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siRNA Mediated Gene Silencing in the Pancreatic Cancer Capan-1 Cell Line

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Gene therapy, c-Myc, siRNA, Capan-1, Real-Time PCR, Pancreatic cancer

Abstract: İngilizce Today, cancer is the second leading among the causes of death. Especially pancreatic cancer which is an aggressive type progressing without giving too many symptoms. In addition, non-response to traditional treatments cause a low survival rate in this type of cancer. Therefore, many target-oriented studies are carried out to develop alternative diagnosis and treatment methods such as gene therapy method. Small non-coding RNA (siRNA) are used in studies as a therapeutic agent by its delivery to the target gene by various mechanisms.
This study aimed to silence the c-Myc gene using gold nanoparticle (AuNP)-siRNA in the Capan-1 cell line. We performed Real-Time PCR analysis, WST-1 *assay* in the *cytotoxicity* analysis, Double Staining method and Agarose gel electrophoresis using gold nanoparticles (2X, 4X, 8X) and siRNA (25 nM) at concentrations determined in the Capan-1 cell line. The data obtained by Real Time PCR were evaluated as relative (Efficiency 2). $2^{\Delta Ct}$ values were calculated. The $2^{\Delta Ct}$ value was 0.352 for the control, and these values were found to be between 0.210-0.027 in AuNP / siRNA applications. Considering these values, it was observed that the gene expression decreased the most for 8XAuNP/siRNA.
The averages of the OD values (438 nm) read in the WST-1 analysis were calculated. These values were calculated as 0.180 for the control group, 0.194 for 2XAuNP/siRNA, 0.293 for 4XAuNP/siRNA, and 0.277 for 8XAuNP/siRNA.
The data obtained from the analysis suggested that the nanoparticle siRNA complex could be used effectively in silencing the target gene. Nevertheless, more knowledge is needed on this subject.

Pankreas Kanseri Capan-1 Hücre Hattında siRNA Aracılı Gen Susturulması

Anahtar Kelimeler
Gen terapi, c-Myc, siRNA, Capan-1, Real-Time PCR, Pancreas kanseri

Öz: Günümüzde kanser ölüm nedenleri arasında ikinci sırada yer almaktadır. Özellikle agresif bir tip olan Pankreas kanseri çok fazla belirti vermeden ilerlemektedir. Ayrıca geleneksel tedavilere yanıt alınamaması bu kanser türünde düşük sağkalım oranına neden olmaktadır. Bu nedenle gen tedavisi gibi hedef odaklı alternatif tanı ve tedavi yöntemleri geliştirmeye yönelik çalışmalar yapılmaktadır. Küçük kodlayıcı olmayan RNA (siRNA), çeşitli mekanizmalarla hedef gene ulaştırılmasıyla çalışmalarda terapötik ajan olarak kullanılmaktadır.
Küçük kodlayıcı olmayan RNA (siRNA), çeşitli mekanizmalarla hedef gene ulaştırılmasıyla çalışmalarda terapötik ajan olarak kullanılmaktadır.
Bu çalışmada; Capan-1 hücre hattında, altın nanopartikül (AuNP)- siRNA kullanılarak c-Myc geninin susturulması amaçlandı. Capan-1 hücre hattında belirlenen konsantrasyonlarda altın nanopartiküller (2X, 4X, 8X) ve siRNA (25 nM) kullanılarak Real-Time PCR analizi, sitotoksisite analizinde WST-1 testi, Double Staining yöntemi ve Agaroz jel elektroforezi gerçekleştirdik. Real-Time PCR ile elde edilen veriler göreceli olarak değerlendirildi (Verimlilik 2). 2ΔCt değerleri hesaplandı. Kontrol için 2ΔCt değeri 0,352 olup, AuNP/siRNA uygulamalarında bu değerler 0,210-0,027 arasında bulunmuştur. Bu değerlere bakıldığında gen ekspresyonunun en çok 8XAuNP/siRNA için azaldığı gözlendi. WST-1 analizinde OD değerlerinin (438 nm) ortalamaları

hesaplandı. Bu değerler kontrol grubu için 0,192, 2XAuNP/siRNA için 0,194, 4XAuNP/siRNA için 0,293 ve 8XAuNP/siRNA için 0,277 olarak hesaplandı.
Analizlerden elde edilen verilere göre; Nanopartikül siRNA kompleksinin hedef genin susturulmasında etkili bir şekilde kullanılabileceği düşünülmektedir. Ancak bu konuda daha fazla bilgi birikimine ihtiyaç vardır.

1. INTRODUCTION

Ana başlıklar büyük harf ile yazılmalıdır. Bu alandaki Cancer is among the most important causes of morbidity and mortality worldwide. According to GLOBOCAN, a subsidiary of the World Health Organization (WHO), 19.3 million new cases and 10 million deaths were detected in 2020. 233 thousand new cancer cases and 126 thousand cancer-related deaths were reported in 2020 in Turkey. The number of cancer-related deaths worldwide in 2020 reported that pancreatic cancer ranked seventh in the overall rating of the highest percentile. [1] Furthermore, this type of cancer is among the most aggressive. The five-year survival rate is less than 5%, which is due to its progress without symptoms, difficulty in early diagnosis, and traditional treatment methods being not as effective as desired. [2, 3]

Primarily the mutations that inhibit DNA repair mechanisms, mutations in tumor suppressor genes, disorders in RNA function and faults in the control mechanism of the cell cycle are effective in cancer formation. [4-6]

The MYC proto-oncogene is involved in the expression of genes normally involved in cell proliferation. In many types of cancer, it turns into an oncogene with the formation of multiple copies of the MYC gene due to faults in DNA replication. Since the increase in cell proliferation by transcriptional activation of MYC oncogene increases the malignant potential of tumor cells, it is considered to be effective in ovarian, breast, colon, pancreatic, stomach and uterine cancers. Buchholz et al. [7] reported that ectopic activation of NFATc1 and Ca2+/calcineurin signaling pathway is an important mechanism of oncogenic c-Myc activation in pancreatic cancer. [8, 9]

Apoptosis and necrosis mechanisms are vital in cancer. It is a common knowledge that cancer develops due to an increase in cell proliferation and a decrease in apoptosis. [10] Apoptosis is programmed cell death where the nucleus is affected, and changes begin at this stage; the nucleus shrinks, and the chromatin condenses. Necrosis, on the other hand, is characterized by disruption of cell integrity and leakage of its contents. [11, 12] One of the methods used to detect apoptotic and necrotic cells is the HO/PI staining method. It is a method that allows the observation and counting of viable, apoptotic and necrotic cells in a single preparation by staining cells with two fluorescent dyes (Hoechst 33258 and propidium iodide). While Hoechst 33258 stains the nuclei of all cells, propidium iodide cannot pass through the membrane and stains late apoptotic and necrotic cells with impaired membrane integrity. [13, 14]

Today, studies on new therapeutic methods have increased, especially due to the inefficacy of traditional ones. The most prominent among these new methods is the gene therapy method. Gene therapy can be performed by increasing or inhibiting the expression of defective genes responsible for the formation and development of the disease. [15] It has two types: germline and somatic gene therapies. The most crucial factor in gene therapy depends on the effective delivery and expression of the nucleic acid-based molecule to the target cell. However, when these molecules are administered, they attach to many biological barriers in the bloodstream. [16] In gene therapy, gene transfer systems have been developed for nucleic acids to cross barriers and modulate their expression in the target tissue. These systems, called gene transfer vectors, are recognized by the specific receptors of the cells in the target tissue and pass through the cell membrane and reach the nucleus and provide a therapeutic effect. [17, 18]

The gene silencing approach, which occurs at the transcription or translation stage, includes Antisense, RNA interference (RNAi), and aptamer and ribozyme technologies. RNA interference (RNAi) pathway is the pathway where RNA inhibition is achieved by microRNA (microRNA; miRNA) and small interfering RNA (small interfering RNA; siRNA). siRNA contains 20-24 nt long sequence. In the siRNA-mediated pathway, the double-stranded RNA molecule (dsRNA) released into the cytoplasm is cut by the Dicer enzyme, and double-stranded siRNA is formed. The guide sequence, which directs the RNAi mechanism with the base pairing of siRNA, is included in the structure of the RNA-induced silencing complex (RISC). The RISC complex binds to the target mRNA, causing it to be destroyed, resulting in the reduction of proteins and specific mRNA. Although RNA interference is a natural mechanism, the expression of endogenous genes can be suppressed by using siRNAs synthesized in vitro. Therefore, siRNA has a wide application in gene therapy. [19-22]

In gene therapy, carrier molecules called vectors are used to transport the gene to the targeted cell. These are classified into two categories as biological and physical/chemical vectors. The biological vectors are plasmid or viral ones. Physical/chemical vectors, which are non-viral, are transferred to the cell by physical or chemical means. Molecules such as lipid-DNA complex or nanoparticles can also be used. Of these, nanoparticles are colloidal particles ranging from 10-100 nm, which release the absorbed active substance in a controlled manner. These particles are advantageous as they are targeted by ligands, encapsulated and stored in a lyophilized form. [23, 24] Nanoparticles are prevalently preferred in the release of targeted drugs in medicine and biotechnology, such as biosensors, and in gene therapy,

especially in the diagnosis and treatment of cancer.[25-27]

Gold nanoparticles (AuNP) are effective inorganic structures for gene therapy and drug delivery applications. Their biocompatibility, stability, physical size and low cytotoxicity are their most significant advantages, but their low DNA binding capacity reduces transfection efficiency. Gold nanoparticles can be visualized by techniques such as dark field microscopy and transmission electron microscopy, and their uptake into the cell can be observed. [28]

In this study, it was aimed to silence the c-Myc gene using gold nanoparticle (AuNP)-siRNA. Using gold nanoparticles-siRNA complex in the Capan-1 cell line. Real-Time PCR, Dual Staining and gel electrophoresis were performed for this aim. According to the data obtained from the analysis; it is thought that the nanoparticle siRNA complex can be used effectively in silencing the target gene. However, it is thought that effective results can be obtained with more knowledge on this subject.

2. MATERIAL AND METHOD

2.1. Cell Line, Chemicals and Devices Used

Human pancreatic cancer cell line Capan-1, RPMI-1640 medium (Roswell Park Memorial Institute-1640), Fetal Bovine Serum (FBS; Biological Industries), ethanol, PBS (Biological Industries) with Trypsin-EDTA Solution C) and trypan blue dye was used for analysis.

The siRNA (c-Myc) Human siRNA Oligo Duplex (Locus ID 4609) used for the target c-Myc gene was obtained from ABM. Polyethylene imine-coated gold nanoparticle, a polymeric nanoparticle, to provide controlled delivery of silencing siRNA to the target gene.

WST-1 analysis kit (Biovision), which is applied as a standard for cytotoxicity tests, Ribonuclease A (Serva), Hoechst 33342 (Serva), Propidium Iodide (Serva) in the preparation of the dual dye solution.

High Pure RNA Isolation Kit (Roche) for RNA isolation; Transcriptor High Fidelity cDNA Synthesis Kit (Roche) for cDNA synthesis from the obtained RNAs and LightCycler® 480 system (Roche) to investigate the expression of the c-Myc gene. Housekeeping GADPH and c-MYC R5'-CAACATCGATTTCTTCCTCATCTT-3', c-MYC F5'-TGAGGAGACACCGCCCAC-3' primers (Sybrgreen, Roche) in RT-PCR analysis; High Pure RNA Isolation Kit (Roche) for RNA isolation; Transcriptor High Fidelity cDNA Synthesis Kit (Roche) for cDNA synthesis from the obtained RNAs; LightCycler® 480 system (Roche) to investigate the expression of the c-Myc gene.

Cell culture study was performed in culture dishes and multi-well plates (Corning, USA) using 6-well plate, 48-well plate, 25 cm² cell culture flask, cryo tube 1 ml (BD), 15 ml centrifuge tube (Nunc), 0.2 µm filter (Sartorius), 96 e-plate (RTCA Resistor) plate 96, (Roche) disposable pipettes (2ml, 5ml, 10ml), micropipette (20µm-100µm-1000µm; Scaltec) and various glassware.

Laminar Flow Cabinet (ESCO class ll BSC Laminar Flow Cabinet, Labor Ildam, Turkey), refrigerated centrifuge (ROTINA 380R Hettich), inverted microscope (Leica DM6000B), vortex, Elisa plate reader (BIOTEK GEN5 Elisa Reader PowerWave XS2), carbon dioxide oven (Binder CB150) and LightCycler® 480 system (Roche, Germany) device used were.

2.2. Real Time PCR Assay and Cytotoxicity Analysis

Cell culture by culturing CAPAN-1 pancreatic cancer cells frozen in liquidnitrogen was prepared by culturing. c-Myc-siRNA at 25 nM in 2X, 4X and 8X concentrations from 50% polyethylene coated gold nanoparticle (AU-PEI) solution provided for use in the studies. The resulting solutions were sterilized by keeping them under UV light for 15 minutes. siRNA-Au nanoparticles were incubated at room temperature for 30 minutes for Capan-1 cell interaction; then performed WST-1 test for cytotoxicity analysis of the specified doses; and examined the effect of combined applications on cells.

In order to examine whether siRNA increases or maintains its single and double effects on cells by double staining an inverted microscope (Leica DM6000, Sweden) was used to morphologically determine which death pathway the cells chose; and evaluated the data obtained.

In the last part, RT-PCR was applied to demonstrate the expression level of the c-Myc gene suppressed by siRNA and performed mRNA isolation for different Au nanoparticle and siRNA applications. Then, we conducted cDNA synthesis from the isolated RNA. After applying different concentrations prepared with Au nanoparticle and siRNA to the cells, the expression levels of the c-Myc gene was analyzed with the LightCycler 480® instrument. In addition, PCR products were run on 1.7% agarose gel electrophoresis and determined band profiles by Gel Imaging.

3. RESULTS

3.1. Necrotic Index Results Obtained by Double Staining

We specified the irradiance obtained with the PI fluorescent dye used in the double staining method under fluorescent light (with FITC filter) at a 480-520 nm wavelength in Capan-1 necrotic cells of siRNA-nanoparticle applications. It was observed that the applied nanoparticle siRNA complex increased cell death in a necrotic pathway. Some of the obtained necrosis microscope images are given in Figure 1.

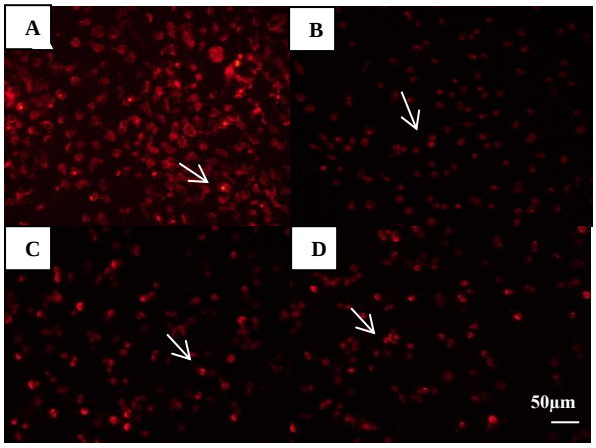

Figure 1. Photographs of necrotic cells using PI fluorescent dye (Leica DM6000, Sweden; 200X magnification) **A:** Control group **B:** Capan-1 cells interacting with 2X PEI-coated AuNP-siRNA **C:** Capan-1 cells interacting with 4X PEI-coated AuNP-siRNA **D:** Capan-1 cells interacting with 8X PEI coated AuNP-siRNA. (BAR= 50µm)

3.2. Real Time PCR (RT-PCR) Results and Agarose Gel Electrophoresis Evaluation

First of all, we performed mRNA isolation from Capan-1 cells, then cDNA synthesis from the isolated RNA samples. We oxidized these cDNA samples with the synthetic primer sequences synthesized for the c-Myc gene with the LightCycler480® analyzer. The data obtained by Real Time PCR were evaluated as relative. Furthermore, we ran PCR products in agarose gel electrophoresis (1.7%) and assessed band profiles. Figure 2 shows the obtained band profile.

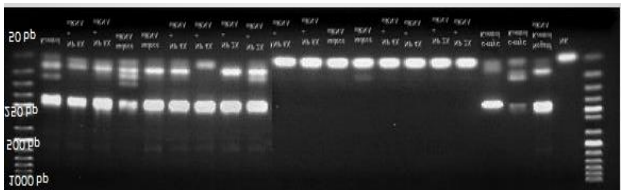

Figure 2. Agarose gel image of the products obtained in RT-PCR analysis (1.7%) (1 and 23 lined 50 bp DNA ladder)

In addition, in WST-1 analysis, it was observed that the viability values in % were higher than the control group. The data of OD values (438 nm) are given Table 3.1. The averages of the OD values (438 nm) read in the WST-1 analysis were calculated. These values were calculated as 0.192 for the control group, 0.194 for 2XAuNP/siRNA, 0.293 for 4XAuNP/siRNA, and 0.277 for 8XAuNP/siRNA. WST-1 analysis results are shown in Figure 3.

Table 1. OD values (438 nm) for WST-1 analysis

Groups/ OD values	1	2	3	Average
Control	0,24	0,162	0,175	0,192
siRNA	0,193	0,136	0,113	0,147
Np 2X	0,29	0,266	0,211	0,256
Np 4X	0,324	0,296	0,198	0,272
Np 8X	0,336	0,357	0,357	0,350
NP2X+siRNA	0,212	0,184	0,186	0,194
NP4X+siRNA	0,366	0,265	0,249	0,293
Np8X+siRNA	0,273	0,261	0,297	0,277

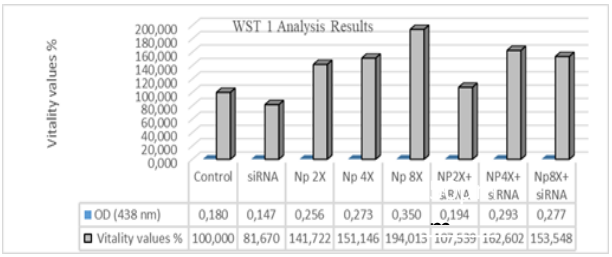

Figure 3. The results of WST 1 analysis

4. DISCUSSION AND CONCLUSION

Today, the importance of new therapeutic methods such as gene therapy is increasing. Gene therapy enables a targeted approach to the disease process. It is a mechanism frequently used in RNAi gene silencing studies. In such studies, it is important to effectively transport the gene to the target cell. Among the vectors used for this, gold nanoparticles have been demonstrated to be effective. According to the study, it is thought that the AuNp-siRNA complex is effective in silencing the target gene. Dykman et al. [29] reported that AuNPs enabled the efficient transport of siRNA, at organismal and cellular levels, and this facilitated the clinical application of siRNA treatments for genetic diseases. In another study, Daniels et al. [30] demonstrated that PEG-AuNP was effective in siRNA-mediated gene silencing in the MCF-7 cell line. Ku et al. [31] suggested that nanoparticles affected the transport of siRNA to the target tissue in cancer therapy, while we observed in this study that AuNPs did the same. Shaat et al. [32] used gold AuNPs (siRNA/bPEI/AuNP) modified with cationic polyethyleneimine (bPEI) to ensure efficient and reliable intracellular delivery of siRNA targeting the c-Myc gene in human hepatocellular carcinoma cells. They used comparative semi-quantitative and quantitative real-time PCR to quantify c-Myc gene expression, reported successful c-Myc protein translation in transfected HuH7 cells with pure siRNA and achieved siRNA/bPEI/AuNPs. Similar results were obtained in this study, necrosis was observed in Capan-1 cells interacting with siRNA and siRNA-PEI-AuNP, and a decrease in cell proliferation was detected.

While obtaining DNA band in the expected range for the GAPDH housekeeping gene used in the study, we observed no amplification for the targeted region for siRNA, AuNP-siRNA and NK.

In addition, it was observed that the vitality values in % in WST-1 analysis were higher than the control. It is considered that the reason for this may be due to the increase in values by affecting the absorbance of the residues remaining on the surface during the application of Au nanoparticle particles.
The fact that the viability values were higher in the WST-1 analysis compared to the control is thought to be due to the fact that the residues on the surface of AuNPs increase the values by affecting the absorbance.

This study evinced that AuNP-siRNA could be effectively used therapeutically to silence the target gene. As a consequence of the increase of similar studies, it is vital to develop target-oriented methods alternative to traditional ones which cause several adverse effects. However, further knowledge is needed for the effective application of such methods.

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