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Downregulation of Tetraspanin 8 and Carbonic Anhydrase 9 Gene Expression Sensitized Pancreatic Cancer Cells to Cisplatin.

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Abstract

In this context, we used the combined siRNA and chemotherapeutic drug treatment strategy for minimizing the side effects of chemotherapy agents in pancreatic cancers.We used the gene silencing approach with siRNA to target CA9 and TSPAN8 genes, which are overexpressed in pancreatic cancer.Then expression of the targeted CA9 and TSPAN8 geneswere determined by western blot analysis. Effect gene silencing on the proliferation of Panc-1 and MiaPaca-2 cells were detected by MTT assay after transfection with ControlsiRNA, CA9siRNA and TSPAN8 siRNA. MTT cell proliferation assay was also performed to determine the effect the combined siRNA and chemotherapeutic drug treatment as to evaluate cisplatin and epirubicinsensitivity.The results demonstrated that targeted CA9siRNA and TSPAN8 siRNA have increased the sensitivity of pancreatic cells to cisplatin and epirubicin that can conclude;CA9 and TSPAN8 silencing may be a suitable candidate for therapeutic applications.

Keywords: CA9, TSPAN8, siRNA, pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the 4thmajor reason for cancer-related death in Europe [1].Pancreatic cancer is an aggressive disease and has a highly poor prognosis [2].Chemotherapy is still the first approach in the treatment of metastatic pancreatic cancer although it has insufficient effects on patients' survival[3].Development of new chemotherapy strategies specifically in combination with molecular targeted therapies are increasing progressively[4].TSPAN8, member of the tetraspanin superfamily, is a tumor-associated gene and overexpressed in several types of cancersincluding pancreatic cancers[5-7]. TSPAN8 has a pivotal role in many cancer cell vital functions, such as cancer cell migration, metastasis and tumor angiogenesis [7-12]. CA9 is hypoxic tumor marker protein that catalyzes the reversible hydration of carbon dioxide to bicarbonate and proton on the cell membrane[13]. CA9 is responsible for the cancer cell survival, as well as to several other biological processes, such as the maintenance of migration and invasion. Targeting of CA9 using therapeutic drugs is a current approach for the treatment of hypoxic solid tumors and clinically useful biomarker of the broad range of hypoxic tumors[14].

In the present study, weinvestigated the cytotoxic effects of cisplatin and epirubicin treatment with siRNA mediated gene silencing of TSPAN8 and CA9 genes in pancreatic cancer cells.

MATERIALS AND METHODS

ICell culture and transfection of siRNA

Panc-1 and MiaPaca-2 were cultured in Dulbecco's modified essential medium (Biological Industries) supplemented with 10% fetal bovine serum (Biological industries) at 37˚C in 5% carbon dioxide incubated (Nuaire). Cells were subcultured when a confluence of 80-90% was grown in T-75 flask. MiaPaca-2 cells were transfected withfinal concentrationof 100-50-10-5 nM of CA9siRNA and TSPAN8 siRNA (Santa Cruz). siLentFect™ Lipid Reagent (Biorad) is used for transfections according to the manufacturer's instructions.

Cell proliferation assay (MTT)

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Panc-1 cells or MiaPaca-2 cells(10000 cells/well) seeded in 96 wellplates and transfected with 100 nM siRNA as described above. 24h and 48hafter transfection,MTT added to cells at a final concentration of 0.5 mg/ml and 4 h incubated at 37°C, the medium was discarded and dissolve the cells in 100 µl in 2-propanol containing0.004M HCl and the absorbance was measured with aspectrophotometer at 550 nm.

Combinational treatment of cells with siRNA and chemotherapy agents

Cells were seeded (5,000 cells/well) in a 96 well plate of O/N and transfected with 50 nM siRNA and incubated 24 hours. Different concentrations of cisplatin (0.5-5-25-50-100 µg/ml) or epirubicin (0.1-1-10-100-500 µg/ml) were added and incubated for 24 hours. Non-transfected cells were also treated withthesame concentration of cisplatin and epirubicin. Cell proliferation wasmeasured by MTT assay as described above.

Western blot

Panc-1 and MiaPaca-2 cells cultured in 6 well plates 5x105cells/well. After 48-hourtransfection, medium was discarded and cells were washed with PBS. Cells were scraped with RIPA buffer, transferred to eppendorf tubes and incubated on ice for 30 minutes. The supernatant was collected by centrifugation at 12000 g,for10minat + 4oC. The amount of total protein was determined using Qubit system. 50 µg total proteinswas loaded to 12.5%SDS-PAGE and separated by electrophoresis. Separated protein was transferred to a PVDF membrane.Membrane blocked with 5% non-fat milk powder in 1xTBS-Tween 20 buffer

for1 hour at room temperature. Then membrane labeled with specific antibodies.CA9 antibody (Abcam), TSPAN8 antibody (Abcam), β-actin antibody (Sigma), horseradish peroxidase-conjugated secondary antibody (Abcam) was used in specific protein labeling. The protein bands were then visualized using the Fusion FX Vilber Lourmat imaging system. Densitometric analyses were performed with the ImageJ software program.

RESULT

Optimization of CA9 and TSPAN8 siRNA dose in MiaPaca-2 cells

MiaPaca-2 cells were transfected with CA9, TSPAN8 and control siRNAs (siCA9, siTSPAN8andsiCont) at a concentration of 50-10-5 nM. MiaPaca-2 cells were harvested 48 hours after the transfection.Western blot analysis was performed as described in the material method section and normalizedresults using β-actin bands are given in Figure 1.A for CA9 and Figure 1.Bfor TSPAN8. Protein expression level is decreased relative to the amount administered siRNA shown in Figure 1 B, D.

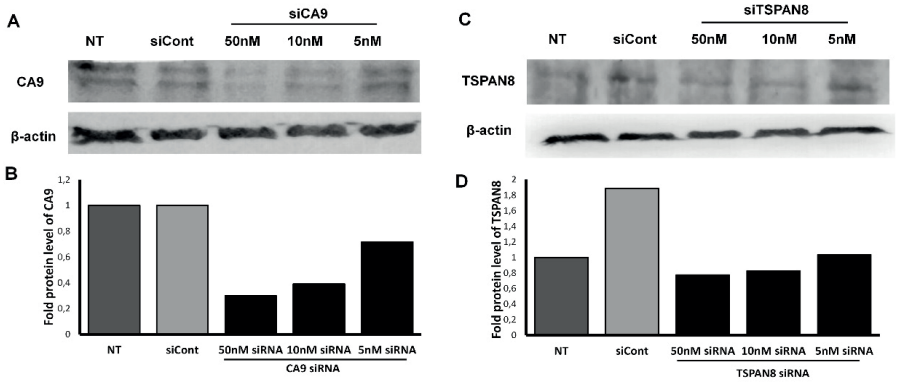

Figure 1. The dose-dependent effect of silencing CA9 and TSPAN8 siRNAs in MiaPaca-2 cells.
The effect of CA9 and TSPAN8 suppression on different concertation was examined in MiaPaca-2 cells. (A) MiaPaca-2 cells were transfected with control siRNA (siCont) concentration of 50 nM then CA9 and TSPAN8 siRNAs (siCA9 and siTSPAN8) and at a concentration of 50-10-5 nM. Cells were harvested 48 hours after the transfection, and the silencing effect at the CA9 (A) and TSPAN8 (C) protein level was determined using western blot in MiaPaca-2 cells. Western blot was performed 50 µg whole cell lysate run on 12.5% SDS-PAGE after transferred to PVDF membrane on ice for 2 h at 120V. CA9 antibody (Abcam), TSPAN8 antibody (Abcam) was 1/500 diluted by 10% BSA in PBS. Protein level CA9 (B) and TSPAN8 (D) showed bands densitometric values were calculated with ImageJ software.

Silencing of CA9 and TSPAN8 genes decreased the cell proliferation of Panc-1 and MiaPaca-2 cells.

MTT assay was performed to evaluate the effect of gene silencing on Panc-1 and MiaPaca-2cell proliferation. Cells were transfected with Control siRNA, CA9siRNA and TSPAN8 siRNA for 24 and 48 h. As seen in the Figure2 decrease in the absorbance of 550 nm for CA9 and TSPAN8 siRNA treated groups indicates the reduction of the cell viability compared to control siRNA treated cells and non-treated cells. Control siRNA (siCont) treatment didn't show statistically significant effect on cell proliferation compared non- treated cells (NT) at all-time points (Figure 2).

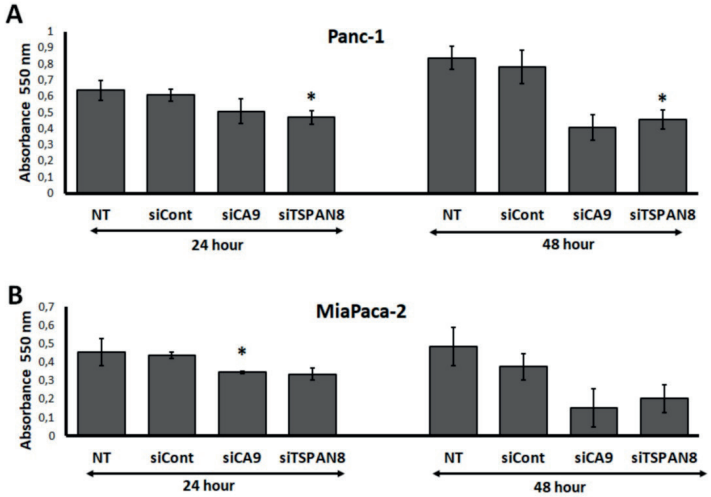

Figure 2. Effect of CA9 and TSPAN8 gene silencing on MTT cell viability assay in Panc-1 and MiaPaca-2 cells.
The time-dependent proliferation of Panc-1 and MiaPaca-2 cells was determined by MTT assay after transfection with control, CA9 and TSPAN8 siRNA. (A) Panc-1 and (B) MiaPaca-2 cells were transfected withCA9 and TSPAN8 siRNAs (siCA9 and siTSPAN8) and control siRNA (siCont) at a concentration of 50 nM. Cell proliferation was detected 24 and 48 hours after transfection with MTT. 24 and 48 hours later, MTT solution was added for 96 wells, followed by incubation at 37C° for 4 hours. Absorbance was then measured at 550 nm using a spectrophotometer

The cumulative effect of targeted gene silencing and chemotherapeutic drugs on the proliferation of pancreatic cells.

Here in we investigated the response of pancreatic cancer cells to cisplatin and epirubicin after silencing of CA9 and TSPAN8 genes. Panc-1 cells and MiaPaca-2 cells were transfected with 50 nM CA9 or TSPAN8 siRNA or control siRNA for 24 h, followed by incubation with different concentrations of cisplatin or epirubicin. After 24 hours of incubation, cell viability was measured by the MTT assay. Silencing of CA9 gene enhanced the cytotoxic effect of cisplatin on Panc-1 and MiaPaca-2 cells. Furthermore, downregulation of TSPAN8 gene dramatically sensitized the pancreatic cancer cells to cisplatin compared to CA9 downregulation (Figure 3A). The cumulative cytotoxic effect of TSPAN8 gene silencing with epirubicin treatment was only observed in Panc-1 cells (Figure 3). The effect of CA9 silencing with epirubicin was found similar in Panc-1 and MiaPaca-2 cells (Figure 3).

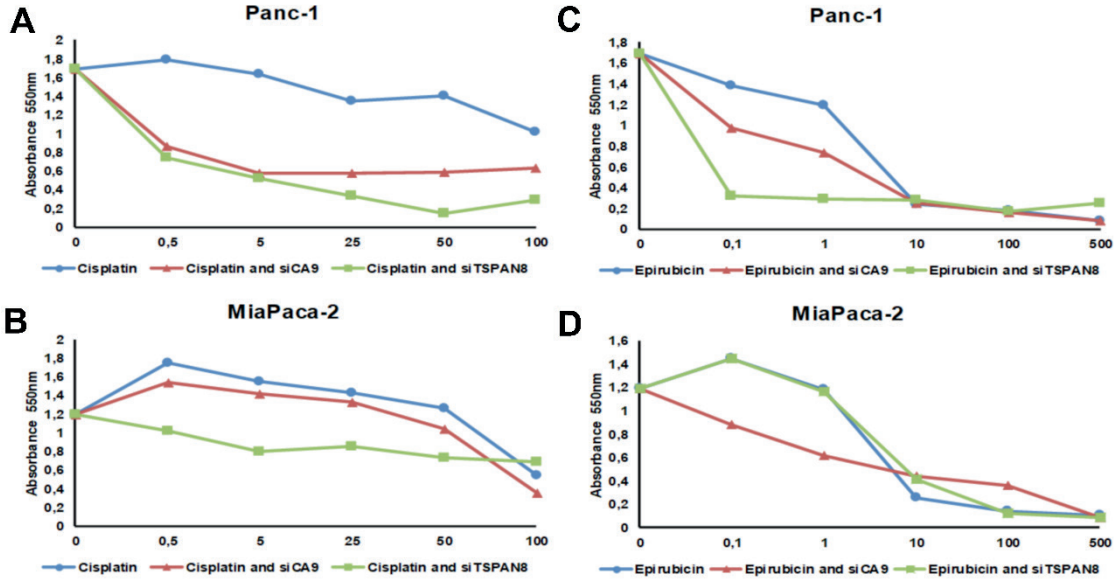

Figure 3. Silencing of CA9 and TSPAN8 sensitized Panc-1 cells and MiaPaca-2 cells to cisplatin and epirubicin.
24 hours after the siRNA transfection, (A), (C) Panc-1 cells and (B), (D) MiaPaca-2 cells were treated with (A), (B) cisplatin (0.5-5-25-50-100 µg/ml) or (C), (D) epirubicin (0.1-1-10-100-500 µg/ml) for 24 hours, and the cell viability was detected withMTT assay.

DISCUSSION

Pancreatic cancer aggressiveness is generally accompanied by overexpression of some proteins involved in tumor growth, metastasis and survival.Therefore,it is not only difficult to diagnose pancreatic cancer; it is also hard to overcome resistance to chemotherapy. Consequently, in combination with the chemotherapy agents, target siRNAs may play a role in sensitizing cancer cells[15, 16]. CA9 plays an important role in the regulation of acid base balance of cancer cells[14].Moreover, it has an important role in the development of the tumor and at the same time it participates in the formation of cell differentiation, migration and invasion[17].CA9 is overexpressed in many types of cancer, and its overexpression is declared to solid tumor aggressiveness and poor outcome[18].TSPAN8 is responsible for cell mobility and it has a role in cell adhesion and motility[6].

MiaPaca-2 cells were transfected with CA9 and TSPAN8 siRNA to achieve a dose-dependent silencing of total CA9 and TSPAN8 respectively, which did correlate with a decrease in their specific proteins (Figure 1). Accordingly, transfection for 48 hours was found more effective in decreasing CA9 and TSPAN8 siRNA treated cell proliferation in comparison to 24 hours (Figure 2).Researchers studied the effect of STEAP1 silencing on LNCaP cells.After 24 and 48 hours' transfection for STEAP1 silenced cells were compared with the control cell group, cell viability decreased by 33% and 44%, respectively [19].

Whitehurstand coworkers found that silencing of several genes sensitized lung cancer cells to a paclitaxel concentration much less than the dose needed that required for a significant response. These researchers also demonstrated that decrease in cell number was attributable to cell death[20].Cisplatin resistance in resistant pancreatic cancer cell lines were known to be associated with drug inactivation, drug transport, DNA damage response, DNA repair and the modulation of apoptosis[21]. Epirubicin (4'-epidoxorubicin), an analogue of doxorubicin (Adriamycin), has been used alone or in combination with other cytotoxic agents in the treatment of a variety of malignancies[22].

Co-delivery of TSPAN8or CA9 targeting siRNA and cisplatin to Panc-1 cells reduced the proliferation of cells more than 50% compared to only cisplatin treated cells. This effect seems to be cell specific as the response of MiaPaca-2 cells to CA9 targeting siRNA with cisplatin was not found significant. Similarly, treatment of TSPAN8 targeting siRNA with epirubicin also did not reduce the cell viability compared to only epirubicin treated cells (Figure 3).Many recent reports also show the similar results for the combination of chemotherapy agents and siRNAs. Their results underline that the combination of chemotherapy agent and siRNA offers superior anticancer effects when compared to chemotherapy agent alone or siRNA alone [23-25]. Beh and coworkers found that the combination of Bcl-2 siRNA

and paclitaxel treatment clearly chemo sensitized HeLa cells, resulting in significantly reduced of cell proliferation in a HeLa cell compared with untreated cells[23].

CONCLUSION

The present study demonstrates that silencing of TSPAN8 and CA9 genes sensitized pancreatic cancer cell to cisplatin and epirubicin.Our findings suggest that combinational treatment of cisplatin and TSPAN8 siRNA may be considered as a novel approach for pancreatic cancer and needs to be further analyzed in vitro and in vivo.

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