The solution was then sonicated. Exosomes were measured by Zetasizer Nano ZS software (Malvern Instruments, UK).

Total RNA isolation and qRT-PCR

Since exosomes are a rich and protected source of miRs, it is presumed that exosomal transfer of miRs from tumor cells to their adjacent cells may be a factor in modulating target genes' expression and regulating tumor progression. We, therefore, sought to provide a molecular justification for the effect of tumor exosomes on its microenvironment.

Total RNA was isolated with quite similar to manufacturer's instructions, briefly as follows: Trizol (Invitrogen/Life Technologies) was initially admitted and then the sample was treated with RNase-free DNase (Fermentase, Lithuania). Complementary DNA (cDNA) was synthesized by reverse transcription of 2 μg total RNA using random hexamers. Then, poly-(A)-tailed RNAs were reverse-transcribed. Quantitative real-time PCR was

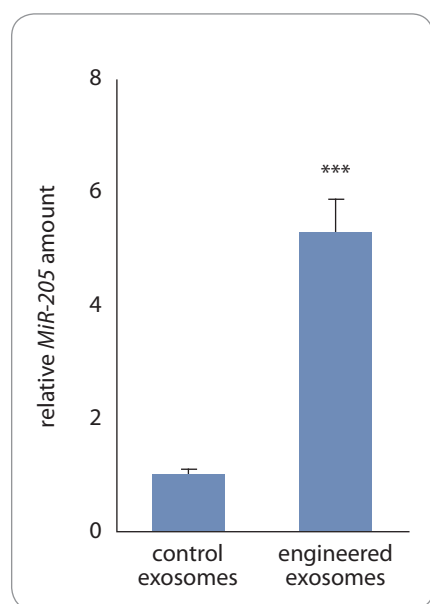


Fig. 3. Evaluation of *MiR-205* expression levels in both engineered and control exosomal groups indicates the presence of significant amounts of *MiR-205* in engineered exosomes (exosomes derived from cells transfected with the *MiR-205* precursor sequence).

For a complete figure, see an online version of this article.

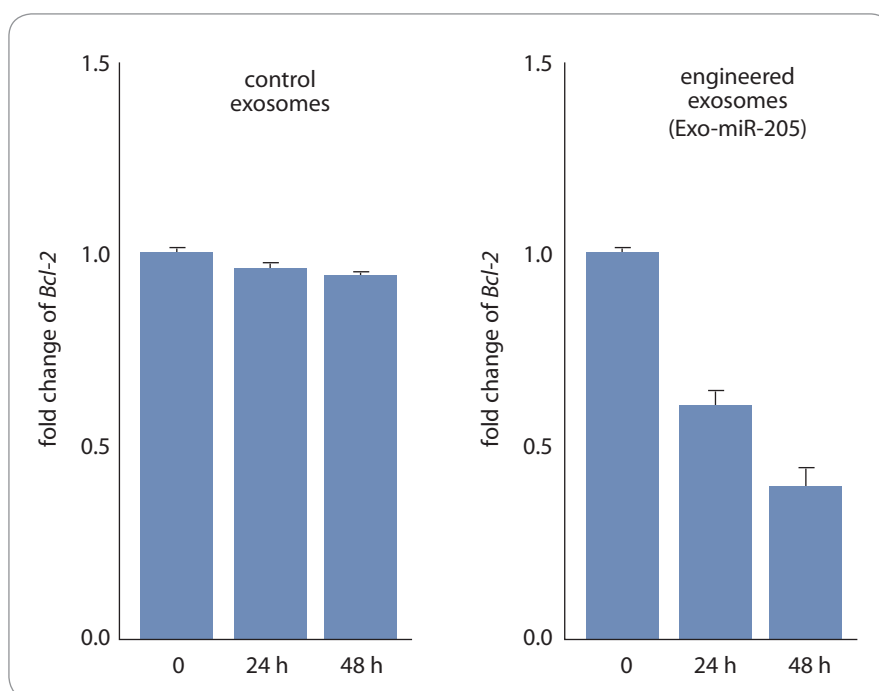


Fig. 4. Evaluation of *MiR-205* expression levels in both engineered and control exosomal groups indicates the presence of significant amounts of *MiR-205* in engineered exosomes (exosomes derived from cells transfected with the *MiR-205* precursor sequence). As expected, only the treated engineered exosomes containing *MiR-205* (right figure) resulted in a significant reduction in the expression of *Bcl-2* anti-apoptotic gene transcript in a time-dependent manner. The results are normalized to glyceraldehyde-3-phosphate dehydrogenase reference gene expression.

performed by ABI Step One Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBRGreen[®] Mastermix[™] II (TAKARA, Japan). Real-time PCR was used to measure the effect of *MiR-205*-containing exosomes of engineered breast cancer cells on the induction of apoptosis in breast cancer cells. The expression level of each gene and miR were subsequently assessed by using the $2^{-\Delta\Delta C_t}$ method, in which $\Delta\Delta C_t = (C_{t_{miR}} - C_{t_{U6snRNA}})_{target} - (C_{t_{miR}} - C_{t_{U6snRNA}})_{control}$. The expression level of *MiR-205* was normalized to U6 small nuclear RNA (U6snRNA). To calculate the relative fold change values, the C_t value data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequence was as follows: 5'-CGG GAT TTC AGT GGA GTG AAG TTC-3' (*miR-205*); 5'-CTCGCTTCGGCAGCACATATACT-3' (U6snRNA, sense) and 5'-ACGCTTCACGAATTTGCGTGTC-3' (U6snRNA, antisense); 5'-ACCACTCCTCCACCTTTGA-3' (GAPDH, sense) and 5'-CTGTTGCTGTAGCCAAATTCGT-3' (GAPDH, antisense).

Overexpression of miR

The tube containing the lyophilized miR precursor sequence was briefly centrifuged to collect all material at the end of the tube. The precursor sequence was dissolved in 330 μ L of nuclease-free water according to the manufacturer's protocol, increasing this amount of water to form a 10 μ M solution. The tube was placed at room temperature for a few minutes, and then the contents of the tube were mixed with a gentle pipette. The resulting suspension was stored at -20°C .

Data analysis

The data were presented as mean standard deviation (SD) from two or three independent experiments and the t-test was used for statistical analysis of data changes. P-values < 0.05 were considered statistically significant.

Results

The size and morphology of the exosomes were assessed by Scanning

Electron Microscopy. The results showed that the isolated exosomes have a spherical appearance with a size range of 100 nm, which is shown in Fig. 1.

Using Dynamic Light Scattering (DLS) technique, particles' motion was measured and the size of the exosomes was measured by a Zetasizer. The following diagram (Fig. 2) is based on two parameters: size and percentage of vesicles. This diagram shows that the maximum vesicle population is about 60 nm. Another short peak is observed within 120–100 nm. As we know, vesicles below 200 nm are evidence of the presence of exosomes.

Exosome size measurements by DLS showed a bell-shaped size distribution with a peak of about 60 nm. What is important at this stage is the determination of *MiR-205* levels in engineered exosomes (exosomes derived from transfected breast cancer cells) with the precursor sequence (*MiR-205* compared with the exosomal control group (exosomes derived from uninfected breast

cancer cells)). As it can be seen in Fig. 3, engineered exosomes showed a much higher expression of *MiR-205* compared to control exosomes ($P < 0.001$).

To investigate the effects of exosomal transmission of *MiR-205* to breast cancer cells, these cells were compared with engineered exosomes (exosomes derived from cancer cells transfected with the *MiR-205* precursor sequence) compared with the control group's exosomes derived from untransfected breast cancer cells. The real-time PCR results as shown in the following figure showed that exosomes derived from untransfected breast cancer cells (exosomal control group) had no significant effect on the expression of *Bcl-2* anti-apoptotic gene transcript. In contrast, engineered exosomes containing *MiR-205* resulted in a significant reduction in the expression of *Bcl-2* anti-apoptotic gene transcript in a time-dependent manner. The idea is demonstrated in Fig. 4.

To investigate the effects of exosomal transfer of *MiR-205* on the induction of apoptosis in breast cancer cells, these cells were treated with the *MiR-205* derived exosomes. Flow cytometry results, as shown in Fig. 5, presented that engineered exosomes containing *MiR-205* were able to induce apoptosis in breast cancer cells compared to the control group.

Discussion

A report of the presence of mRNA in the exosomes refers to the study by Posner et al. The group showed that human and mouse mast cell lines contain about 1,300 different mRNAs, many of which are not present in the cytoplasm of exosome-secreting primary cells. Translation of these mRNAs *in vitro* showed that they could be converted into functional proteins. This group was also able to prove the presence of miRs in the studied exosomes [12]. In 2017, Kalli et al examined microparticles in mesenchymal stem cell secretions and confirmed the presence of RNA in these secretions. They suggested that RNAs in the environment of mesenchymal stem cells are located in phospholipid vesicles and that these RNAs are often small RNAs, especially miRs [13]. In 2004, Varadharajan

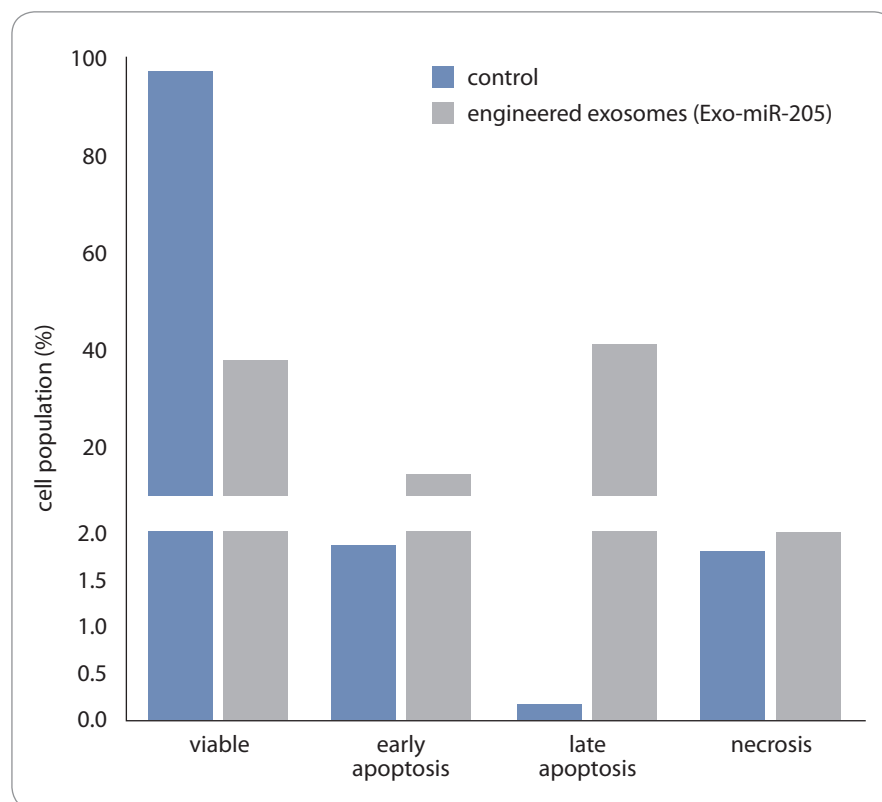


Fig 5. Analysis of annexin V positive / PI positive breast cancer cells treated with engineered exosomes containing *MiR-205* in a selected assay. Dead cells are considered necrotic (annexin V negative / PI positive) or apoptotic (annexin V positive / PI negative and annexin V positive / PI positive). The x-axis represents fluorescein isothiocyanate-labelled annexin V and the y-axis represents PI.

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PI – propidium iodide

et al examined bone marrow mesenchymal stem cells and tissue-specific stem cells and demonstrated that the secretion of miRs has a selective pattern, indicating a dynamic regulation of the presence of miRs in microvesicles. Based on their observations, the group stated that stem cells exert at least part of their trophic activity through the exchange of microvesicles between cells [14]. In 2017, Tamimi et al concluded that the cellular separation of cultured breast cancer cells from the substrate, when rapid, results in the release of exosomes and has significant effects on the cellular process, which clinically leads to the metastasis of cancer [15]. In line with previous findings, this paper found that exosomes' secretion could have a direct effect on its cancer.

Due to the attractive properties of exosomes as carriers of drugs and genes, many studies have been conducted in

this regard and very satisfactory results have been obtained. In 2018, Varadharajan et al reported that overexpression of miR-10b initiates invasion and metastasis in breast cancer and that overexpression in early breast cancer is associated with clinical progression of the disease [14]. It has been reported that MiR-31 was highly expressed in mouse lung cancer cells [15]. A further study on the relationship between expression mRNAs and the progression of lung cancer have discovered a large number of miRs that are closely related to lung cancer. Some of them contained miR overexpression in lung cancer, and the rest had less miR than normal lung tissue or cells. However, downregulation and silencing methods are commonly used for overexpression in lung cancers, especially RNA intervention technology [16].

Apoptosis could have been induced to the cells via their adjacent cells, in which

many molecular pathways are involved. Exosome transfer in the microenvironment intercellular communication is to name but a few, which has been found in this study. In line with our study, other previous papers also support the idea. The pathway of cell death activation varies depending on how the death message is transmitted to the cell [17,18]. If the messages are internal, the first activated organ will be the mitochondria, and if the message is expressed through cell surface receptors, the message will be transmitted from the adapter to the caspase cascade through adaptive molecules. The rate of receptor-induced cell death is more severe than the mitochondrial pathway. Apoptosis receptors are cell surface receptors that transmit messages through specific ligands and activate the caspase cascade [19,20].

Conclusion

As presented in the Results section, *MiR-205* was overexpressed in tumor cells. Interestingly, exosomes derived from the engineered cells, which contained significant amounts of *MiR-205*, reduced the expression of the anti-apoptotic *Bcl-2* gene and induced apoptosis in breast cancer cells. Importantly, not only does *MiR-205* induce apoptosis by targeting *Bcl-2* gene transcripts, but also exosomal nanocarriers were able to deliver this miR to the cell, and so in this study, a platform is proposed that is able to effectively deliver nucleic acid to the cell.

Since the use of exosomes secreted by cells in gene delivery has recently been proposed as a new approach in gene therapy, the targeted placement of *MiR-205* in exosomes of breast cancer cells following the induction of overexpression of this miR in cells and the effect of exosomal treatment on the key gene involved in apoptosis, *Bcl-2*, was an innovative and targeted approach to this study.

Alterations in the natural expression of miRs are a common feature of cancers, including breast cancers. Because miRs can act as oncogenes or tumor

suppressors, understanding the biology of miRs and modulating their expression and activity could create new opportunities for future cancer treatment. Also, since the anti-apoptotic gene *Bcl-2* acts as a tumor promoter in breast cancer, it can be an attractive target for the treatment of this malignancy.

Overall, the findings of the present study showed that exosomes containing *MiR-205* can significantly reduce the expression of *Bcl-2* anti-apoptotic gene transcript and induce apoptosis in breast cancer cells. However, the tumor exosomes of the control group were not able to produce this feature. The results also show that not only tumor cell-derived exosomes with *MiR-205* overexpression can cause cell apoptosis in breast cancer cells, but also this platform delivers nucleic acid to the cell in terms of performance efficiency with minimal necrosis for the cell, and this is very noteworthy in gene therapy.

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Author's contributions

Narges Maleki and Sadegh Babashah contributed equally to this work as corresponding authors. The corresponding authors of this article justify that ALL the mentioned individuals in this article are members of this research team and had had substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, revising it, and final approval of the version to be published.

Availability of data and materials

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

Consent for publication

All the authors confirm that the manuscript represents their honest work.

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Fig. 2. Investigation of isolated exosomes by dynamic light scattering

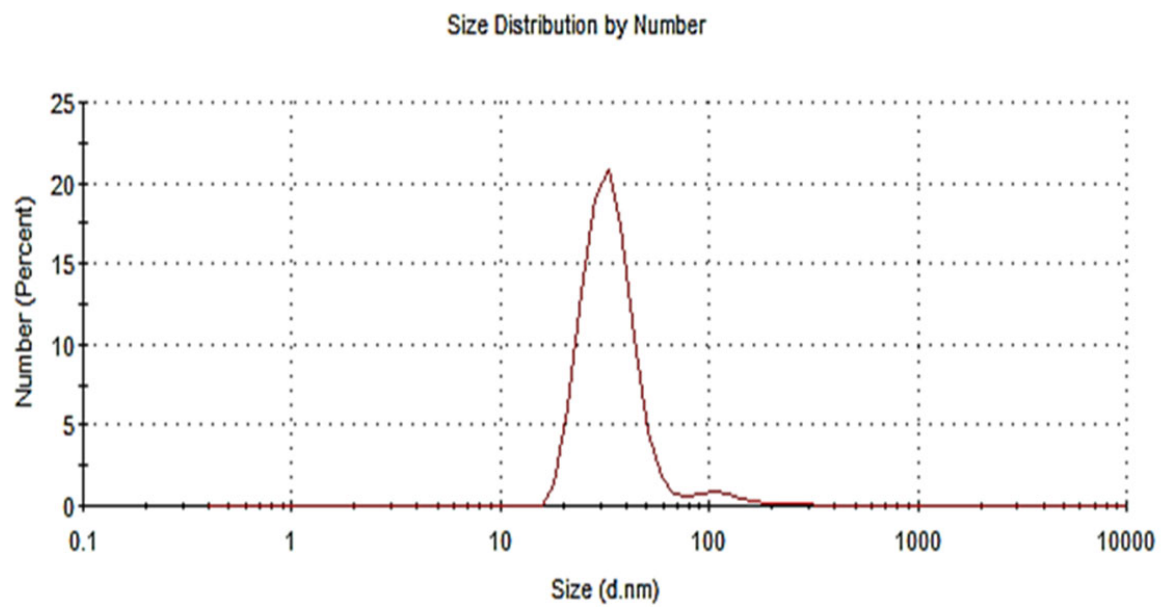


Fig3. Evaluation of miR-205 expression levels in both engineered and control exosomal groups indicates the presence of significant amounts of miR-205 in engineered exosomes (exosomes derived from cells transfected with the miR-205 precursor sequence).

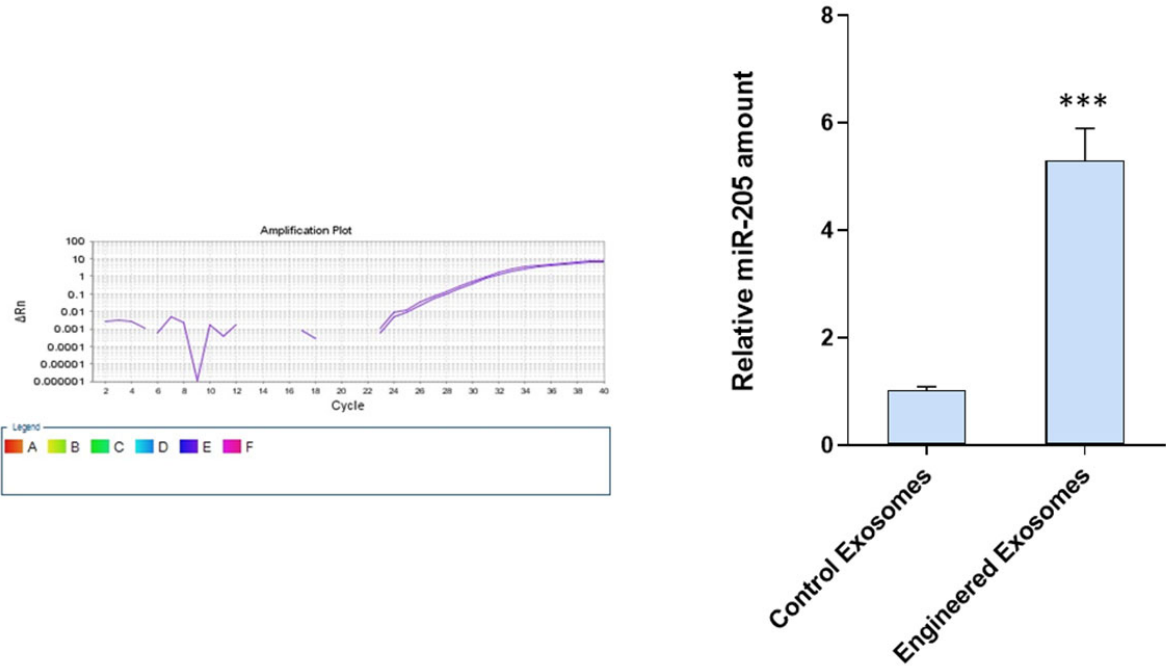
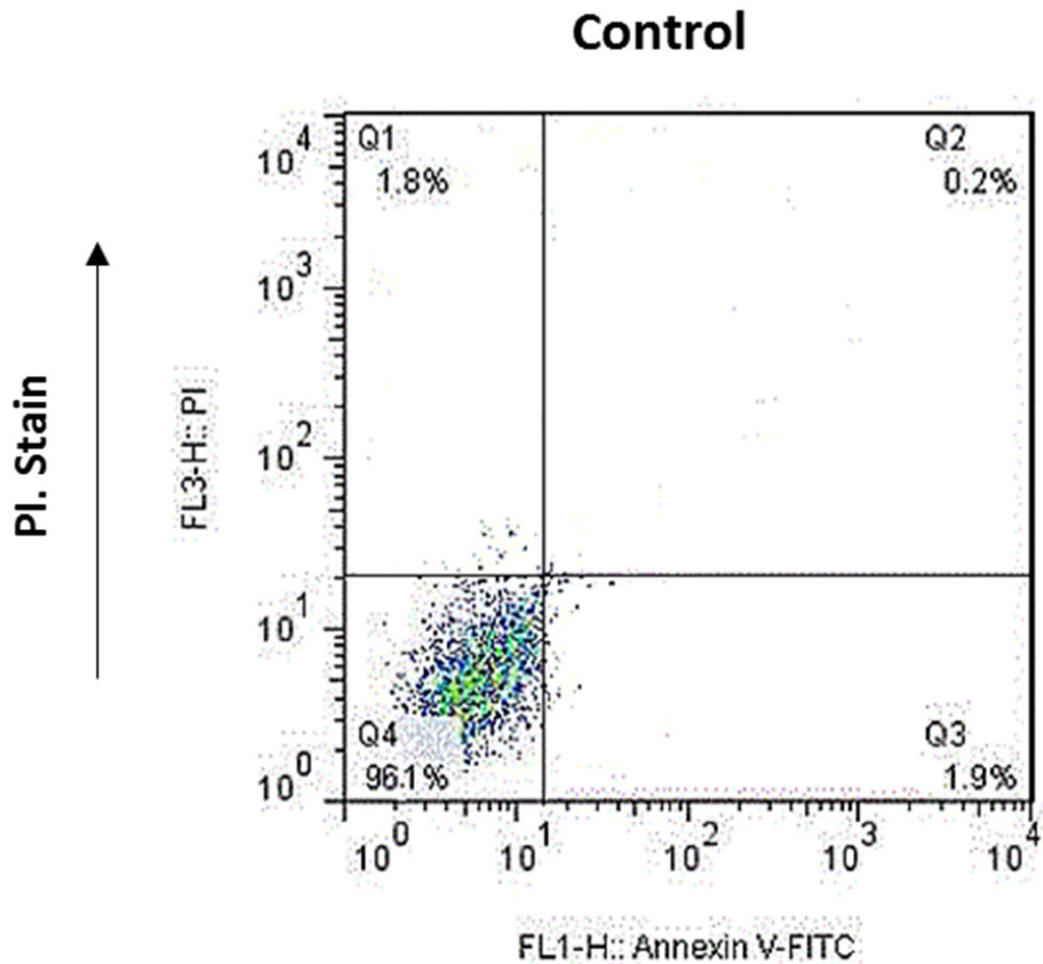


Fig. 5. Analysis of Annexin V + / PI + breast cancer cells treated with engineered exosomes containing miR-205 in a selected assay. Dead cells are considered necrotic (Annexin V negative / PI-positive) or apoptotic (Annexin V positive / PI-negative and Annexin V positive / PI-positive). The x-axis represents Annexin-V-FITC and the y-axis represents Propidium iodide (PI).



Exo-miR-205

