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The effects of chitosan/miR-200c nanoplexes on different stages of cancers in breast cancer cell lines☆,☆☆

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ABSTRACT

Dysregulation of miR-200c in breast cancer has been associated with migration, epithelial mesenchymal transition (EMT), angiogenesis and metastasis of the tumor cells. Therefore, the modulation of miR-200c offers a promising therapeutic approach in breast cancer. However, the major obstacles in the usage of miRNAs in therapy are their low stability, rapid clearance, and poor cellular uptake. The development of efficient and safe delivery systems is important in effective therapy with miRNA. The purpose of this study was to investigate the therapeutic role of chitosan/miR-200c nanoplexes in angiogenesis, EMT, invasion, and apoptosis in breast cancer cell lines. We found that miR-200c levels were downregulated in various breast cancer cell lines by qRT-PCR. After transfection with chitosan/miRNA nanoplexes in the appropriate size (294 nm) and zeta potential (12.3 mV), levels of miR-200c increased and reached the endogenous miR-200c levels in the MCF-7, MDA-MB-231, and MDA-MB-435 cells. While the chitosan/miR-200c nanoplexes decreased angiogenesis, invasion, EMT, and metastasis in the cells, the apoptosis levels increased by 3.1, 1.3, and 3 fold in the MCF-7, MDA-MB-231, MDA-MB-435 cell lines, respectively. Consequently, chitosan is a suitable carrier for miR-200c and formed stable nanoplexes with miR-200c. The effect of the chitosan/miRNA nanoplexes on tumor angiogenesis, EMT, invasion, metastasis, and apoptosis, changed depending on the cell-types. Therefore, during the treatment with the chitosan based miR-200c nanoplexes in breast cancers, the type of tumor cells must be considered.

1. Introduction

The progression, invasion, angiogenesis, and metastasis of breast cancer, are a complicated process comprised of many steps. It is a complex, heterogenous, and multifactorial disease, which interplays between both genetic and environmental factors. Novel therapeutic approaches are necessary due to this complex nature of cancer. Recently, many researchers have been focusing on nucleic acid-based therapeutics in cancer therapy: including miRNA. Many microRNAs (miRNAs) have an important effect on tumor behavior and progression. They are believed to be the key factors in cancers. Cancer therapy with miRNAs can be accomplished either by direct inhibition or the replacement of miRNAs, or by targeting specific genes (Deng et al., 2014; Rothschild, 2014).

The miR-200 family is an important target in cancer (Zhang et al., 2014). The miR-200 family consists of the 5 miRNAs, which form two clusters located in two different genomic regions. Cluster II miR-200s, contains miR-200c/141. The miRNAs in each cluster are likely to be co-regulated (Park et al., 2008). Several studies have shown that miR-200s play a suppressive role in breast cancer development (Howe et al., 2011; Rhodes et al., 2015). The delivery of the miR-200 members into the tumor endothelium resulted in the marked reductions in metastasis and angiogenesis, and induced vascular normalization (Pecot et al., 2013). The expression of miR-200c inhibits the clonal expansion of the stem cells and represses the breast cancer stem cells. The miR-200c has potent effects on migration, EMT, angiogenesis, and metastasis (Le et al., 2014; Pecot et al., 2013; Schliekelman et al., 2011; Tang et al., 2016). EMT is necessary for carcinoma cells in order to be able to start the metastatic cascade. E-cadherin is one of the key downstream regulators of miR-200c contributing to EMT (Gravgaard et al., 2012; Yeh et al., 2014), and miR-200c is also important in inhibiting tumor invasion and proliferation, as well as inducing cell apoptosis (Chen et al., 2013). In addition, researchers found that miR-200c decreases the invasiveness ability of breast cancer cells in vitro (Sundararajan et al., 2015; Tang et al., 2016). However, the effects of the miR-200c on EMT, invasion, angiogenesis, and the apoptosis of the breast cancer cells have not been fully characterized yet. Metastatic colonization is the major cause of cancer mortality and the final step of metastasis (Fazilaty et al., 2013). Recently, Korpal et al. (2011) reported miR-200c promote metastatic colonization

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through the direct targeting of Sec23A-mediated secretion of metastasis suppressive proteins such as Igfbp4 and Tinagl1.

The most important challenges for the miRNA-based therapeutics are low stability and poor cellular uptake. For effective therapy, the miRNA must penetrate the target cell and tissue, so the development of efficient and safe delivery systems is important. Viral and non-viral vectors have been extensively used in nucleic acid-based therapy. Compared to viral vectors, non-viral vectors have several advantages such as a lack of immunogenicity, low or no integration in the genome, large scale production, and size limitations of nucleic acids. Cationic polymers are widely used as gene delivery systems (Buschmann et al., 2013; Neuberg and Kichler, 2014; Salva et al., 2013). Chitosan is a promising non-viral vector in gene delivery because of properties such as its cationic structure, biodegradability, biocompatibility, and non-toxicity.

There are a few studies related to chitosan-based systems for miRNA delivery in cancer therapy. Chitosan-molecular beacon complexes were used for miR-155 detection and imaging in lung cancer (Zhu et al., 2014). In another paper, Chen et al. (2014) used the Achilles tendon injured rat model and investigated the role of miRNAs on the effects of chitosan on tendon healing via the regulation of the TGF-β1/Smad3 pathway. They suggested that chitosan markedly increased the expression of miR-29b in both the rat repaired tendon tissue and the fibroblasts.

Doxorubicin and miR-34a encapsulated hyaluronic acid-chitosan nanoparticles were prepared and the therapeutic effects in vitro and in vivo in the triple negative breast cancer model were investigated. Co-delivery of doxorubicin and miR-34a could achieve synergistic effects on tumor suppression (Deng et al., 2014).

Santos-Carballal et al. (2015) prepared chitosan/miR-145 nanocomplexes using chitosans with a different molecular weight and degrees of acetylation (12% and 29%) and then conducted in vitro characterization studies. These nanocomplexes did not have any cytotoxic effect on the MCF-7 cells. The miRNA-145, which is an oncomiR, downregulates junction adhesion molecule A (JAM-A) mRNA. The dose-dependent downregulation of JAM-A mRNA following transfection with chitosan/miR-145 nanocomplexes were reported in this study.

Although the biological functions of miR-200c have been studied, there has been no report to date related to the development and progress pathways of cancer by using chitosan/miR-200c nanoplexes. In this study, we investigated the therapeutic role of chitosan/miR-200c nanoplexes in angiogenesis, epithelial mesenchymal transition (EMT), invasion, and apoptosis, in various breast cancer cell lines including MCF-7, MDA-MB-231 and MDA-MB-435.

2. Materials and Methods

2.1. Materials

Hsa-miR-200c and negative control (cel-miR-67) were obtained from Dharmacon (miRIDIAN™, UK). Chitosan (75 kDa; 75%–85% deacetylation degree) were purchased from Sigma (St. Louis, MO, U.S.A.). All the substance used in the study were of molecular grade.

2.2. Preparation and Control of Chitosan/miRNA Nanoplexes

Chitosan was dissolved in 40 mM acetate buffer (pH 5.4) and the final concentration was adjusted to 1%. Chitosan solution was mixed with the solution of miR-200c (0.5 μg/μl) at different ratio (+/−) (10/1 to 50/1). Formation of nanoplexes were checked by agarose gel electrophoresis using 2% of gel at 80 mA and zeta potential and size of nanoplexes were characterized with ZetaSizer NanoZS (Malvern Instruments, UK) in PBS pH 7.4 at 25 °C. miRNA was visualized under UV light as a result of reaction of miRNA with RedSafe™ (Intron Biotech, South Korea) nanoplexes were prepared in triplicate (n = 3).

2.3. Cell Lines and Cell Culture

The breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-435 (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, penicillin and streptomycin antibiotic solution (Biological Industries, USA) and breast cell line MCF-10A (ATCC, Rockville, MD) were maintained in Mammary Epithelial Cell Growth Medium (MEGM™) Bulletkit (Lonza, USA). All cell lines were cultivated in incubator at 37 °C with 5% CO_2 (Sanyo CO_2 Incubator, Japan) in a humidified atmosphere. T-25 flasks and 6-well plates were used for studies. Cells were passaged every three days with 1:4 split ratios. Breast cancer cell lines were transfected with chitosan/miR-200c (20/1) nanoplexes containing 750 ng, 100 ng and 750 ng miR-200c to MCF-7, MDA-MB-231 and MDA-MB-435, respectively. Transfection studies were done at 70% confluency.

2.4. Determining of miR-200c Levels by Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed to evaluate the expression level of miR-200c after 48 h from chitosan/miR-200c (20/1) transfection in breast cancer cells using StepOne Plus 7500 fast RT-PCR (Applied Biosystem, USA). Total RNA was isolated by miRVana miRNA Isolation kit (Ambion, USA) from cultured cells following the protocols of the manufacturer. For miRNA expression analysis, 10 ng of total RNA was applied for reverse transcription to synthesize cDNA of miRNA using TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR analysis was performed using TaqMan® Universal Master Mix (Applied Biosystems, USA) and TaqMan® microRNA assays (Applied Biosystems, USA) for miR-200c according to manufacturer's instructions. RNU6B was used as housekeeping controls. Each sample was analyzed in triplicate. Relative expressions of miRNAs were calculated using the comparative CT method. Results were expressed as RQ.

2.1. Effects of Chitosan/miR-200c Nanoplexes on VEGF, E-cadherin, Igfbp4 and Tinagl1 Expression Levels

Cell culture supernatants were centrifuged for 5 min to remove cells and cell debris. VEGF-A (Life Technologies, USA), E-cadherin (Cloud-Clone Corp., USA), Igfbp4 (RayBiotech, USA) and Tinag11 (SunRed, Shanghai) proteins that accumulated in the culture medium and dilutions of a recombinant human protein standards were analyzed using sandwich ELISA according to manufacturer protocols. Protein concentrations in the samples were determined spectrophotometrically (UV spectrophotometry, Shimadzu, Japan) at 450 nm. Each test was repeated for three times and data were shown as mean values ± SE.

2.2. Invasion Study

For the invasion study, 1×10^5 cells were suspended in DMEM medium and seeded in the upper chamber with 8 μm pore size polyethylene terephthalate (PET) membrane (Becton Dickinson, USA) and incubated at 37 °C in CO_2 incubator during 1 h. Chitosan nanoplexes containing miR-200c were added and after 24 h of incubation, cells on the upper side of the membrane were removed with a cotton swab, whereas the cells that migrated through the membrane to the underside were fixed with 4% paraformaldehyde and stained with giemsa. Cell numbers were counted under an inverted microscope (Olympus, Japan).

2.3. Apoptosis Study

In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The

human anticoagulant, annexin V, is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with FITC were used as a probe by binding to PS to identify apoptotic cells (Invitrogen, USA). Following the manufacturer procedure, cells were counted and viewed under fluorescence microscope (Olympus, Japan). Apoptotic cells showed green fluorescence while dead cells showed red and green fluorescence.

2.4. Statistical Analysis

A two-tailed Student *t* test was used to estimate intergroup differences if not otherwise stated. All analyses were performed using SPSS Version 20 (Statistical Software for Social Sciences, USA).

3. Results

3.1. Nanoplex Formation and Characterization

Chitosan formed full complexes with miR-200c at 20/1 (+/−) ratio. As seen in the electrophoresis photograph, the chitosan partly complexed with miRNA below this ratio (Fig. 1). Chitosan/miR-200c nanoplexes having 294.8 ± 5 nm size and +12.3 ± 0.8 mV surface charge were used in this study. Chitosan/cel-miR-67 complexes were used at 20/1 ratio as negative control.

3.2. Determination of Endogenous miR-200c Expression Level by qRT-PCR

To investigate the potential importance of miR-200c in the progression of breast cancer, we firstly determined the expression level of the miR-200c in the breast cancer cell lines. The normal (non-cancerous) cells were used as control. This data suggested that the miR-200c expression was significantly downregulated in the breast cancer cell lines in vitro. While the miRNA level decreased by 60% in the MCF-7 cell line, a 99% decrease was measured in the MDA-MB-231 and MDA-MB-435 cells (Fig. 2).

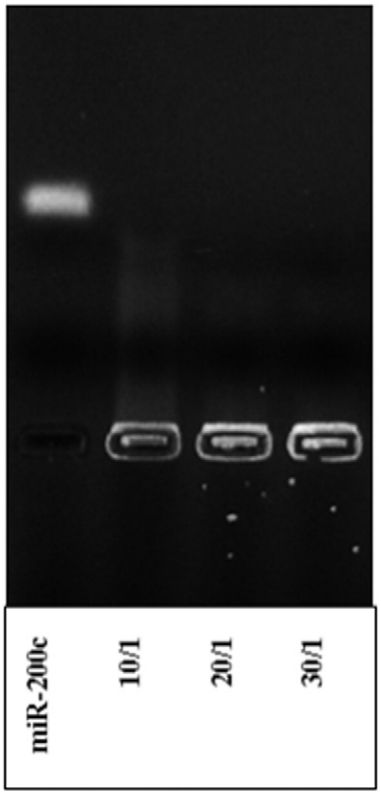

Fig. 1. Agarose gel electrophoresis of free miR-200c and chitosan/miR-200c nanoplexes prepared in the different ratios (10/1, 20/1 and 20/1).

3.3. Determining of miR-200c Levels of Breast Cancer Cell Lines After Chitosan/miRNA Treatments

After the transfection with the chitosan/miRNA nanoplexes of the cell lines, the levels of the miR-200c were determined. As given in Fig. 3, the miR-200c level increased and reached the endogenous miR-200c levels in the MCF-7, MDA-MB-231, and MDA-MB-435 cells.

3.4. Effect of Chitosan/miR-200c Nanoplexes on VEGF Expression

In order to investigate the effect of the miR-200c containing chitosan-based systems on angiogenesis, the VEGF levels of the breast cancer cells were measured in treated and untreated cancer cell lines (Fig. 4). The VEGF expression decreased in all the breast cancer cell lines after the chitosan/miR-200c nanoplexes treatment and was not affected after transfection of the chitosan/cel-miR-67 nanoplexes. In the MCF cell, after treatment with chitosan/miR-200c, the VEGF level is reduced by 24.4%. After the nanoplex treatment, the VEGF production diminished 6.8% in the MDA-MB-231 and 18.1% in the MDA-MB-435 cell lines (Fig. 4).

3.5. Effect of Chitosan/miR-200c Nanoplexes on EMT

In order to study the role of miR-200c in EMT, E-cadherin levels in the MCF-7, MDA-MB-231, and MDA-MB-435, the cells were measured by using ELISA. E-cadherin levels were not changed by chitosan/cell-miR-67 complexes ($p > 0.05$). As seen in Fig. 5, after the chitosan/miR-200c nanoplexes transfection, the E-cadherin levels increased in MCF-7 (15.4%) and MDA-MB-231 (88.7%) cells when compared to the untreated cancer cells. However, after the transfection in MDA-MB-435, the E-cadherin level slightly increased to 1.9% (Fig. 5). In the MDA-MB-435 cell, there was no significant statistical difference between the E-cadherin levels ($p > 0.05$).

3.6. Effect of Chitosan/miR-200c Nanoplexes on Metastasis

To define the relationship between metastasis suppressive proteins such as Igfbp4 and Tinagl1 and chitosan/miR-200c nanoplexes, the Igfbp4 and Tinagl1 levels in the breast cancer cells were determined. There were no significant statistical changes after transfection of chitosan/cell-miR-67 complexes ($p > 0.05$). The Igfbp4 expression slightly increased after the chitosan/miR-200c nanoplexes transfection to the MCF-7 and MDA-MB-231 cells (Fig. 6). The Igfbp-4 level slightly declined after the nanoplex transfection in the MDA-MB-435 cell (Fig. 6). According to our findings, after the chitosan/miR-200c

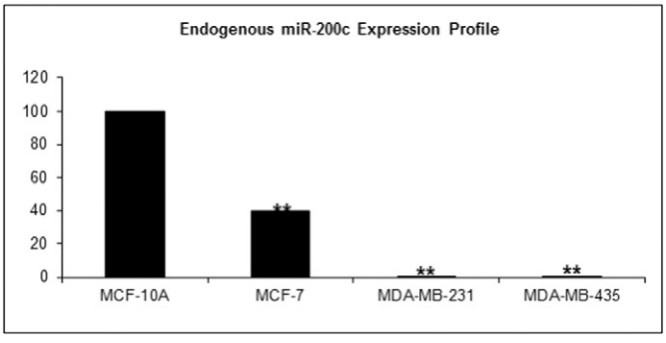

Fig. 2. Comparison of endogenous miR-200c expression levels in healthy normal and breast cancer cell lines by qRT-PCR. *$p < 0.05$, **$p < 0.01$.

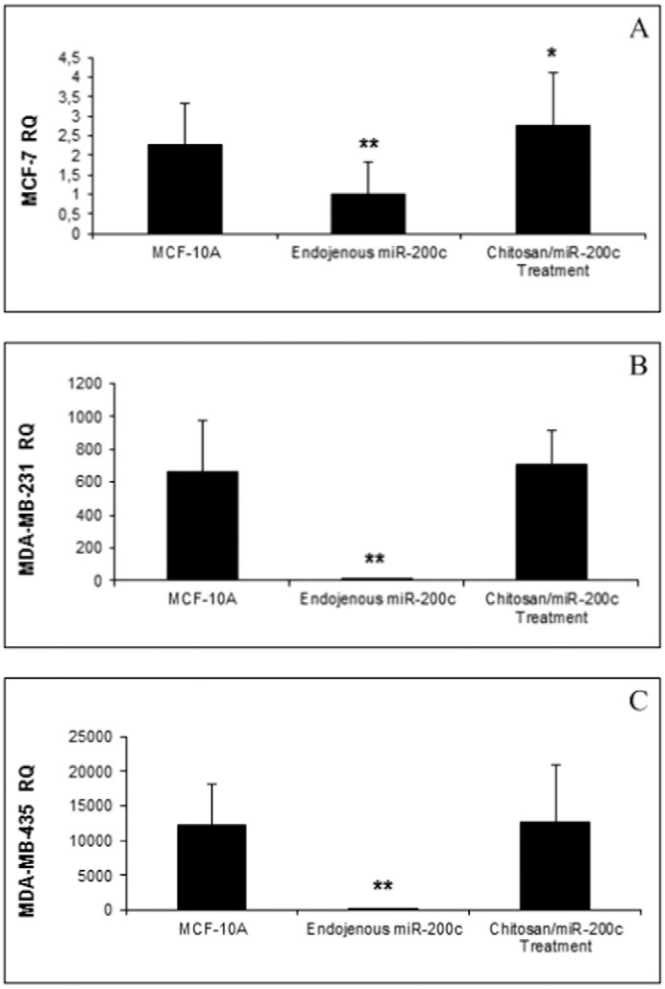

Fig. 3. Determining by qRT-PCR miR-200c levels of breast cancer cell lines after chitosan/miRNA nanoplex treatment A-MCF-7, B-MDA-MB-231, C-MDA-MB-435. *p < 0.05, **p < 0.01.

nanoplexes transfection, the Tinagl1 levels showed an increase depending on the cell type (Fig. 7). The highest increase was seen in MDA-MB-231 (36%) (Fig. 7).

3.7. Effect of Nanoplexes on Invasion

The effect of the miR-200c-containing nanoplexes on the invasion was studied in the breast cancer cell lines. According to the data, the migrating cell numbers significantly decreased in the MDA-MB-231 cell line ($p \leq 0.01$) and in the MCF-7 cell line ($p < 0.05$) after the nanoplex transfection (Fig. 8). As seen in Fig. 8, highest invaded cell numbers were observed at the MDA-MB-435 cell line treated with nanoplexes when compared to the untreated cells ($p < 0.05$).

3.8. Effect of Nanoplexes on Apoptosis

The apoptosis study was performed in order to determine whether or not the chitosan/miR-200c nanoplexes caused an apoptosis induction. The apoptosis levels in the breast cancer cells were evaluated based on the DNA fragmentation. Fig. 9 shows the results of the apoptosis assay in the chitosan/miR-200c nanoplexes treated and untreated cells. The data is summarized as the percentage of positive cells for the apoptosis (Fig. 9). The cells treated with the chitosan/miR-200c nanoplexes showed a 3.1, 1.3, and 3 fold increase in apoptosis levels of the MCF-7, MDA-MB-231, MDA-MB-435 cell lines, respectively.

4. Discussion

Although there have been advancements in diagnostics and therapeutics, breast cancer is still the leading cause of cancer deaths in the world. Because of its resistance to chemotherapy, new approaches are necessary in the therapy of breast cancer. The miR-200s expressed in breast cancer have important roles in tumor initiation and progression (Pereira et al., 2013). It is known that the expression of the miR-200 family is the inhibitor of EMT, migration, and invasion, downregulated in human tumors (Zhang et al., 2014). While the activity of the miR-200c in relation to EMT has been widely studied (Burk et al., 2008; Hilmarsdottir et al., 2015, 2014; Howe et al., 2011; Jurmeister et al., 2012; Rajabi et al., 2014; Sundararajan et al., 2015; Xie et al., 2014), the effects of chitosan/miR-200c nanoplexes on angiogenesis, invasion, EMT, metastasis, and apoptosis, in breast cancer cells have not yet been fully investigated.

The only study related to the physicochemical and biologic characterizations of the chitosan:miRNA-145 nanocomplex was reported by Santos-Carballal et al. They evaluated the chitosan:miRNA-145 nanocomplexes (<200 nm) using chitosan with a low deacetylation degree (12% and 29%) and reported that the target mRNA expression

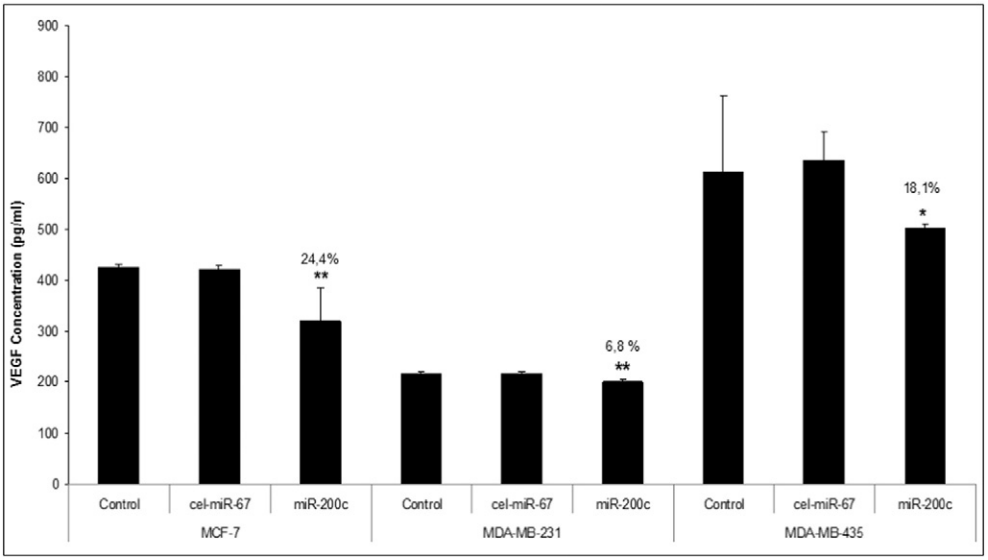

Fig. 4. VEGF levels of breast cancer cell lines after transfection of chitosan/miR-200c nanoplexes. *p < 0.05, **p < 0.01.

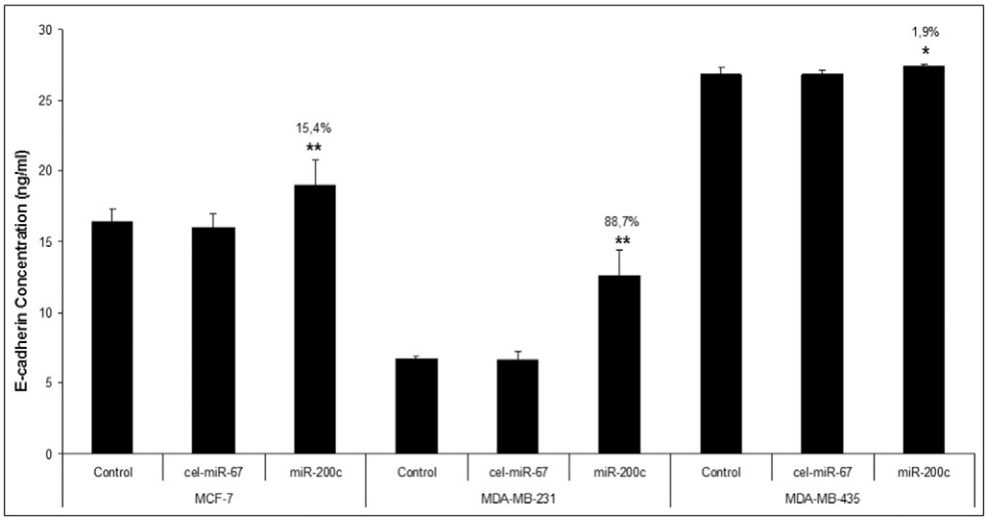

Fig. 5. E-cadherin levels of breast cancer cell lines after treatment with chitosan/miR-200c nanoplexes. *p < 0.05, **p < 0.01.

downregulated in MCF-7 cells after transfection (Santos-Carballal et al., 2015).

In our study, endogenous miR-200c levels of normal and cancerous cells (MCF-7, MDA-MB-231, and MDA-MB-435) were determined. As seen Fig. 2 miR-200c has been downregulated all the breast cell lines. The miR-200c levels in MCF-7, MDA-MB-231, and MDA-MB-435 cells were lower than that in MCF-10A, by 60%, 99%, and 99%, respectively (Fig. 2). This data showed a similarity with previous papers related to the downregulation of miR-200c in cancer cells (Deng et al., 2014; Rhodes et al., 2015). In this study, we tried to increase the miR-200c levels of the cells by transfecting the cancer cells with chitosan nanoplexes containing miR-200c to reach miR-200c level of the normal (healthy) cells (Fig. 3). Dose studies were done all the cancer cell lines (Kaban et al., 2016) and according to data nanoplexes containing 750 ng miR-200c were applied to MCF-7 and MDA-MB-435 cells and 100 ng miR-200c was given to MDA-MB-231 cells for providing non-cancerous cell line endogenous level (Fig. 3). The EMT, angiogenesis, invasion, metastasis, and apoptosis studies were performed in chitosan/miR-200c nanoplexes treated and untreated breast cancer cell lines including MCF-7, MDA-MB-231, and MDA-MB-435.

Angiogenesis plays a critical role in pathological conditions including tumor growth. In solid tumors, this process depends on the expression of angiogenic factors such as the vascular endothelial growth factor (VEGF) (Salva et al., 2014, 2010). Recently, many angiogenic miRNAs have been identified and the mechanism of miRNA in regulating angiogenesis have been elucidated (Li et al., 2014; Mao et al., 2015). They have shown that angiogenesis can be affected by modulating the expression of the angiogenic factors. Recent papers have shown that the miR-200 family can inhibit angiogenesis because they directly target the VEGF receptors (Humphries and Yang, 2015; Shi et al., 2013). In another study, miR-200a and b have been shown to target the proaniogenic ligands in ovarian cancer (Pecot et al., 2013). Therefore, in order to find out the relationship between the chitosan/miR-200c nanoplexes and angiogenesis, we investigated the VEGF profile after the chitosan/miR-200c transfection. As seen in Fig. 4, the VEGF level decreased in the MCF-7, MDA-MB-231, and MDA-MB-435 cell lines at 24.4%, 6.8% and 18.1%, respectively, after transfection. This angiogenesis decreasing effect shows the similarity with an earlier report (Zhang et al., 2014). In addition, the regulatory role of miR-200c in angiogenesis in the pancreatic tumors has been reported by Li et al. (2015).

EMT can be defined by the formation of the mesenchymal cells from the epithelial in the developmental program, and must have critical properties for tumors in cancer invasion and metastasis (Mani et al., 2008; Thiery, 2003). This process is associated by a switch mechanism in mesenchymal and epithelial markers one of which is E-cadherin. The upregulation of E-cadherin by miR-200s inhibited EMT in different cancers (Burk et al., 2008). Members of the miR-200 family had previously been investigated for their ability to inhibit EMT, a developmental process in which epithelial cells acquire the migratory, invasive, and apoptosis-resistant properties of mesenchymal cells (Radisky, 2011). We

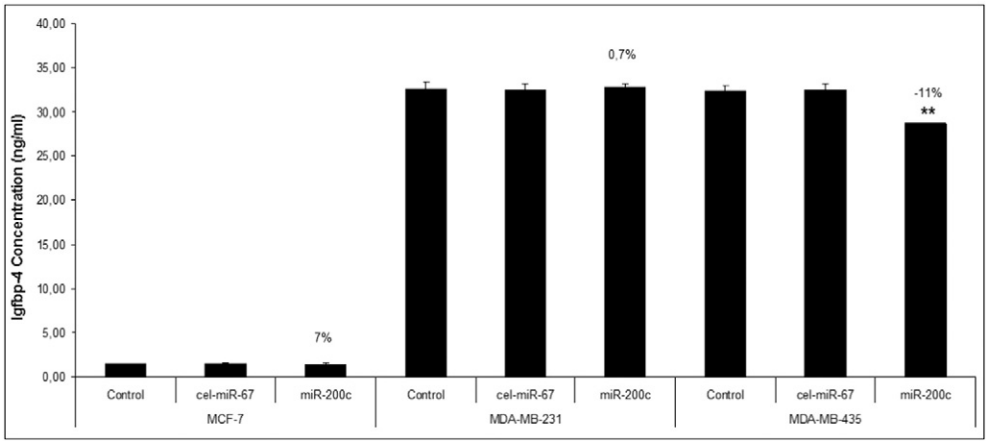

Fig. 6. Igfbp-4 levels of breast cancer cell lines after treatment with chitosan/miR-200c nanoplexes. **p < 0.01.

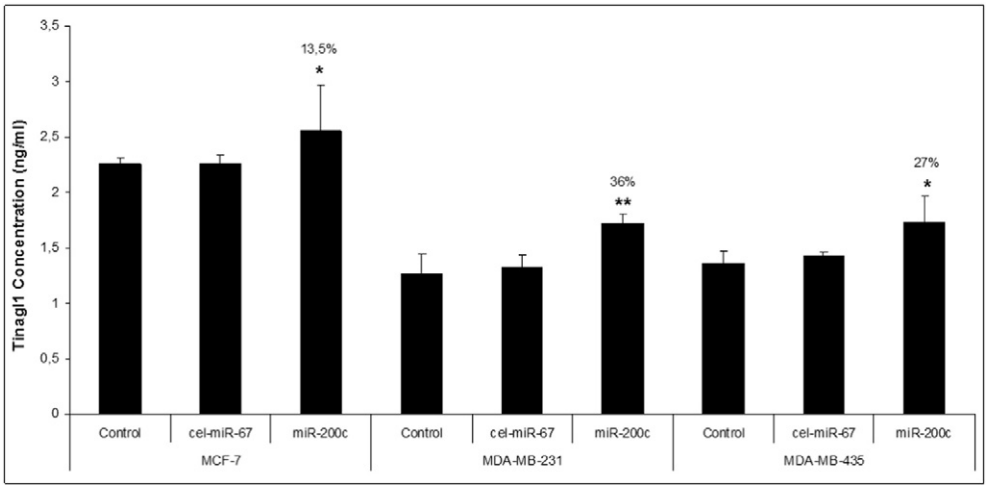

Fig. 7. Tinagl-1 levels of breast cancer cell lines after treatment with chitosan/miR-200c. *p < 0.05, **p < 0.01.

showed that level of E-cadherin, an important epithelial marker of EMT increased in the breast cancer cell lines except MDA-MB-435 cells after transfection of chitosan/miR-200c nanoplexes. Especially, E-cadherin level significantly increased in MDA-MB-231 cells (88.7%) (Fig. 5). Thus, the formation of stable cell-cell contacts increases and impairs migration (Jurmeister et al., 2012). Our data are consistent with that of Jurmeister et al. (2012). Jurmeister et al. (2012) reported that miR-200c could regulate the induction and maintenance of epithelial phenotype through downregulation of ZEB1 and ZEB2 and upregulation of E-cadherin. Humphries and Yang (2015) also noted the restoration of E-cadherin expression by miR-200c.

EMT is an important process in tumor invasion and metastasis. (D'Amato et al., 2013; Kalluri, 2009; Micalizzi et al., 2010). During the EMT process, the epithelial cells are subjected to EMT and lose cell-cell interactions, and other epithelial trails while migrating, and are the invasive phenotype. On the other hand, the first and most critical step in the metastasis is the invasion of the normal host tissues by the malignant cells. In order to test whether or not the administration of miR-200c-based nanoplexes results in the invasion of malignant cells to normal cells, invasion was studied. As seen in Fig. 5, the cell migration lowered by 40% in the MDA-MB-231 cell line after the transfection of the chitosan/miR-200c nanoplexes ($p < 0.01$). In the MDA-MB-435 cell line, the invasive cell number decreased to 15.2% in chitosan/miR-200c group accordingly to untreated tumor group ($p < 0.05$). After the re-upregulation of miR-200c by using chitosan nanoplexes, the breast cancer cell invasion diminished according to our findings, which was similar to earlier data (Pecot et al., 2013; Rhodes et al., 2015). Ulhmann et al. (2010) and Humphries and Yang (2015) reported the reducing effect of the miR-200c family on cancer cell intravasion.

Moreover, the miR-200s inhibit EMT and invasion, and are likely to suppress metastasis (Dykxhoorn et al., 2009; Gibbons et al., 2009). Korpal et al. (2011) showed that the functions of miR-200s related to the metastasis are regulated through not only ZEBs and E-cadherin, but also through the metastasis of suppressive proteins, including Igfbp4 and Tinagl1. Recently, it has been acknowledged that for most human cancers, not only gene mutations, but also nutrition and life style are important factors (Zielinska et al., 2015). Moreover, these later factors are the major determinant insulin-like growth factor bioactivity, and there is also relationship between EMT and Igfbp-4. The IGFBPs have the potential to either inhibit or enhance the IGF functions. According to our data, when compared to the untreated group, the Igfbp-4 levels of the cells did not change, or slightly changed dependent on the cell types (Fig. 6). The other protein related to metastasis is Tinagl1. The level of Tinagl1 increased in all the cells after being given chitosan/miR-200c nanoplexes (Fig. 7). The increase in the levels of two metastasis suppressor proteins, Igfbp-4 and Tinagl1, showed that chitosan/miR-200c nanoplexes have an effect on metastatic behavior of breast cancer cell lines. Gibbons et al. (2009) reported that tumor cell metastasis is regulated by miR-200s expression and miR-200s are likely to be important mediators of EMT and metastasis.

The miR-200s family has been shown as an important target for the promotion of invasiveness (Zhang et al., 2014). In our study, an inhibitor effect of chitosan:miRNA polyplexes has been observed in MCF-7, MDA-MB-231, and MDA-MB-435 cell lines. However, as seen in Fig. 8, this effect is not strong in MDA-MB-435 cell line.

The cancer cells that have undergone EMT gained resistance to the apoptosis. Schickel et al. (2010) reported the EMT regulator miR-200 regulates the apoptosis sensitivity of the cancer cells. Researchers have

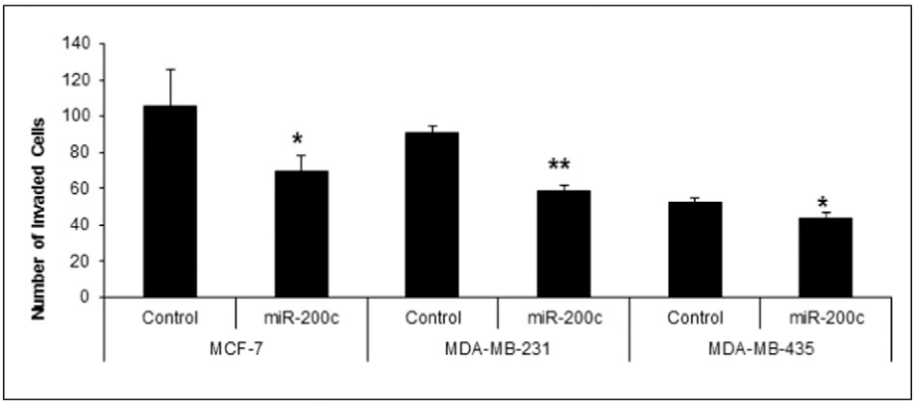

Fig. 8. The effect of chitosan:miR-200c nanoplexes on the invasiveness of breast cancer cells after 24 h (cell numbers/inserts). *p < 0.05, **p < 0.01.

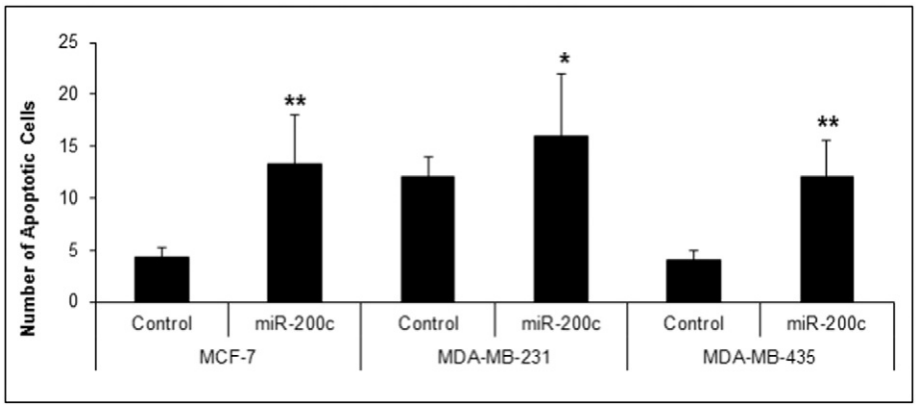

Fig. 9. The effect of chitosan/miR-200c complexes on apoptosis in breast cancer cell lines (cell number/well). $^{*}p < 0.05$, $^{**}p < 0.01$.

suggested that the reexpression of the miR-200c in different cancers is affected by decreasing the number of the death cells, EMT ability and initiates apoptotic cell death (Cochrane et al., 2009; Howe et al., 2011; Radisky, 2011; Schickel et al., 2010). To determine whether miR-200c is involved in the regulation of the apoptosis, we transfected chitosan/miR-200c nanoplexes to the breast cancer cells. In our study, as seen in Fig. 9, the enhanced expression of the miR-200c by the nanoplexes resulted in a marked increase in apoptosis in all the breast cancer cells compared with their negative control. Our data correlated with other apoptosis studies (Howe et al., 2011; Schickel et al., 2010).

5. Conclusion

In conclusion, chitosan is a suitable carrier for miR-200c, and formed stable nanoplexes with miR-200c. In this study, the role of chitosan/miR-200c nanoplexes in tumor development and angiogenesis progression was studied in three breast cancer cell lines. The effect of the chitosan/miRNA nanoplexes on the tumor angiogenesis, EMT, invasion, metastasis, and apoptosis, changed depending on the cell-types. Therefore, during treatment with chitosan based miR-200c nanoplexes in breast cancers, the type of tumor cells must be considered.

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