

***In vitro* gene silencing effect of chitosan/shRNA PDGF-D nanoparticles in breast cancer**

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ABSTRACT

Breast cancer is the most common cancer worldwide in women and it is highly malignant and fatal. PDGF-D plays role in regulation of many cellular processes such as angiogenesis. PDGF-D is overexpressed in many types of cancers and promote tumor growth and metastasis. Silencing of PDGF-D gene by using shRNA with an appropriate carrier system may decrease tumor growth and metastasis. In our study, we prepared chitosan nanoparticles loaded with five different shRNA plasmids targeting different exons of PDGF-D gene. Then, nanoparticles were characterized *in vitro* and transfection efficiency of these nanoparticles were investigated in breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-435). The effects of single

and multiple shRNA sequences, molecular weight of chitosan (150 kDa and 400 kDa) and the amount of shRNA (100 and 500 µg) on the characterization and transfection efficiencies of nanoparticles have been studied. Size of nanoparticles changed between 200-400 nm and approximately 95-100% encapsulation efficiency were obtained. Release of shRNA changed with the molecular weight of chitosan. It was obtained that formulation containing shRNA plasmid targeting PDGF-D exon 6 (NP1) has the highest silencing efficiency in MDA-MB-231 cell line. It was also evaluated that chitosan can be a suitable gene delivery system for shRNA targeting PDGF-D.

Key words: Breast cancer ; chitosan ; shRNA ; gene silencing ; RNAi.

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Submitted / Gönderilme: 05.05.2017 **Revised / Düzeltme:** -
Accepted / Kabul: 15.06.2017

How to cite this article : Ekentok C, Özbaş Turan S, Akbuğa J. *In vitro* gene silencing effect of chitosan/shRNA PDGF-D nanoparticles in breast cancer. Marmara Pharm J 2017; 21 (4): 793-803

1. Introduction

Breast cancer is one of the most common, highly malignant and fatal disease. Angiogenesis, the process of new blood vessel formation, plays a critical role in solid tumor formation and growth. Thus, angiogenesis is an important target for cancer drug development.

Development and metastasis of tumors in the breast are dependent on angiogenesis. Various molecules such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) play roles in regulation of angiogenesis. Among them, VEGF and FGF are the most studied molecules [1].

In recent years, there are publications related to the effect of PDGF/PDGFR on tumor angiogenesis [2]. PDGFs belong to growth factor family that consist of 5 disulfide-linked dimmers (PDGF-A to D). PDGF molecules are mitogen for most cells and their expression increase in some malignant situations [3]. Platelet-derived growth factor signaling pathway is well-defined and recent years it has been discussed

that PDGF signaling pathway is different in normal and cancer cells [4].

PDGF family consist of 4 different PDGF chains (-A, -B, -C, -D). PDGF-A and -B have been known since 1970, on the other hand PDGF-C has been found next years and finally PDGF-D has been found recently [5-7].

PDGFs regulate different cellular processes like cell proliferation, transformation, migration and viability during growth or pathogenesis [8, 9]. Studies show that PDGF signal has an effect on growth, invasion and metastasis of cancer cells [10]. Increase of PDGF expression is generally related to tumor growth, angiogenesis and metastasis in different types of cancer. Different studies reported that PDGF-D expression increases in prostate, lung, kidney, ovary, brain, pancreas and especially breast cancer [10, 11-14]. There are limited publications about PDGF-D and molecular mechanism of inducing tumor growth of PDGF-D is not clear [15]. It is known that PDGF-D affect cell invasion, angiogenesis and metastasis and also regulate VEGF signal in different cancer types. Another important effect of PDGF-D on tumor growth and metastasis is having role in epithelial-mesenchymal transmission (EMT) [16].

RNA interference (RNAi) is a sequence-specific, post-transcriptional gene regulation mechanism that is based on double-stranded RNA (shRNA, siRNA, miRNA) and respect to these properties it is a new potential therapeutic group [17, 18]. On the other hand, because of rapid degradation by nucleases, biological instability, inefficient cellular uptake and off-target effects, clinical use of RNAi molecules is limited and an appropriate delivery system is necessary [19-21]. Even viral vectors frequently are used as delivery system there are several disadvantages like immune reaction and carcinogenic effect and because of these problems, non-viral vectors are gaining popularity as delivery system. Cationic polymers are less toxic compared to liposomes [22]. Chitosan, in addition to its cationic characteristic, is a biodegradable, biocompatible, less immunogenic and safe biopolymer. In spite of viral vectors have high transduction efficiency, they have serious problems such as potential mutation, recombination and oncogenic effects and beside this lipid-based non-viral vectors have also serious problems like toxicity and stability [23, 24].

Chitosan, deacetylated form of chitin, is a natural polysaccharide and a proper delivery system [25-31]. Molecular weight and deacetylation degree of chitosan are important for shRNA delivery. In the previous study, our group investigated effect of VEGF targeted shRNA on breast

cancer [31]. Xu *et al.*, [32] researched tumor growth and lung metastasis in human kidney carcinoma cell line (SN12-C) by inhibition of PDGF-D with shRNA and observed that this can be a new therapy approach.

In summary, RNAi based therapeutics are promising for breast cancer therapy and it is known that expression of different growth factors increase in breast cancer metastasis. In this *in vitro* based study, shRNA targeting PDGF-D gene silencing, was encapsulated into chitosan nanoparticles and transfection efficiency of these nanoparticulate system was investigated in three different breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-435).

2. Results and Discussion

The purity, topological forms and accuracy of restriction map of isolated plasmid DNA was determined by agarose gel electrophoresis. As seen in Figure 1, shRNAs are in 95-100% supercoiled form. After *pst*I restriction enzyme application, shRNA forms are given in Figure 2. The purity was checked spectrophotometrically and 260/280 absorbance rate value was found 1.85.

As mentioned above 5 different shRNA plasmids targeting different exons of PDGF-D gene were used for nanoparticle preparation (NP1-5). Also, mix of them were used for comparison (NPK1-4). Single and multiple PDGF-D

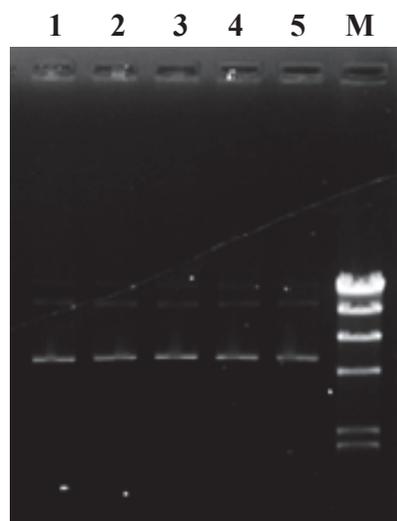


Figure 1. Agarose gel electrophoresis shRNA vectors [1- PDGF-D shRNA-1 (sh-1), 2- PDGF-D shRNA-2 (sh-2), 3- PDGF-D shRNA-3 (sh-3), 4- PDGF-D shRNA-4 (sh-4), 5- PDGF-D shRNA-5 (sh-5), M- Lambda DNA/Hind III marker]

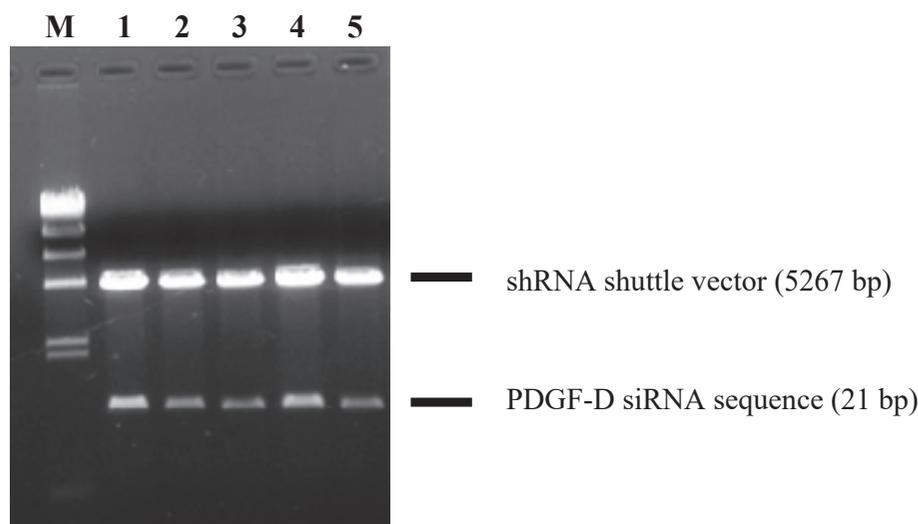


Figure 2. Agarose gel shRNA vectors restriction control [1- PDGF-D shRNA-1 (sh-1), 2- PDGF-D shRNA-2 (sh-2), 3- PDGF-D shRNA-3 (sh-3), 4- PDGF-D shRNA-4 (sh-4), 5- PDGF-D shRNA-5 (sh-5), M- Lambda DNA/Hind III marker]

targeted shRNA sequences were encapsulated in chitosan nanoparticles prepared with different molecular weight chitosan (NP1-5 and NPK1-2 with MMWC, NPK3-4 with LMWC). Two different plasmid amount were used in nanoparticle preparation (NP1-5 and NPK3 with 100 μ g DNA, NPK2 and NPK4 with 500 μ g DNA). Shape of nanoparticles and their surface morphology were shown in Figure 3. Nanoparticles have porous surface as seen in SEM photography.

The characteristics of shPDGF-D loaded CS NPs (encapsulation efficiency, size, zeta potential) were given in Table 1. Encapsulation efficiency of shPDGF-D containing chitosan nanoparticles were about 95.82 \pm 1.07-100%. The particle size of nanoparticles containing single sequence (NP1-NP5) changed between 203.5 \pm 1.5 nm and 406.1 \pm 15.9 nm and particle size of nanoparticles containing multiple sequences (NPK1-NPK4) was 256.1 \pm 0.9 nm- 400.4 \pm 10.4 nm

($p < 0.05$). The surface charge of all formulations was about neutral and zeta potential values were between 1.04 \pm 0.09 mV and 5.03 \pm 0.35 mV.

To determine whether the chitosan nanoparticles efficiently protected the shRNA from serum degradation, samples was applied agarose gel electrophoresis after serum application. It observed that chitosan nanoparticles protect shPDGF-D from serum during 96 hours (Figure 4).

Release of shRNA from nanoparticles prepared with different sequences was observed during 168 hours. Release profile of nanoparticles with different sequences did not change ($p > 0.5$) (Figure 5).

Release of shRNA from nanoparticles prepared with low molecular weight chitosan (NPK3, NPK4) was faster than nanoparticles prepared with medium molecular weight chitosan (NP1-5, NPK1, NPK2). shRNA release from

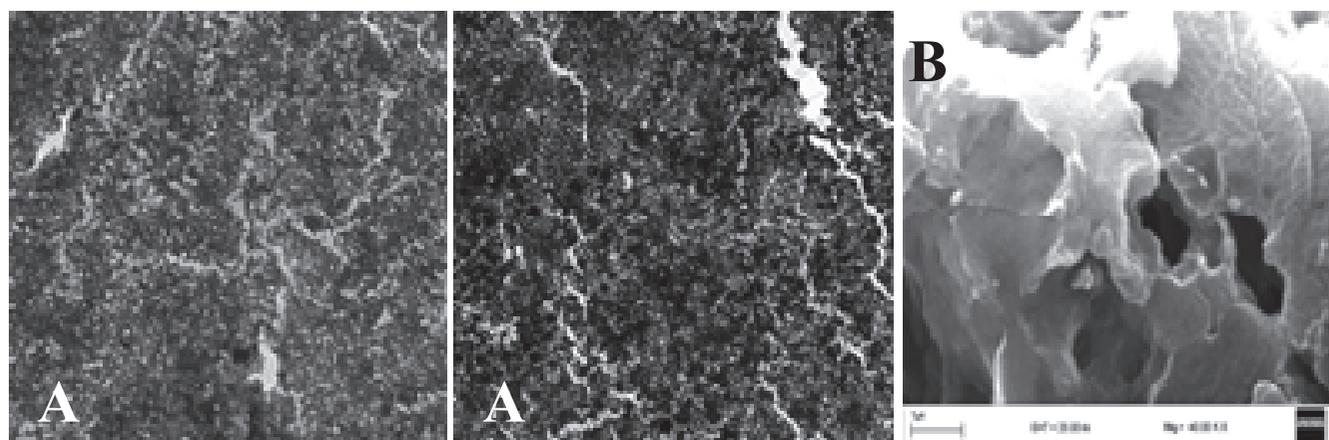
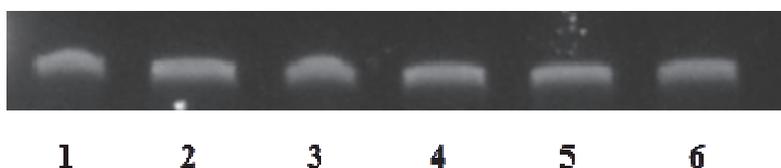
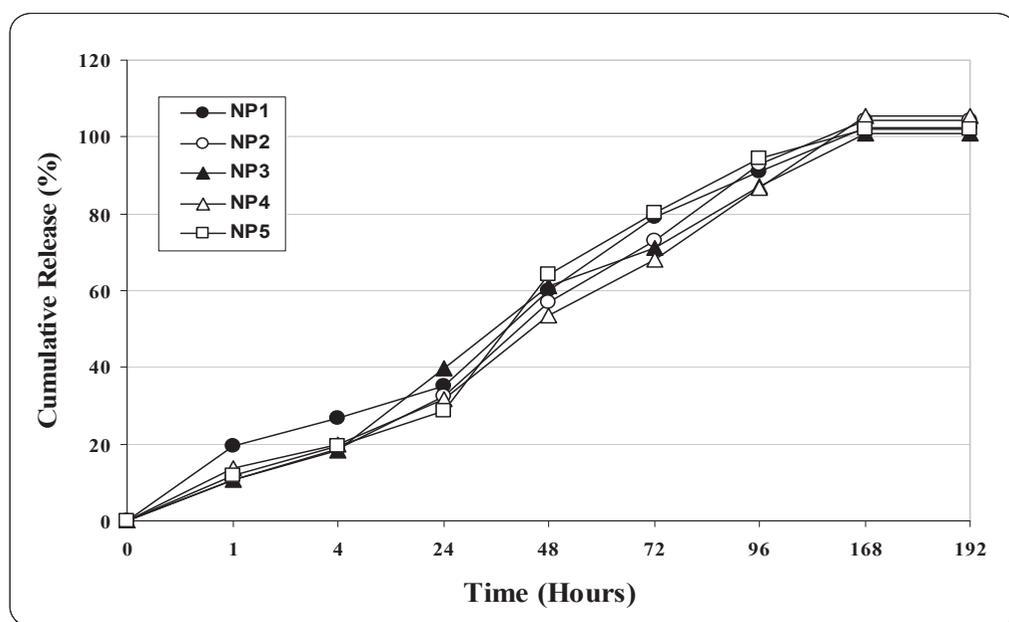


Figure 3. A; Transmission (TEM) and B; Scanning (SEM) electromicrographs of shRNA-CS NPs.

Table 1. Encapsulation efficiency, zeta potential and size values of shRNA loaded chitosan nanoparticles (shRNA-CS NPs).

Codes	Encapsulation efficiency (% \pm SD)	Zeta Potential (mV \pm SD)	Particle Size (nm \pm SD)
shRNA	-	-19.85 \pm 0.1	-
Chitosan	-	28.45 \pm 0.1	-
NP1	100.0 \pm 5.1	5.03 \pm 0.4	247.5 \pm 16.5
NP2	100.0 \pm 2.4	5.29 \pm 0.1	341.7 \pm 2.8
NP3	100.0 \pm 2.0	2.61 \pm 0.5	203.5 \pm 1.5
NP4	95.82 \pm 1.1	1.94 \pm 1.2	311.3 \pm 3.1
NP5	97.44 \pm 1.1	1.04 \pm 0.1	406.1 \pm 15.
NPK1	97.44 \pm 0.8	1.15 \pm 0.1	256.1 \pm 0.9
NPK2	99.20 \pm 4.0	1.87 \pm 0.1	295.6 \pm 1.2
NPK3	96.09 \pm 0.2	1.24 \pm 0.1	400.4 \pm 10.4
NPK4	98.57 \pm 3.6	1.27 \pm 0.1	308.2 \pm 4.9

**Figure 4.** Serum stability of shRNA-CS NPs (Lane 1; initial, lanes 2-6; after 4, 24, 48, 72 and 96 hours)**Figure 5.** Release profiles of PDGF-D shRNA from CS NPs.

nanoparticles prepared with low molecular weight chitosan completed in 96 hours and release from nanoparticles prepared with medium molecular weight chitosan was observed during 168 hours (Figure 6).

Release profiles of nanoparticles prepared with different amount of shRNA (100-500 µg) and single/multiple sequences did not change ($p > 0.05$) (Figure 7). By these results, while molecular weight of chitosan has an effect on release profile of nanoparticles, shRNA amount and different sequences have not.

To determine effect of encapsulation process and release studies on plasmid integrity, agarose gel electrophoresis was applied to the samples after release studies. As shown in Figure 8 shRNA remained stable during nanoparticle preparation procedure, centrifugation, lyophilisation and *in vitro* release studies. This result showed similarity with our earlier studies.

Cellular uptake of shPDGF-D/chitosan nanoparticles controlled with GFP marker gene which is encoded by shRNA plasmid vector. To determine cellular uptake and transfection efficiency, GFP expression was observed by fluorescence microscopy. As shown in Figures 9 and 10, cells were transfected successfully and 80-85% transfection efficiency was observed and GFP expression increased with time.

Three different breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-435) were used for *in vitro* transfection studies

and PDGF-D amount after transfection was measured in cell homogenate using ELISA. Endogen PDGF-D expression and gene silencing efficiency at 3 different cell lines were shown in Figure 11. As shown in Figure 11, PDGF-D expression reduced in each breast cancer cell line at 168 hours after transfection. PDGF-D silencing values changed between 69.17-82.20% in MCF-7, 47.5-92.14% in MDA-MB-231 and 10.44-88.44% in MDA-MB-435 after 168 hours. Either cell line type or nanoparticles with different sequences affect the knock down of PDGF-D gene level. Different silencing efficiency was also observed with nanoparticles containing single and multiple sequences of shRNA. In MCF-7, silencing effect of formulations prepared with single sequences (NP1-NP5) was between 69.17 and 80.87% and difference between NP4, NP5 and NP1, NP2, NP3 was significant particularly ($p < 0.05$) (Figure 11). In MDA-MB-231 cell line, a significant difference was found between NP1 and other formulations ($p < 0.05$) (Figure 11). However silencing difference between formulations containing single sequences (NP1-NP5) was not significant in MDA-MB-435 cell line ($p > 0.05$) (Figure 11).

When a comparison was made between formulations containing single and multiple sequences, a significant difference was observed between NPK1 and other formulations except NP4 and NP5 in MCF-7 cell line ($p < 0.05$) (Figure 11). Different gene silencing effect in MDA-MB-231 was highly significant ($p < 0.001$) and in MDA-MB-435 while difference between NP3, NP4 and NPK1 was highly

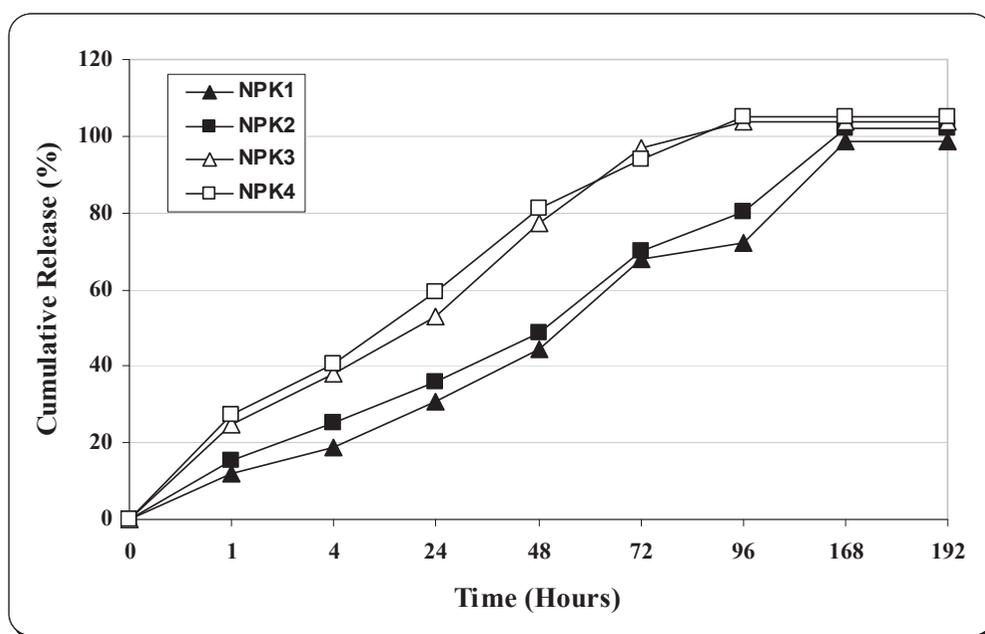


Figure 6. Effect of molecular weight of chitosan on release profiles of shRNA CS NPs.

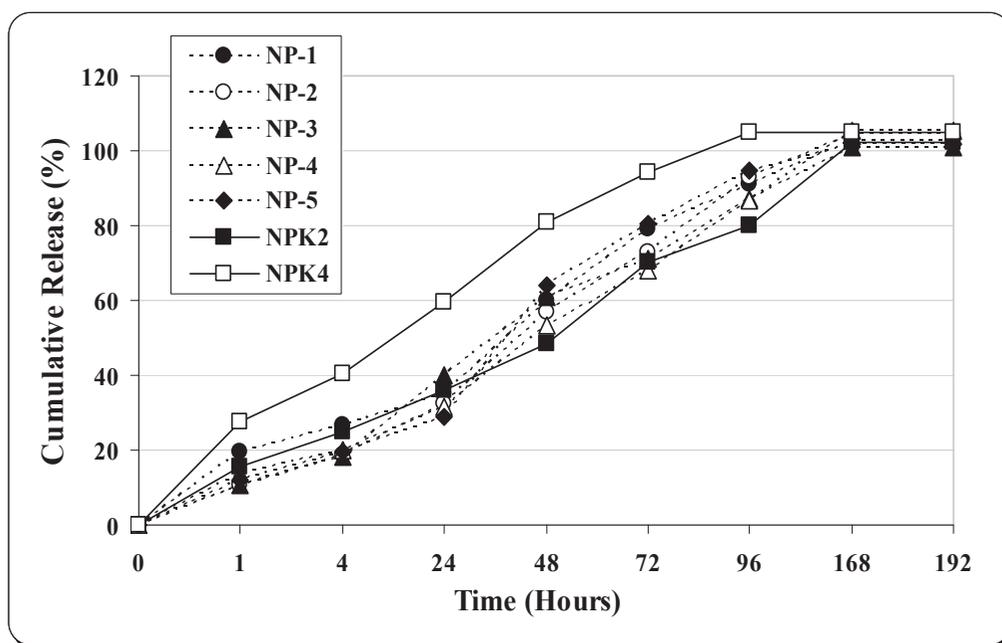


Figure 7. Effect of shRNA amount on release profiles of shRNA CS NPs.

significant ($p < 0.001$), difference between other formulations and NPK1 was significant ($p < 0.05$) (Figure 11).

When gene silencing effect of formulations containing different molecular weight chitosan (NPK1/NPK3 or NPK2/NPK4) compared, a highly significant difference was found at PDGF-D inhibition level in each 3 cell lines ($p < 0.001$) (Figure 11).

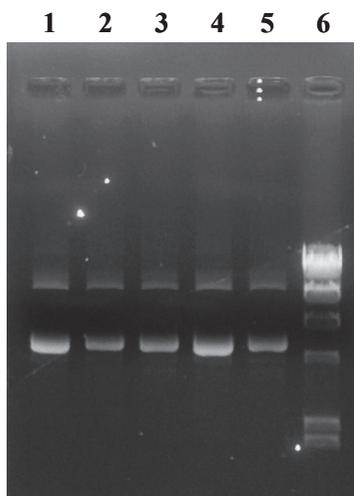


Figure 8. Agarose gel shRNA after release studies. (Lane 1, NP5; Lane 2, NP4; Lane 3, NP3; Lane 4, NP2; Lane 5, NP1; Lane 6, marker)

Effect of shRNA amount (NPK1-NPK4) on PDGF-D inhibition in MCF-7 cell line was not significant ($p > 0.05$) (Figure 11). In MDA-MB-231, shRNA amount affected PDGF-D silencing in nanoparticles only containing low molecular weight chitosan ($p < 0.05$) and in MDA-MB-435 cell line, shRNA amount affected silencing in formulations only containing medium chitosan ($p < 0.05$) (Figure 11).

MDA-MB-231 cell line has the highest endogen PDGF-D amount while MDA-MB-435 cell line has the lowest. When the PDGF-D inhibition effect of nanoparticles compared at different breast cancer cell lines it showed the following rank (MDA-MB-231 > MCF-7 > MDA-MB-435) (Figure 11). Based on this, MDA-MB-231 cell line used in gene silencing studies at different time intervals trying to find the time dependent effect of gene inhibition (Figure 12). As shown in Figure 12, shRNA-CS NPs showed PDGF-D gene silencing affect in a time-dependent manner. While silencing of single sequence containing formulations (NP1-NP5) was 53.65-75.11% at 72 hours, silencing effect of NP1-NP5 was between 92.14 and 84.68% at 168 hours ($p < 0.05$). NP1 had the highest silencing effect at 168 hours in MDA-MB-231 cell line and difference between NP1 and other formulations was statistically significant ($p < 0.05$) (Figure 12). Formulations prepared with different single sequences (NP1-NP5) had higher silencing effect than

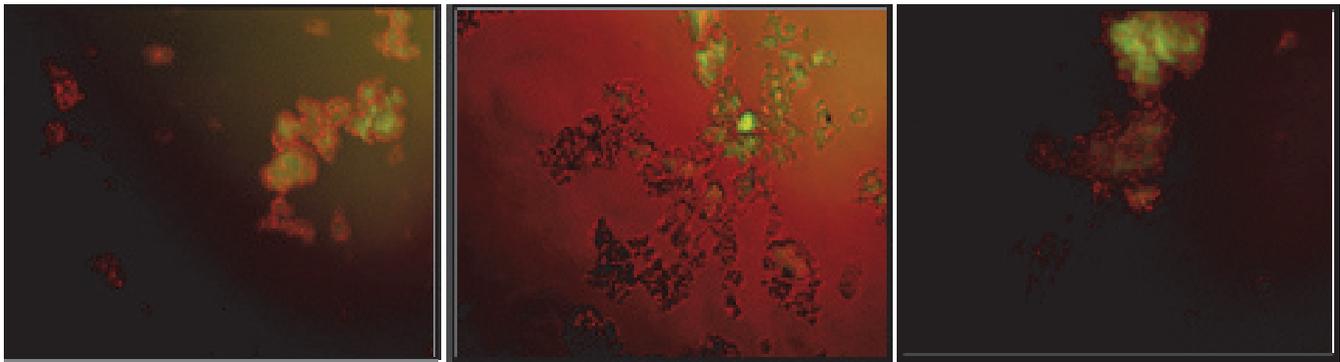


Figure 9. Cellular uptake of shRNA CS NPs 24 hours after transfection

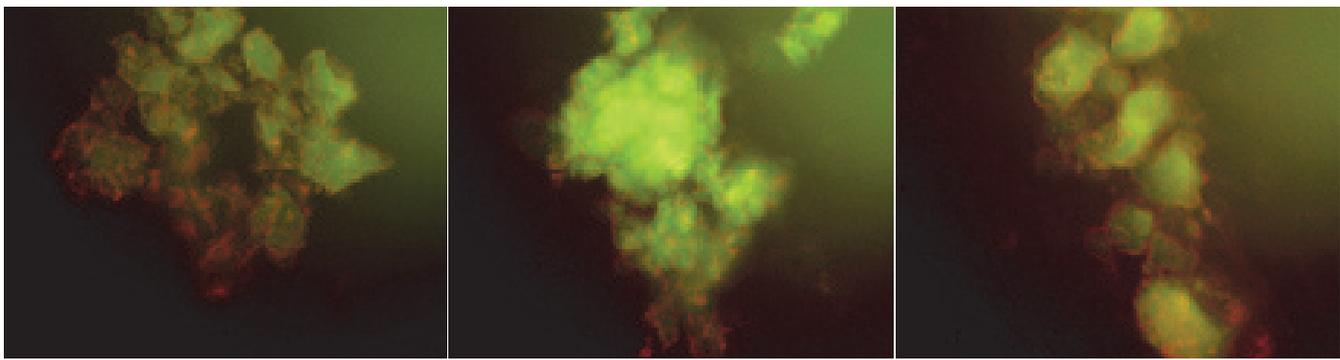


Figure 10. Cellular uptake of shRNA CS NPs 48 hours after transfection

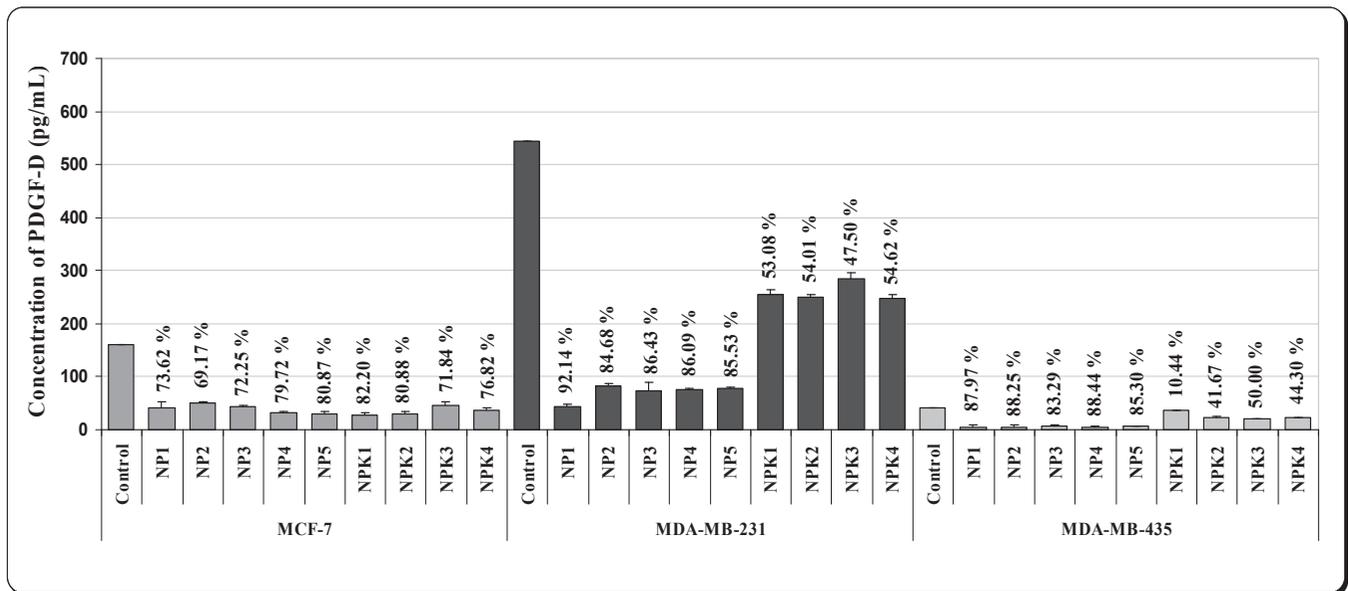


Figure 11. PDGF-D silencing effect of shRNA-CS NPs 168 hours after transfection at different breast cancer cell lines.

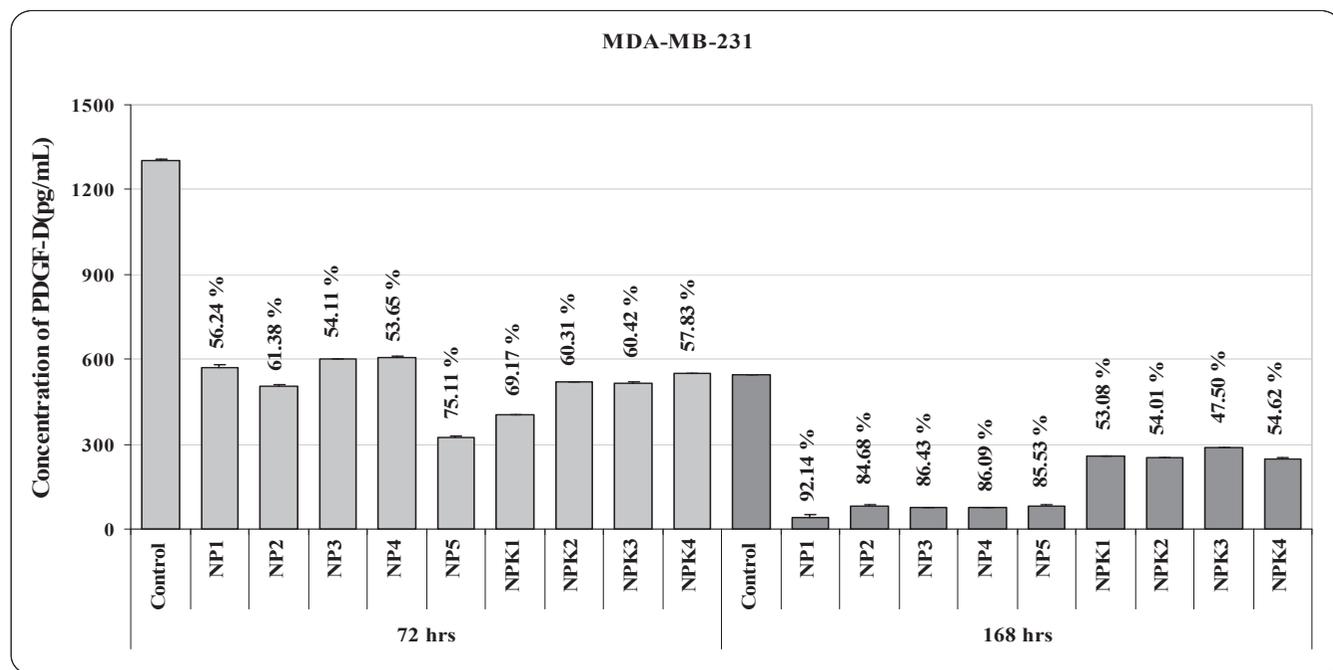


Figure 12. Time-dependent PDGF-D inhibition effect of shRNA-CS NPs in MDA-MB-231 cell line.

formulations prepared with multiple sequences (NPK1-NPK4) and difference between formulations prepared with different single sequences was significant ($p < 0.05$) (Figure 12). When effect of shRNA amount in silencing efficiency investigated, difference between formulations prepared with medium molecular weight chitosan was not significant ($p > 0.05$) while difference between formulations prepared with low molecular weight chitosan was significant ($p < 0.05$).

We concluded that difference between silencing efficiency of formulation related to plasmids that they contain targeting different exons of PDGF-D gene and formulation containing plasmid targeting exon 6 of PDGF-D gene (NP1) has the highest silencing efficiency. These results have similarity with study carried out with siRNA VEGF targeting different isoforms of VEGF [36].

3. Conclusions

In this study, 5 plasmids targeting different exon regions of PDGF-D gene were used and it was obtained that formulation containing shRNA plasmid targeting PDGF-D exon 6 (NP1) has the highest silencing efficiency. It was also evaluated that chitosan can be a suitable gene delivery system for shRNA targeting PDGF-D.

4. Materials and Methods

4.1. Cell lines and reagents

Three different breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-435 were obtained from American Type Culture Collection (ATCC). Cell lines were cultured in Dulbecco's minimum essential medium (DMEM) (Biological Industries, Israel) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel), 100 mM glutamin and 100 mM antibiotics solution. Two types of chitosan with different molecular weight (low molecular weight, 150 kDa, DD 93; medium molecular weight, 400 kDa, DD 87) were purchased from Fluka, Germany. Human PDGF-D ELISA kit (USCN Life Science, USA) was used in determination of PDGF-D level. All other chemicals and reagents were of molecular grade and purchased from Sigma-Aldrich.

4.2. PDGF-D shRNA plasmid

pGeneClip hMGFP expression vector, encodes human PDGF-D, contains CMV and humanU1 promoter and GFP and ampicillin selection marker genes (5.2 kb) (SureSilencing KH00048G, Qiagen, USA). Studies were carried out with five different shRNA plasmids targeting different exons of PDGF-D gene. The plasmids were transformed into *Escherichia coli* DH5 α bacterial strain by using classical CaCl₂ method and isolated by the alkaline-lysis technique [33].

4.3. Spectrophotometric and electrophoretic controls of shRNA

The quantity and quality of isolated plasmid DNA were determined by spectrophotometrically at 260 and 280 nm (Shimadzu UV-Biospec 1601, Japan). The purity and forms of plasmid DNA was analyzed by electrophoresis in agarose gel. For this purpose, plasmid DNA was applied into 0.8% agarose gel containing tris-boric acid-EDTA (TBE, pH 8.0) and ethidium bromide at constant voltage (80mA, 200 V) during 1 hour. Conformation of DNA was checked using DC290 Camera and 1D Image Analysis Software system (Kodak, USA) under UV light. The percentage of supercoiled DNA was determined by using following equation [34]:

$$SC(\%) = \frac{V_{sc} - V_0}{[V_{sc} + V_L + V_{oc}] - (3 \times V_0)}$$

(V_{sc} , volume of supercoiled DNA; V_L , volume of linear DNA; V_{oc} , volume of open

Circular DNA; V_0 , volume of background)

4.4. Preparation of shRNA loaded nanoparticles

shRNA loaded chitosan nanoparticles were prepared by ionic gelation method as previously described [35]. Briefly, tripolyphosphate solution (0.25 % w/v) containing plasmid dropped into chitosan solution (0.25 % w/v) under constant stirring. After one hour stirring, nanoparticles were separated by centrifugation at 12.000xg for 15 minutes. The supernatant was discarded and particles were washed with bidistilled water three times. Nanoparticles were stored at +4° C after freeze-drying. In order to study effect of formulation variables [molecular weight of chitosan (150 and 400 kDa), plasmid type (5 different plasmid and mix of these) and plasmid concentration (100 and 500 µg)] different nanoparticle formulations were prepared (Table 2).

4.5. Characterization of nanoparticles

The morphological examination and size control of nanoparticles were performed by transmission electron microscopy (TEM) (JEM 1200 EXII, Jeol, Japan) and scanning electron microscopy (SEM) (JSM-5910 LV, Jeol, Japan).

Encapsulation efficiency, the amount of DNA encapsulated in nanoparticles, was calculated by measuring the difference between the total DNA amount added in nanoparticle solution and the amount of not-entrapped DNA remaining in the supernatant after centrifugation. For this purpose, the supernatant after centrifugation was measured spectrophotometrically at 260 and 280 nm.

Measurements of size and zeta potential of nanoparticles were performed by Zetasizer (NanoZS ZEN3500, Malvern Instrumentals Ltd., UK). The samples were measured in phosphate saline buffer (PBS pH 7.4) and each measurement was done in triplicate.

4.6. Serum stability of nanoparticles

Stability of shRNA-chitosan nanoparticles against serum was studied by incubating samples in PBS containing 10% inactivated fetal bovine serum at 37° C. Samples were taken at different time intervals. The integrity of shRNA was examined by using agarose gel electrophoresis.

4.7. In vitro release studies

The release of shRNA from nanoparticles was determined in shaking water bath at 37° C. For *in vitro* release studies, nanoparticles containing 7 µg shRNA were suspended in 2 ml PBS (pH 7.4) and incubated at 37° C. Samples were removed at different time intervals and centrifuged for 15 minutes at 14.000 g and the release medium was replaced with fresh

Table 2. Composition of shRNA loaded chitosan nanoparticles (shRNA-CS NPs).

Codes	PDGF-D shRNA*	Chitosan Mol wt / Conc. (%)	Amount of shRNA (µg)
NP1	1	400 / 0.25	100
NP2	2		
NP3	3		
NP4	4		
NP5	5		
NPK1	1-5	150 / 0.25	500
NPK2			100
NPK3			500
NPK4			500

buffer. The amount of shRNA released in the supernatant was measured spectrophotometrically at 260 nm. Additionally, the structural integrity of released shRNA from nanoparticles was controlled with agarose gel electrophoresis.

4.8. *In vitro* transfection studies

In vitro transfection studies were performed in MCF-7, MDA-MB-231 and MDA-MB-435 cell lines. The cells were cultured in DMEM containing 10% FBS, 100 mM L-glutamine and 100 mM antibiotic solutions and maintained at 37°C in a humidified 5 % CO₂ incubator. For transfection studies, the cells were seeded in 24-well plate at a density 2 x 10⁴ cells/well and incubated overnight. Transfection was performed on cells that were approximately 70% confluent. shRNA loaded nanoparticles were suspended in the serum-free medium and added to the cells (5µg shRNA/well). The cells were incubated for 4 hours at incubator and then serum-containing fresh medium was added to the wells. Cells were incubated at incubator for 7 days.

4.9. Cellular uptake of nanoparticles

In order to investigate the cellular uptake of PDGF-D targeting shRNA loaded nanoparticles, cells were observed with GFP marker gene encoded by shRNA plasmid by using fluorescence microscopy at different time intervals.

4.10. Determination of PDGF-D levels in cells by ELISA

Three and seven days after transfection the medium was discarded and the cells were washed with PBS. Then the cells were lysed in 200 µl Ripa Buffer by 3 freezing and thawing cycles. The expression of PDGF-D was determined in cell lysate by ELISA kit according to manufacturer's instructions and read spectrophotometrically at 450 nm.

4.11. Statistical analysis

All results related to *in vitro* studies were evaluated and differences were compared using SPSS-12. Statistical significance was determined when p<0.05.

Acknowledgement

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK), grant no:113S003 and this study was also presented as a poster in ESGCT

Congress in 23-26 October 2014, The Hague, Holland and the poster abstracts were published in Human Gene Therapy.

Authorship statement

Author contributions: Concept – J.A., S.Ö.T.; Design – J.A., S.Ö.T., C.E.; Supervision – J.A.; Resource – J.A., S.Ö.T.; Materials – J.A., S.Ö.T.; Data Collection and/or Processing – S.Ö.T, C.E.; Analysis and/or Interpretation – J.A., S.Ö.T., C.E.; Literature Search – J.A., S.Ö.T., C.E.; Writing – C.E., J.A.; Critical Reviews – J.A., S.Ö.T., C.E.

Conflict of interest statement

The authors declared no conflict of interest.

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