

# Investigation of the Role of Encapsulated miR-372 in Chitosan on Induction of Apoptosis in MCF-7 Cancer Cells

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## Abstract

In recent years, various efforts have been made to improve the functional potency of cancer drugs. Due to the fact that microRNA in the cell can act as a tumor inhibitor, it can be used as a suitable treatment for most cancers. In this study, chitosan coating is considered a factor that enhances function and effectiveness of microRNAs. MCF-7 cell line was divided into four experimental groups, including an untreated MCF-7 cell group, MCF-7 cell group with miR encapsulated with chitosan, MCF-7 cell group with chitosan, and MCF-7 cell group with doxorubicin as a positive control group. The effect of different concentrations of miR-372 was first evaluated, and the optimal dose was selected to evaluate the following parameters: the induction of cell death by applying flow cytometry, the cell survival by MTT, and the level of P53 protein by immunocytochemistry. The results showed that the dose of 1500 ng/ $\mu$ l of miR-372 coated with chitosan could induce cell death up to 50% in 24 and 72 hours of treatment. In addition, the rate of induction of cell death in the group treated with miR-372 coated with chitosan for 72 hours was statistically significant compared with the control group. In addition, the expression level of P53 protein in the same group was statistically significant compared with the control group. According to the results, the use of cell proliferation cycle regulators such as miR-372 can control the process of proliferation and thus improve the treatment of cancer.

**Keywords:** Chitosan nanoparticles, Breast cancer cells, miR-372, Apoptosis, Cell Proliferation

## Introduction

Cancer is mainly caused by mutations in genes that are present in the nuclei of all cells in the body. Cancer may be benign or malignant (Osborne et al., 2004). They are similar to the cells of the organism from which they originated and have the same DNA and RNA, which is why they are not recognized, especially if the immune system is weak (Sharma et al., 2010).

Uncontrolled proliferation of breast cells which is mainly caused by inherited mutations in the BRCA1 and BRCA2 genes (Friedman et al., 2007; Bogdanova et al., 2013), accounts for 11% of cancers each year and is one of the leading causes of death in women (Friedman et al., 2007) and the second most common type. Symptoms of breast cancer can include as a mass in the breast, deformity of the breast skin, and nipple fluid secretion (Osborne et al., 2004). Breast cancer is divided into two types of carcinoma—lobular or ductal—depending on the origin (Friedman et al., 2007). According to the WHO report in 2020, there were 2.3 million women diagnosed with breast cancer and

685 000 deaths globally. As of the end of 2020, there were 7.8 million women alive who were diagnosed with breast cancer in the past 5 years, making it the world's most prevalent cancer.

Normally, cells undergo apoptosis at the end of their life cycle when they are no longer needed by the body. Before apoptosis, they are protected by various pathways and proteins. These pathways include AKT/PI3K and RAS/MEK/ERK. Sometimes genes related to these pathways mutate and cause permanent activation of these pathways, leading to continuous cell division and cell proliferation. Normally, the PTEN protein is responsible for silencing the PI3K/AKT pathway during cell apoptosis (Majeed et al., 2014).

Breast cancer treatment strategies include: hormone therapy that reduces hormone-sensitive cancer cells by inhibiting the production of estrogen and progesterone by the ovaries in the body as well as inhibiting the hormonal function (Dunnwald et al., 2007); Auxiliary therapy for prevention after surgical treatment and may include radiotherapy, chemotherapy, and hormone therapy (Burstein et al., 2010; Regan et al., 2011) Immunotherapy,

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which includes the use of monoclonal antibodies and vaccines (Ascierto et. al., 2012; Soliman et. al., 2013); Nano-medicine in the treatment of breast cancer in which doxorubicin was delivered into cancer cells using pH-sensitive liposomes and estrone as a biological ligand (Paliwal et. al., 2012); nanotherapy-based miRs in which some miR inhibitors are used as therapeutic drugs in the treatment of breast cancer (Zhang et. al., 2016).

The role of the disorder in the regulation of miRs in breast cancer was first reported in 2005 and has been followed by numerous studies showing changes in miRs in breast cancer (Iorio et. al., 2005). The decrease of miR-372, which is a member of the miR family in the breast cancer cell, causes significant inhibition of cell proliferation and cessation of the cell cycle in the G1-S phase and the increase of apoptotic cancer cells (Cheng et. al., 2018). Nanoparticles improve the delivery of miR agents in vivo, protect their charge and increase target specificity (Velpurisiva et. al., 2017).

In recent years, new drug delivery systems have received much attention. Polysaccharide is a natural linear compound that is mainly produced by alkaline distillation of chitin (Elieh-Ali-Komi et. al., 2016). Polymeric drug delivery systems are very diverse. Degradable polymers are suitable options for transporting drugs, macromolecules and enzymes. The Carbohydrate polymer chitosan is considered one of the most desirable polymers in the preparation of drug delivery systems due to its properties such as biocompatibility, and biodegradability. Chitosan can increase the absorption of insoluble drugs. The presence of amine groups in chitosan allows the creation of transverse connections in the nanosystem network. Chitosan-based nanoparticles can inhibit tumor cell growth by inducing apoptosis due to their high permeability and cell persistence (Majeed et. al., 2014).

In the present study, we investigated the induction of apoptosis in MCF-7 cells by encapsulating miR-372 in chitosan and determining its effective dose for inducing apoptosis and increasing P53 expression.

## Materials and Methods

### Cell culture

The MCF-7 cells were purchased from the Pasteur Institute. Cells (10000 cells/cm<sup>3</sup>) were cultured in RPMI culture medium containing 10% bovine fetal serum and the antibiotics penicillin and streptomycin in 96-well plates. Temperature 37 °C

and 5% CO<sub>2</sub>). After 24 and 72 hours of treatment with MRNC, 100 µl of MTT stock was added to each well with a final concentration of 0.5 mg/ml. After three hours of incubation, formazan blue precipitate was observed. Then, 100 µl DMSO solvent was added to each well to dissolve Formazan crystals and the solution of each well was absorbed by the ELISA reader applying 570 nm.

### Preparation of miR-372

The sequence was designed using the datamiRbase site and synthesized by the Pishgaman Company.

### Preparation of mRNC (miR-372 Nanoparticle Chitosan)

The required amount of chitosan was added to 25 cc of 2% acetic acid and after 12 hours of stirring, the prepared miR-372 solution was added dropwise at 37 °C and stirred for one hour. In the next step, 25 cc of TTP 0.4 % weight-volume was added dropwise to the above solution at 4 °C and stirred for one hour and centrifuged at 4500 rpm. This step was repeated several times with the addition of deionized water to keep the pH of the solution neutral.

The experiments were performed on four cell groups of the MCF-7 cell line based on the following treatments:

- 1-The first group of MCF-7 cells without any treatment (Control)
- 2-The second group of MCF-7 cells was treated with mRNC (miR-372)
- 3-The third group of MCF-7 cells was treated with chitosan (Sham)
- 4-The fourth group of MCF-7 cells was treated with Doxorubicin as positive control group (Dox)

### Evaluation of the effect of mRNC on apoptosis using flow cytometry

Cells were cultured at a density of 10,000 cells/cm<sup>3</sup>. After reaching logarithmic growth, one group of cells was treated with mRNC for 24 hours and the other group was treated with mRNC for 72 hours. One group was considered a control. Then, 1 cc of 100x stock containing annexin and PI was added so that their final concentrations were 0.25 and 0.1 micromolar, respectively, and then apoptosis was determined by flow cytometry.

### Evaluation of P53 gene expression by fluorescent method

The cell suspension was cultured on a gelatin slide. After 24 hours, it was washed with PBS and

fixed for 20 minutes with paraformaldehyde at 4% at room temperature. The samples were then treated for 72 hours on 24-plate plates using special p53 antibodies. The percentage of p53 expression was examined by an inverse fluorescence microscope. The results were plotted using quantitative Image J software and the resulting pattern in PRISM5.

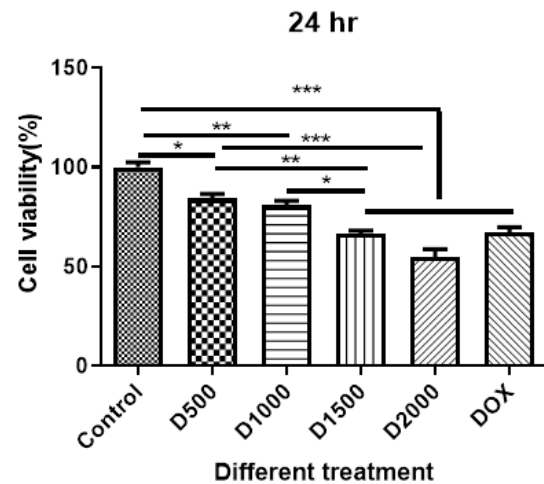
**Information analysis method**

In order to analyze the data, SPSS software version 23 and descriptive and inferential statistics were used, and in order to analyze the data, One-way ANOVA was used. The values  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  were considered meaningful.

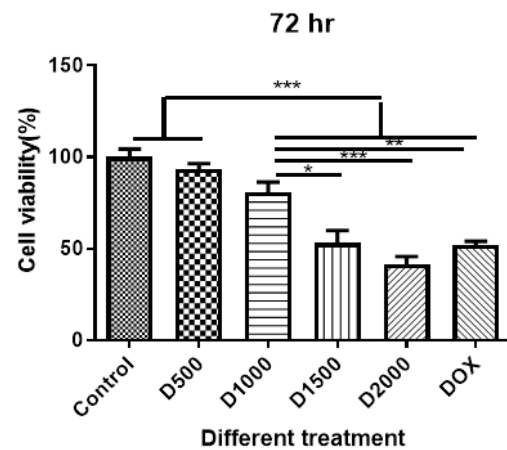
**Result and Discussion**

In this part of the study, to evaluate the effective dose of mRNC on the rate of induction of apoptosis, MCF-7 cells were treated with different concentrations of 500, 1000, 1500 and 2000 ng/μl of mRNC and then the level of apoptosis in these cells was assessed after 24 and 72 hours. The results showed that after 24 and 72 hours, the dose of 500 ng/μl had a higher survival rate than other concentrations. In addition, the results showed that the dose of 2000 ng/μl had the highest induction of apoptosis. However, the concentration of 1500 ng/μl similar to doxorubicin, a known effective treatment in this regard, could lead to a significant reduction in the induction of apoptosis. Thus, according to figures 1 and 2, the results were shown. Comparison of the results of two time periods of 24 and 72 hours showed that although the effect of these concentrations on the induction of apoptosis was parallel, but at 72 hours a stronger induction effect was observed. The results show that with increasing concentration, the rate of apoptosis increased.

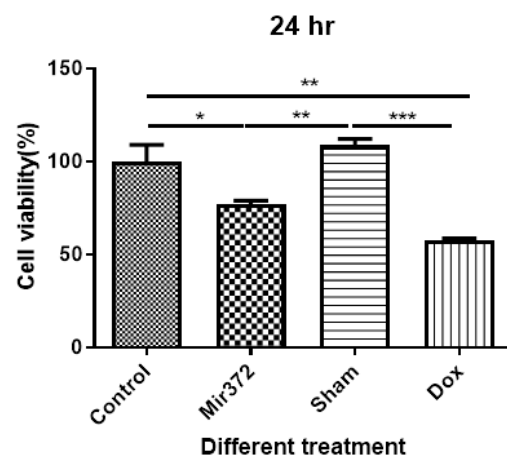
Since the concentration of 1500 ng/μl was similar to that doxorubicin in inducing apoptosis in half of the cells IC50, this dose was evaluated as the optimal dose. After 24 hours, the concentration of 1500 ng/μl resulted in a significant decrease ( $p < 0.05$ ) compared to the control group. In addition, this concentration was not significantly different from doxorubicin as shown in figure 3.



**Figure 1.** Evaluation of the rate of induction of apoptosis after 24 hours of treatment with mRNC (\*\* $p < 0.01$ , \*\*\*  $p < 0.001$ , \*  $p < 0.05$ ).



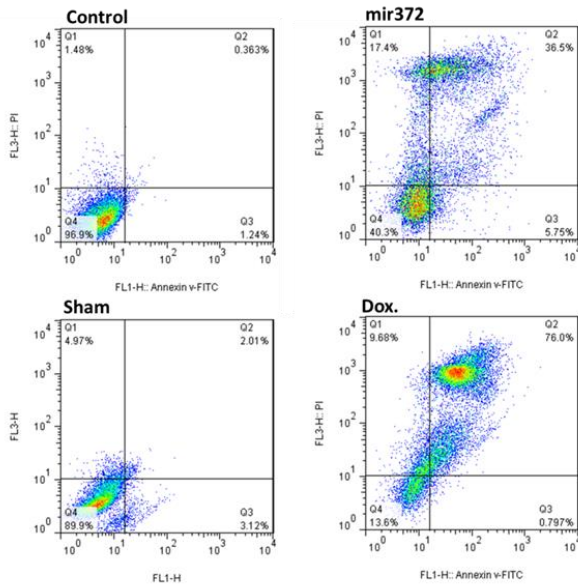
**Figure 2.** Evaluation of the rate of induction of apoptosis after 72 hours of treatment with chitosan-coated miR-372 (\*\* $p < 0.01$ , \*\*\*  $p < 0.001$ , \*  $p < 0.05$ ).



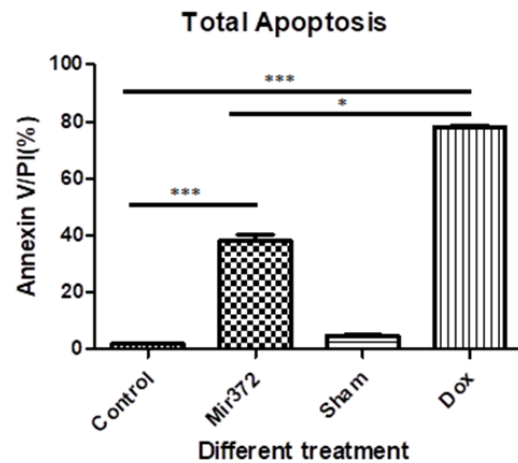
**Figure 3.** Evaluation of the rate of induction of

apoptosis after 24 hours of treatment of MCF-7 cells with miR-372 coated with chitosan at a concentration of 1500 ng/μl by MTT method (\*\*p < 0.001, \*p < 0.01, \*p < 0.05).

After 72 hours, flow cytometry results showed that this concentration was able to induce general apoptosis in MCF-7 cells, so that the highest number of apoptotic cells was observed in the second quarter, which were stained with PI \ Annexin according to figures 4 and 5.

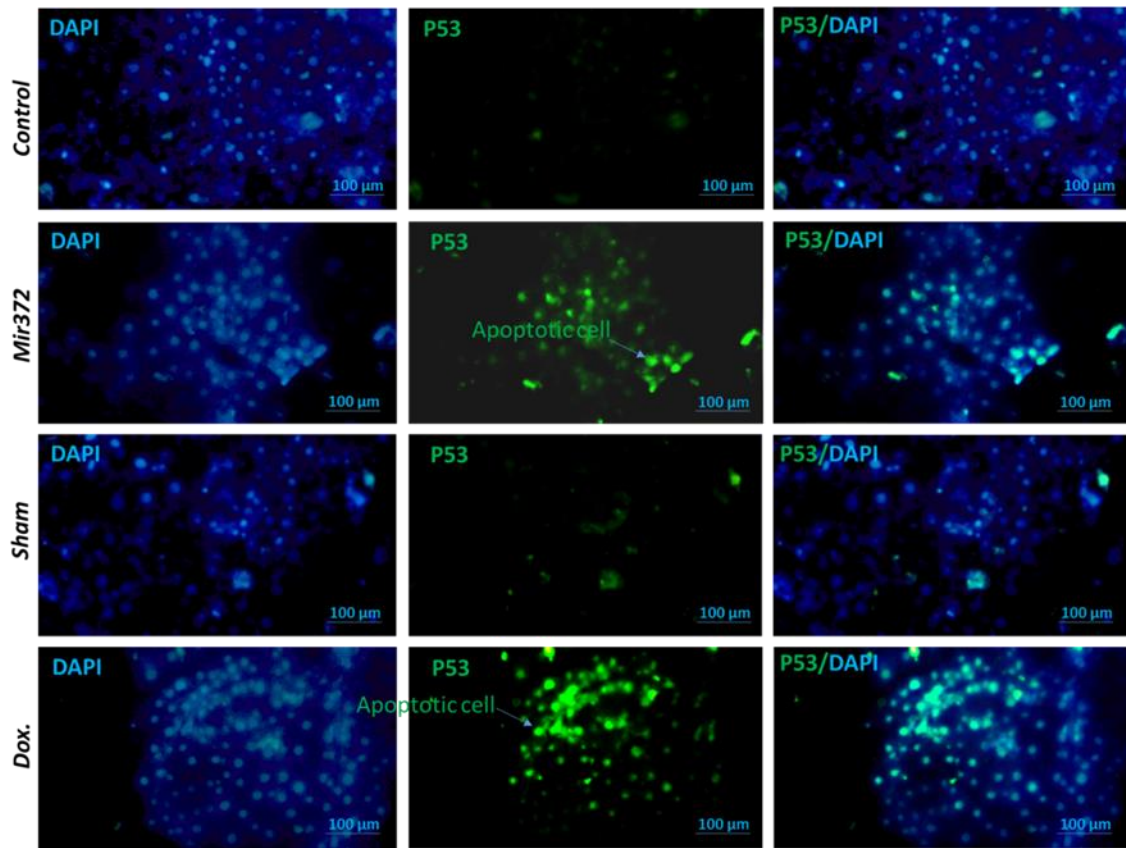


**Figure 4.** Evaluation of induction of apoptosis by flow cytometry and results of Annexin V-PI staining in MCF-7 cells 72 hours after treatment with miR-372 coated with chitosan at a concentration of 1500 ng/μl. As shown in the figure, the cells of the control group with a total apoptosis of  $0.21 \pm 1.76$  were placed in the 4th quarter, which indicates healthy cells that were not stained with PI -V Annexin dyes. In the sham group, the rate of apoptosis was  $4.71. \pm 0.40$ , which did not show a significant difference with the control group, which indicates that the cells in this group are healthy. In the chitosan-coated miR-372 treatment group, a significant population of cells was in the third quadrant, indicating that they were stained with PI -V Annexin, resulting in apoptosis. The rate is  $38.15 \pm 3.77$  and in the doxorubicin treatment group the rate of apoptosis was  $78.07 \pm 0.92$  which indicated the induction of apoptosis in these two treatment groups which showed a statistically significant difference ( $p < 0.001$ ).

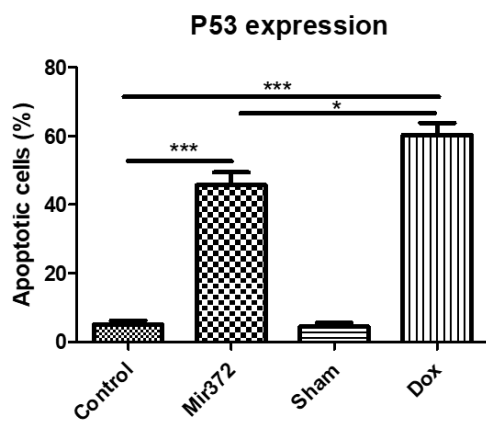


**Figure 5.** Evaluation of the rate of induction of apoptosis after 72 hours of treatment with miR-372 coated with chitosan at a concentration of 1500 ng/μl by flowcytometry. The rate of total apoptosis is obtained from the sum of primary and delayed apoptosis in the second and third quarters. (\*\*p < 0.001, \*p < 0.01, \* p < 0.05).

The P53 gene is a tumor suppressor gene that inhibits cancer by increasing the rate of apoptosis. DAPI is a fluorescent dye that stains DNA and the nucleus of a cell and shows healthy and unhealthy cell nuclei in blue. Image integrated p53 DAPI staining shows cells with apoptosis. The results of fluorescent microscopy showed that the expression of this protein in cells treated with mRNC at a dose of 1500 ng / μl was significantly increased compared to the control group according to figures 6 and 7.



**Figure 6.** Evaluation of p53 protein expression in MCF-7 cells treated with miR-372 coated with chitosan, doxorubicin and the control groups for 72 hours by ICC method. As shown in the FITC filter images, the expression of p53 protein in chitosan and doxorubicin-coated miR-372-treated cells was significantly increased compared to the control and sham groups, which could be Indicates the induction of apoptosis in these cells by increasing the expression of p53 by treatment with miR-372 and doxorubicin. This image was used to examine the percentage of apoptotic cells using Image J software. Images were presented semi-quantitatively. Image magnification is  $\times 400$ .



**Figure 7.** Evaluation of P53 protein expression in MCF-7 cells after 72 hours of treatment with chitosan-coated miR-372 at a concentration of 1500 ng/ $\mu$ l by immunocytochemistry. Protein expression was presented as a percentage of positive cells that were counted relative to the nucleus of all cells. Semi-

quantitative results were presented in the form of graphs which were reported as mean and standard deviation. (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$ , (\*)  $p < 0.05$ ).

### Discussion

Breast cancer is the most common malignancy in women worldwide and can be treated in 70-80% of patients with premature and non-metastatic diseases, so it is important to suggest new treatment mechanisms or improve existing methods. The miRs have been reported to play an important role in the onset and progression of breast cancer (Cai et. al., 2013; Krell et. al., 2012). The miR-372 is also a type of miRNA that regulates apoptotic cell proliferation, migration, and invasion in many human cancers, and may play an oncogenic or suppressive role in various human cells (Zhao et. al., 2017). Despite the important role of miR in the treatment of cancer, there are challenges in the treatment of miR in vivo.



The basis of these therapies includes the stability of cellular uptake of non-target immunogenic effects and the degree of toxicity. As a result, appropriate delivery systems for the miRs must be established. Among the non-viral gene transfer systems used, chitosan is a very promising non-viral carrier for nucleic acid-based therapies. Chitosan is a biodegradable and low-immunity biopolymer (Özbaş-Turan et. al., 2011; Akbuğa et. al., 2004). In this study, chitosan was used to cover miR-372 to investigate its anti-cancer effect on MCF-7 cells. Its effect can be a hope for the production and improvement of systemic methods of breast cancer treatment. Normally, chemotherapy drugs are able to prevent the process of proliferation and stop the mitotic cycle in these cells, so in this study, doxorubicin was used as a sample of the commercial drug as a positive control. Tumor cells with a concentration of 2000 ng/μl of the drug Doxorubicin was treated and cell survival was assessed after 24 to 72 hours using flow cytometry. The results showed that doxorubicin was able to kill 50% of cancer cells in 24 and 72 hours. Probably, the drug could have caused cell death by the mechanism of cell membrane cleavage and DNA cleavage by binding between DNA pairs and preventing the formation of ester bonds. The results showed that up to 75% of the drug could induce apoptosis, which may have been effective on genes in the apoptosis signaling pathway in cancer cells and increased the expression of these genes. In addition, p53 protein expression was investigated as a mechanism involved in tumor induction. For this purpose, cancer cells were treated with a concentration of 2000 ng/μl of doxorubicin and after 72 hours, p53 expression was measured using a fluorescent inverted microscope, which showed a 70% increase in p53 expression. In the continuation of the study, was used of m RNC. In this part of the study, cancer cells were treated with doses of 500, 1000, 1500 and 2000 ng/μl of mRNC and apoptosis was assessed for 24 and 72 hours. Based on the results obtained dose of 1500 and 2000 ng/μl could induce apoptosis in up to 50% of cells that this rate has been more effective over time, i.e., 72-hour treatments showed more induction of apoptosis. In addition, according to this study, the use of higher doses of mRNC has a greater effect on the induction of apoptosis than lower doses. In addition, in the induction of apoptosis section, the flow cytometry results showed that 40% of the cells underwent apoptosis after 72 hours of treatment with 1500 mg/μl mRNC, which was observed by increasing the expression of p53 by 40% compared

to the control group. According to the results of this study, it was found that controlling the expression of upstream genes responsible for cell proliferation and inhibiting proteins using regulatory molecules can affect cell growth and development and increase the likelihood of cancer cells.

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**Conflict of Interest** The authors declare no financial or non-financial conflict of interest.

## References

- Akbuğa J., Ozbaş-Turan S. and Erdoğan N. (2004) Plasmid-DNA loaded chitosan microspheres for in vitro IL-2 expression. *European journal of pharmaceuticals and biopharmaceuticals* 58(3):501-507.
- Ascierto M.L., Kmiecik M., Idowu M.O., Manjili R., Zhao Y., Grimes M. et. al. (2012) A signature of immune function genes associated with recurrence-free survival in breast cancer patients. *Breast cancer research and treatment* 131(3):871-880.
- Bogdanova N., Helbig S. and Dörk T. (2013) Hereditary breast cancer: ever more pieces to the polygenic puzzle. *Hereditary cancer in clinical practice* 11(1):1-16.
- Burstein H.J. and Griggs J.J. (2010) Adjuvant hormonal therapy for early-stage breast cancer. *Surgical oncology clinics of North America* 19:639-647.
- Cai J., Guan H., Fang L., Yang Y., Zhu X., Yuan J., et al. (2013) MicroRNA-374a activates Wnt/β-catenin signaling to promote breast cancer metastasis. *The Journal of clinical investigation* 123(2):566-579.
- Cheng X., Chen J. and Huang Z. (2018) miR-372 promotes breast cancer cell proliferation by directly targeting LATS2. *Experimental and therapeutic medicine* 15(3):2812-2817.
- Dunnwald L.K., Rossing M.A. and Li CI. (2007) Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. *Breast cancer research* 9(1):1-10.
- Elieh-Ali-Komi, D. and Hamblin M.R. (2016) Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. *International*

Journal of Advanced Research 4(3):411-427.

Friedman A.E. (2007) Can a single model explain both breast cancer and prostate cancer? Theoretical biology & medical modelling 4(28):28.1-13.

Iorio M.V., Ferracin M., Liu C-G., Veronese A., Spizzo R., Sabbioni S., et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65(16):7065-7070.

Krell J., Frampton A.E., Jacob J., Pellegrino L., Roca-Alonso L., Zeloof D., et al. (2012) The clinico-pathologic role of microRNAs miR-9 and miR-151-5p in breast cancer metastasis. Molecular diagnosis & therapy 16(3):167-172.

Majeed W., Aslam B., Javed I., Khaliq T., Muhammad F., Ali A. et al. (2014) Breast cancer: major risk factors and recent developments in treatment. Asian Pacific Journal of Cancer Prevention 15(8):3353-3358.

Osborne C., Wilson P. and Tripathy D. (2004) Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. The oncologist 9(4):361-377.

Özbaş-Turan S. and Akbuğa J. (2011) Plasmid DNA-loaded chitosan/TPP nanoparticles for topical gene delivery. Drug delivery 18(3):215-222.

Paliwal S.R., Paliwal R., Pal H.C., Saxena A.K., Sharma P.R., Gupta P.N., et al. (2012) Estrogen-anchored pH-sensitive liposomes as nanomodule designed for site-specific delivery of doxorubicin in breast cancer therapy. Molecular pharmaceutics 9(1):176-186.

Regan M.M., Neven P., Giobbie-Hurder A., Goldhirsch A., Ejlertsen B., Mauriac L., et al. (2011) Assessment of letrozole and tamoxifen alone and in sequence for postmenopausal women with steroid hormone receptor-positive breast cancer: the BIG 1-98 randomised clinical trial at 8.1 years median follow-up. The Lancet. Oncology 12(12):1101-1108.

Sharma G.N., Dave R., Sanadya J., Sharma P. and Sharma K. (2010) Various types and management of breast cancer: an overview. Journal of advanced pharmaceutical technology & research 1(2):109-126.

Soliman H. (2013) Immunotherapy Strategies in the Treatment of Breast Cancer. Cancer Control 20(1):17-21.

Velpurisiva P., Gad A., Piel B., Jadia R. and Rai P.

(2017) Nanoparticle Design Strategies for Effective Cancer Immunotherapy. Journal of biomedicine 2(2):64-77.

Zhang T., Hu Y., Ju J., Hou L., Li Z., Xiao D., et al. (2016) Downregulation of miR-522 suppresses proliferation and metastasis of non-small cell lung cancer cells by directly targeting DENN/MADD domain containing 2D. Scientific reports 6:1-12.

Zhao Y.X., Liu H.C., Ying W.Y., Wang C.Y., Yu Y.J., Sun W.J., et al. (2017) microRNA-372 inhibits proliferation and induces apoptosis in human breast cancer cells by directly targeting E2F1. Molecular medicine reports 16(6):8069-8075.

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