



Investigation of the effects of the toll-like receptor 4 pathway on immune checkpoint vista in pancreatic cancer

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Received: 12 November 2021 / Accepted: 28 December 2021 / Published online: 3 February 2022
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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common malignant tumors of the pancreas. Preclinical studies show that it evades the immune system with immune checkpoints and promotes tumor development. V-domain Ig suppressor of T cell activation (VISTA) is a new immune-check point from the B7 family and is highly expressed in cancer cells. Overexpression of toll like receptor 4 (TLR4) in pancreatic adenocarcinoma is associated with induced tumorigenesis, tumor growth, resistancy to chemotherapy. Naloxone is an opioid and inhibits TLR4-ligand association. In this study, we investigated the relation of TLR4 and downstream pathways with immune-check point VISTA in pancreatic cancer proliferation. We initially collected pancreatic cancer-related datasets using the GEPIA2 and UALCAN databases. Based on this data obtained the effect of various concentrations and incubation times of naloxone were used on PANC-1 cells proliferation. A combination of naloxone and VISTA-siRNA were applied, and the effect of both naloxone and combined treatment on TLR4, Interleukin 1 receptor associated kinase 4 (IRAK4) and VISTA gene expression were analyzed in pancreatic cancer cells. As a result of analysis with Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), gene expression levels of TLR4, IRAK4 and VISTA were significantly suppressed and cell proliferation was significantly reduced. We found that administration of naloxone and VISTA-siRNA in combination with PDAC cells suppressed signaling. Therefore, we considered that the relationship between VISTA and TLR4 signaling pathways and the other possible associated signal molecules may be an important marker in determining the response of immune checkpoint inhibitors in cancer treatment.

Keywords Pancreatic ductal adenocarcinoma · siRNA · TLR4 · VISTA · Immunotherapy

Abbreviations

ANOVA	Analysis of variance	FBS	Fetal bovine serum
cDNA	Complementary DNA	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
CO ₂	Carbon dioxide	GEPIA2	Gene expression profiling interactive analysis 2
DAMPs	Damage-associated molecular pattern	GTex	Genotype-tissue expression
DMEM	Dulbecco's modified eagle's medium	HepG2	Hepatocellular carcinoma
DNA	Deoxyribonucleic acid	HUVEC	Human umbilical vein endothelial cells
		IC50	50% Inhibitory concentration
		IL-6	Interleukin 6
		IRAK4	Interleukin-1 receptor-associated kinase 4
		LPS	Lipopolysaccharides
		MD2	Myeloid differentiation factor 2
		MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
		Myd88	Myeloid differentiation factor 88
		NF-κB	Nuclear factor kappa B; nm, nanometer; nM, nanomolar
		NO	Nitric oxide
		NOS	Nitric oxide synthase
		NSCLC	Non-small cell lung cancer
		NT-siRNA	Non target small interfering RNA

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PANC-1	Pancreatic cancer cells-1
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PD-L1	Programmed cell death-1 ligand 1
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interfering RNA
TCGA	The cancer genome atlas
TLR4	Toll like receptor 4
VISTA	V-domain Ig suppressor of T cell activation
VSIR	V-set immunoregulatory receptor
μL	Microliter

Introduction

Cancer occurs as a result of continuous cell proliferation upon the damage of genetic material by transforming normal cells into malignant over time with the effect of mutations [1]. According to GLOBOCAN data, approximately 19.3 million new cancer cases and roughly 10 million cancer-related deaths were detected worldwide in 2020 [2]. PDAC is one of the most common malignant tumors of the pancreas [3]. The cause of this common cancer type is yet not known, but risk factors have been determined as age, smoking, obesity, diabetes, chemical carcinogens (gasoline, pesticides), gender, *Helicobacter pylori* infection, chronic pancreatitis and family history. According to GLOBOCAN 2020 estimates, pancreatic cancer is the 12th most common cancer, causing 466 003 deaths and 495 773 new cases worldwide [2]. Pancreatic cancer does not show any symptoms in the early stages, it spreads rapidly and therefore it is very difficult to diagnose. Due to the highly heterogeneous and complex nature of pancreatic cancers, it is thought that the development of targeted and combined treatment strategies may increase the survival rate [4].

Cancer cells manage to protect themselves from immune system cells by expressing or stimulating immune checkpoints that regulate immunity in the cell membrane for suppression of autoimmunity. The VISTA is a Type 1 transmembrane protein that functions as a negative immune checkpoint molecule. It is localized on CD4+ and CD8+ T helper cells as an inhibitory receptor and it strongly suppresses T cell activation and cytokine production [5]. The toll was first identified as the gene encoding the transmembrane protein in the species *Drosophila melanogaster* [6]. In recent studies, the homolog of the protein encoded by the toll gene was detected in mammals and named as toll-like receptor (TLR) [7]. TLR4, a member of the TLR family, is known as a cell membrane receptor that activates with

both LPS (Lipopolysaccharides) and DAMPs (Damage-associated molecular pattern) and causes proinflammatory cytokine synthesis. It is expressed in innate immune cells and many tumor cells. It is especially involved in triggering tumor formation, tumor growth, inducing apoptosis and resistance to chemotherapy [8]. RNA interference (RNAi) is known as a highly specific approach used to suppress or silence gene expression. It has significant potential for the treatment of various pathological conditions such as cardiovascular diseases, viral infections and cancer. In this approach to treatment, non-coding RNA molecules inhibit target gene expression or translation in a sequence-specific manner [9].

In this study, TLR4 gene expression, which plays an important role in the progression of the pancreatic cancer cell line (PANC-1), was performed to be silenced with the TLR4 antagonist naloxone. At the same time, it was aimed to specifically silence the negative immune checkpoint VISTA gene expression with siRNA and subsequently to evaluate the relationship of the TLR4 signaling pathway with VISTA. VISTA encoded by the VSIR gene and TLR4 signaling pathways showed that both proteins are associated with the NF-κB signaling pathway (<https://www.genecards.org>, Accessed 08.05.2021). However, potential links between VISTA and TLR4 expression have not been yet discovered in pancreatic cancer. Therefore, we think that the findings of the present study will contribute to the literature to consider as a treatment of gene silencing on pancreatic cancer.

Materials and methods

GEPIA2 database analysis

GEPIA2 (Gene Expression Profiling Interactive Analysis 2) database (<http://gepia2.cancerpku.cn/>) is a quotable resource for gene expression analysis based on tumor and normal samples from the TCGA and GTEx databases [10]. It was used to compare the gene expression levels of TLR4, IRAK4 and VISTA in PANC-1 compared to normal cells. The *p*-value was generated using Student's *t*-test and was considered significant for <0.05. |Log₂FC| > 1 indicates the expression of a gene between cancer and normal cells.

UALCAN database analysis

UALCAN (<http://ualcan.path.uab.edu/index.html>) is an effective web resource that can be analyzed based on relevant cancer data using the TCGA database. In this study, UALCAN was used to observe the association of target gene

expression with patient survival [11]. The *p*-value was generated using Student's *t*-test and was considered significant for <0.05 .

Cell culture

The American Type Culture Collection provided the human pancreatic cancer PANC-1 cell line and the human umbilical vein endothelial cell line (HUVEC) cells used in this obtain (ATCC, USA). PANC-1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; FBS), penicillin (100 U/mL), and streptomycin (100 g/mL) at 37 °C in an incubator with 5% CO₂. Cells were grown in an antibiotic-free medium for transfection studies.

Naloxone treatment of PANC-1 cells

Naloxone drug known as TLR4 antagonist was purchased from Hameln Pharma Plus (GmbH, Germany). It was prepared at different concentrations (150–400 μM) and cell viability analysis (MTT analysis) was performed and the IC₅₀ value was determined. In the next step, qRT-PCR was performed to detect the effects of naloxone on TLR4, IRAK 4 and VISTA gene expressions.

siRNA treatment

For transfections, 25×10^3 cells were seeded per well (in a 6-well plate) in an antibiotic-free medium and cells were incubated until they were confluent.

siRNAs specific for target genes were designed and purchased by Dharmacon (GE Healthcare, USA). VISTA, GAPDH and negative control siRNA were used in the study. siRNAs were supplied in powder form and prepared according to the Dharmacon protocol.

To determine the optimum percent silencing of siRNA, it was determined at different concentrations of 5 nM, 10 nM, 25 nM and 50 nM and transfected into cells with Dharmafect (5 μL/well; Dharmacon; GE Healthcare, USA). After determining the effective dose by qRT-PCR, gene expression and viability analysis were performed.

In the next step, to determine the synergistic effect of siRNA and naloxone drug in cells, the initially determined effective doses of siRNA and naloxone drug were given to the cells in combination. Cell viability analysis and qRT-PCR were performed to detect changes in gene expression levels of target genes.

The sequences of siRNA (Dharmacon; GE Healthcare, USA) were as follows (Table 1);

Cell viability assay

About 5×10^3 cells/well were seeded in 96-well plates and incubated for 1 day. To determine the IC₅₀ dose of naloxone, PANC-1 cells were exposed to a range of different naloxone concentrations (175–400 μM) for 24, 48 and 72 h. On the other hand, for siRNA viability analysis, experimental groups were divided into negative control and treated with different concentrations of VISTA-siRNA (5–50 nM) for 24 h. Then, the cells were incubated with 20 μL of MTT reagent (AppliChem, Germany) at 37 °C for 35 min in the dark and analyzed by Nanodrop 2000 (Thermo Fisher Scientific, USA) at 570 nm.

Cell viability was calculated by normalizing relative to the untreated control for naloxone treatment and the non-target siRNA treated group for siRNA treatment.

Quantitative real-time PCR

PANC-1 cells and HUVEC cells (7×10^6) were cultured in 75 cm² flasks for 24 h and then exposed to 271.789 μM (IC₅₀ dose) naloxone drug for 72 h. For siRNA application, were seeded in 6 well plates at a concentration of 2.5×10^4 cells per well and incubated for 24 h.

Total RNA was used with triazole reagent (Sigma-Aldrich®) and isolated from PANC-1 and HUVEC cells according to the manufacturer's protocol. 1 μg of RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific) on ice in the presence of a Random Hexamer primer. Gene expression studies were performed with SYBR Green Master Mix (Roche).

The following primers were used for gene expression analysis;

IRAK4:

F-5'-CGCTTCCTAGTTCGGCTGGTT-3'; R-5'-GTT TTGGGAACAGCATCTGCC-3'

VISTA:

F-5'-GATAGCGGCCTCTACTGCTG-3'; R-5'-TGGATG GTGCATCTTTGCCT-3'

TLR4:

F-5'-AGACCTGTCCCTGAACCCTAT-3'; R-5' CGA TGGACTTCTAAACCAGCCA-3'

GAPDH:

F-5'-GGAGCGAGATCCCTCCAAAAT-3'; R-5'-GGC TGTGTCATACTTCTCATGG-3'

The qRT-PCR cycling conditions were: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 53 °C for 30 s and extension at 72 °C for 60 s. All experiments were carried

out in triplicate using a Quant Studio3 (Thermo Fisher Scientific, USA) Real-Time PCR instrument. Data analysis for naloxone application was done with Qiagen RT² Profiler PCR Data Analysis. For the siRNA application, it was normalized according to the Non-targeting siRNA samples and analyzed through the reference information and template of the manufacturer.

Statistical analysis

Statistical analyses were performed by using “RT² Profiler™ PCR Array Data Analysis” and graphs and statistical analysis were generated using GraphPad Prism software (version 8.0.1; GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between treated groups and untreated controls were performed using the Student’s t-test (two-tailed) or two-way ANOVA. The tests considered a basic significance level of $p < 0.05$.

Results

TLR4 and VISTA gene expression levels are upregulated in pancreatic cancer compared to normal tissue

According to data analysis in GEPIA 2 databases, a high level of gene expression of TLR4 ($p < 0.05$, Fig. 1a) and

Fig. 1 Prognostic analysis of target genes in pancreatic cancer and effect of TLR4 inhibitor naloxone on PANC-1. **a, b** and **c** differential expression analysis of TLR4, IRAK4 and VISTA genes in pancreatic cancer (79 tumor cases and 171 normal cases). GEPIA2 generates a box plot showing gene expressions in pancreatic cancer and normal tissues (TCGA tumor versus TCGA normal + GTEx normal). UALCAN plot showing the patient-survival relationship of **d** TLR4 and **e** IRAK4 gene expressions. Blue line: low-expression groups (132 cases) red line: high-expression groups (45 cases). **f** The effect of naloxone drug applied to PANC-1 at different concentrations on cell viability **g** TLR4, IRAK4 and VISTA mRNA expression in PANC-1 and HUVEC cells following incubation with 271.789 μM naloxone

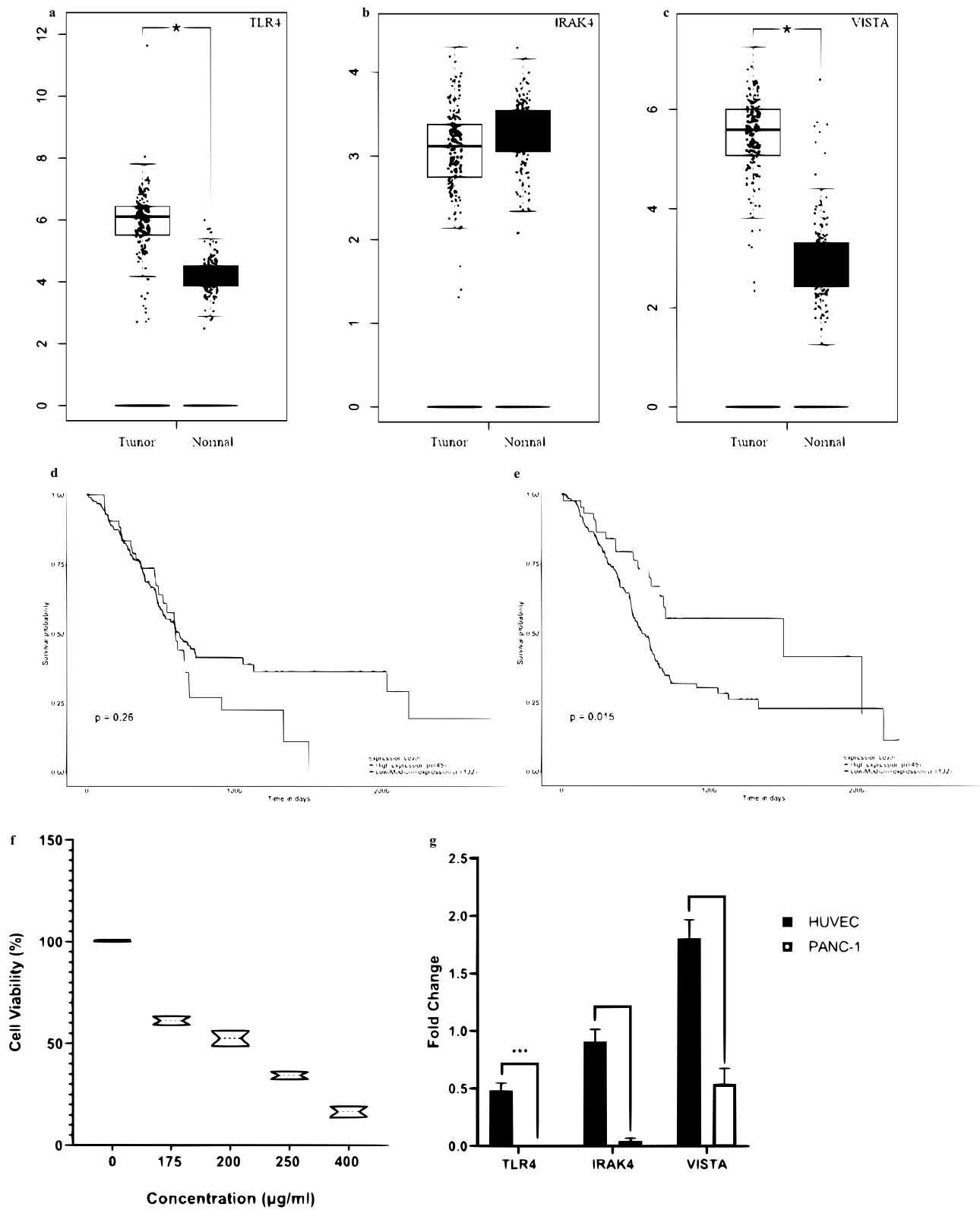
VISTA ($p < 0.05$, Fig. 1c) proteins were observed in cancer tissues compared to normal tissue, while no significant difference was observed in the gene expression change of IRAK4 (Fig. 1b) protein. Comparable data were obtained with MTT and qRT-PCR.

A high level of gene expression of IRAK4 protein is associated with poor prognosis

According to the data analysis in the UALCAN database, TLR4 and IRAK4 gene expressions in pancreatic cancer are decreased in tumor tissue at different cancer stages compared to normal tissue. However, there was no data on VISTA gene expression from pancreatic cancer patients in the database. Bioinformatic analysis showed no significant association between TLR4 ($p < 0.26$ Fig. 1d)

Table 1 siRNA sequence information in the VISTA, NT ve GAPDH siRNA-pool. (a) VISTA; ON-TARGETplus Human EGFR (1956) siRNA – SMARTpool, 5 nmol (Dharmacon, GE Healthcare, L-032651–02-005) (b) Negative control; ON-TARGETplus Human Non-targeting Pool, 5 nmol (Dharmacon, GE Healthcare, D-001810–10-05) (c) GAPDH; ON-TARGETplus Human GAPDH Control Pool, 5 nmol (Dharmacon, GE Healthcare, D-001830–10-05).

Sequence	Sequence information
1. siRNA sequence	ON-TARGETplus SMARTpool siRNA J-032651–21, VISTA GUGAGACAGUGCCGAGAU Mol. Wt: 13,445.1 (g/mol) Ext. Coeff: 367,303 (L/mol.cm)
2. siRNA sequence	ON-TARGETplus SMARTpool siRNA J-032651–22, VISTA CCACAAUGCUUGAUGCCAG Mol. Wt: 13,445.1 (g/mol) Ext. Coeff: 361,073 (L/mol.cm)
3. siRNA sequence	ON-TARGETplus SMARTpool siRNA J-032651–23, VISTA GGACAAAGGGCACGAUGUG Mol. Wt: 13,460.1 (g/mol) Ext. Coeff: 359,738 (L/mol.cm)
4. siRNA sequence	ON-TARGETplus SMARTpool siRNA J-032651–24, VISTA CGGUGGAACUACUACGACUAAA Mol. Wt: 13,430.1 (g/mol) Ext. Coeff: 373,622 (L/mol.cm)
Sequence	Sequence information
1. siRNA sequence	UGGUUUACAUGUCGACUAA
2. siRNA sequence	UGGUUUACAUGUUGUGUGA
3. siRNA sequence	UGGUUUACAUGUUUUCUGA
4. siRNA sequence	UGGUUUACAUGUUUUCUA
Sequence	Sequence information
1. siRNA sequence	GUCAACGGAUUUGGUCGUA
2. siRNA sequence	CAACGGAUUUGGUCGUAU
3. siRNA sequence	GACCUCAACUACAUGGUUU
4. siRNA sequence	UGGUUUACAUGUCCAAUA



gene expression and pancreatic cancer patient survival, whereas up-regulated IRAK4 ($p < 0.015$ Fig. 1e) gene

expression predicted a poor prognosis in pancreatic cancer patient survival.

Table 2 Comparison of mean $2^{-\Delta\Delta CT}$ and *P*-value of TLR4, IRAK4 and VISTA gene expression in PANC-1 and HUVEC cells following incubation with 271.789 μM of naloxone

	VISTA		TLR4		IRAK4	
	$2^{-\Delta\Delta CT}$	<i>P</i>	$2^{-\Delta\Delta CT}$	<i>P</i>	$2^{-\Delta\Delta CT}$	<i>P</i>
HUVEC	1,82	0,65	0,485	0,001	0,91	0,49
PANC-1	0,56	0,02	0,00,585	0,001	0,045	0,001

Effect of different concentrations and incubation times of naloxone on PANC-1 cells proliferation

The drug naloxone dramatically suppressed the proliferation of PANC-1 cells in a concentration- and time-dependent manner. The half-maximum inhibitory concentration (IC₅₀) was 271.789 μM after 72 h of naloxone treatment. ($p < 0.01$; Fig. 1f).

Effect of naloxone treatment on TLR4, IRAK4 and VISTA gene expression in PANC-1 cells

The effects of TLR4 antagonist naloxone on TLR4, IRAK4 and VISTA were compared at the level of gene expressions with $2^{-\Delta\Delta CT}$. According to the study, while TLR4 gene expression was significantly downregulated in PANC-1 cells compared to HUVEC cells, no statistically significant results were obtained in IRAK4 and VISTA gene expressions (Fig. 1g, Table 2).

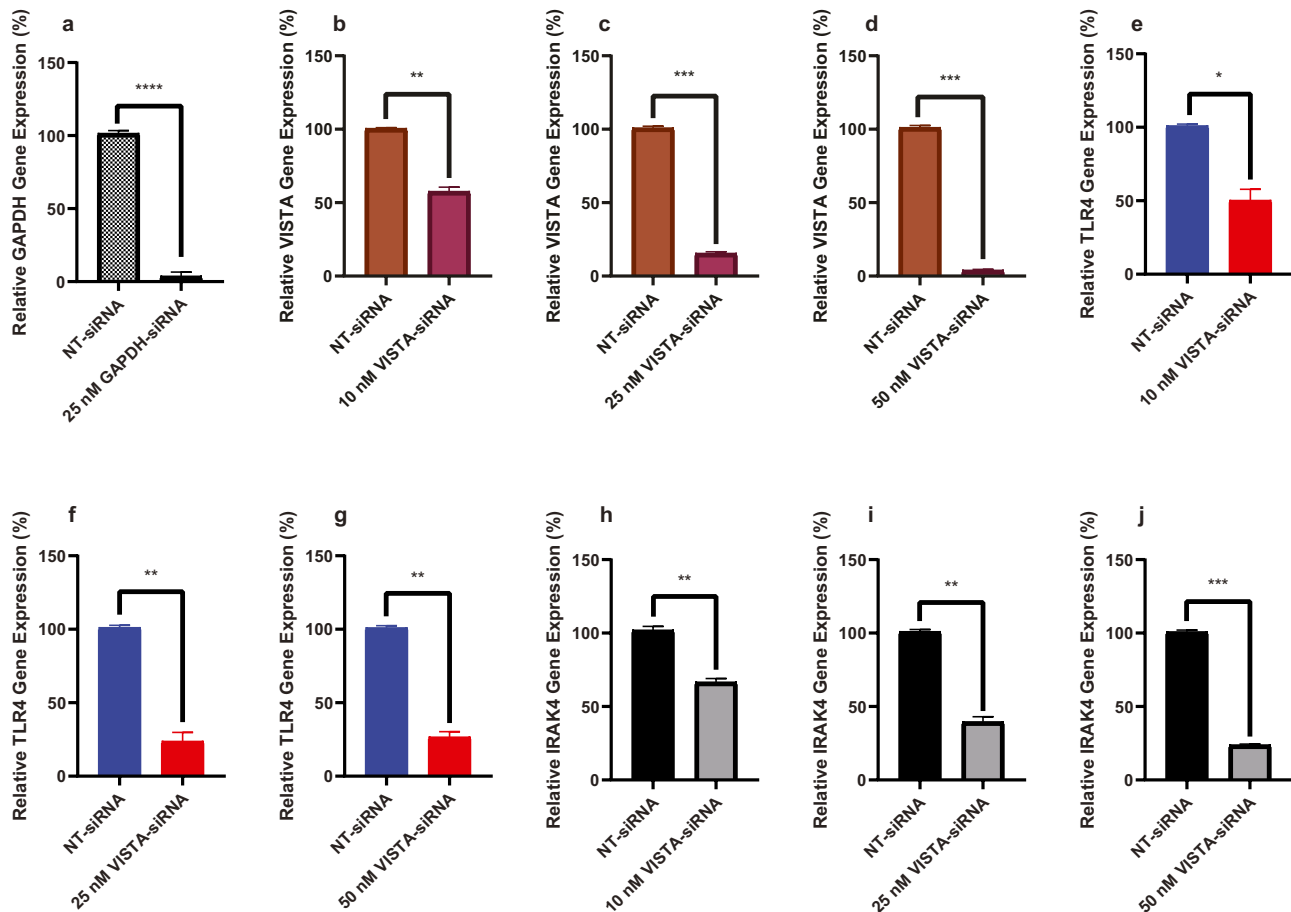


Fig. 2 Effect of various siRNAs transfected into PANC-1 cells on expression of target genes **a** Transfection of 25 nM GAPDH-siRNA into PANC-1 cells for 24 h resulted in 96% suppression of GAPDH gene expression. VISTA gene expression was suppressed by 44% ($p < 0.01$), 84% ($p < 0.001$) and 96% ($p < 0.001$), respectively, by transfection of **b** 10 nM, **c** 25 nM and **d** 50 nM VISTA-siRNA into PANC-1 cells for 24 h. TLR4 gene expression was suppressed by

50% ($p < 0.05$), 70% ($p < 0.01$) and 75% ($p < 0.01$), respectively, by transfection of **e** 10 nM, **f** 25 nM and **g** 50 nM VISTA-siRNA into PANC-1 cells for 24 h. IRAK4 gene expression was suppressed by 33% ($p < 0.01$), 60% ($p < 0.01$) and 75% ($p < 0.001$), respectively, by transfection of **h** 10 nM, **i** 25 nM and **j** 50 nM VISTA-siRNA into PANC-1 cells for 24 h

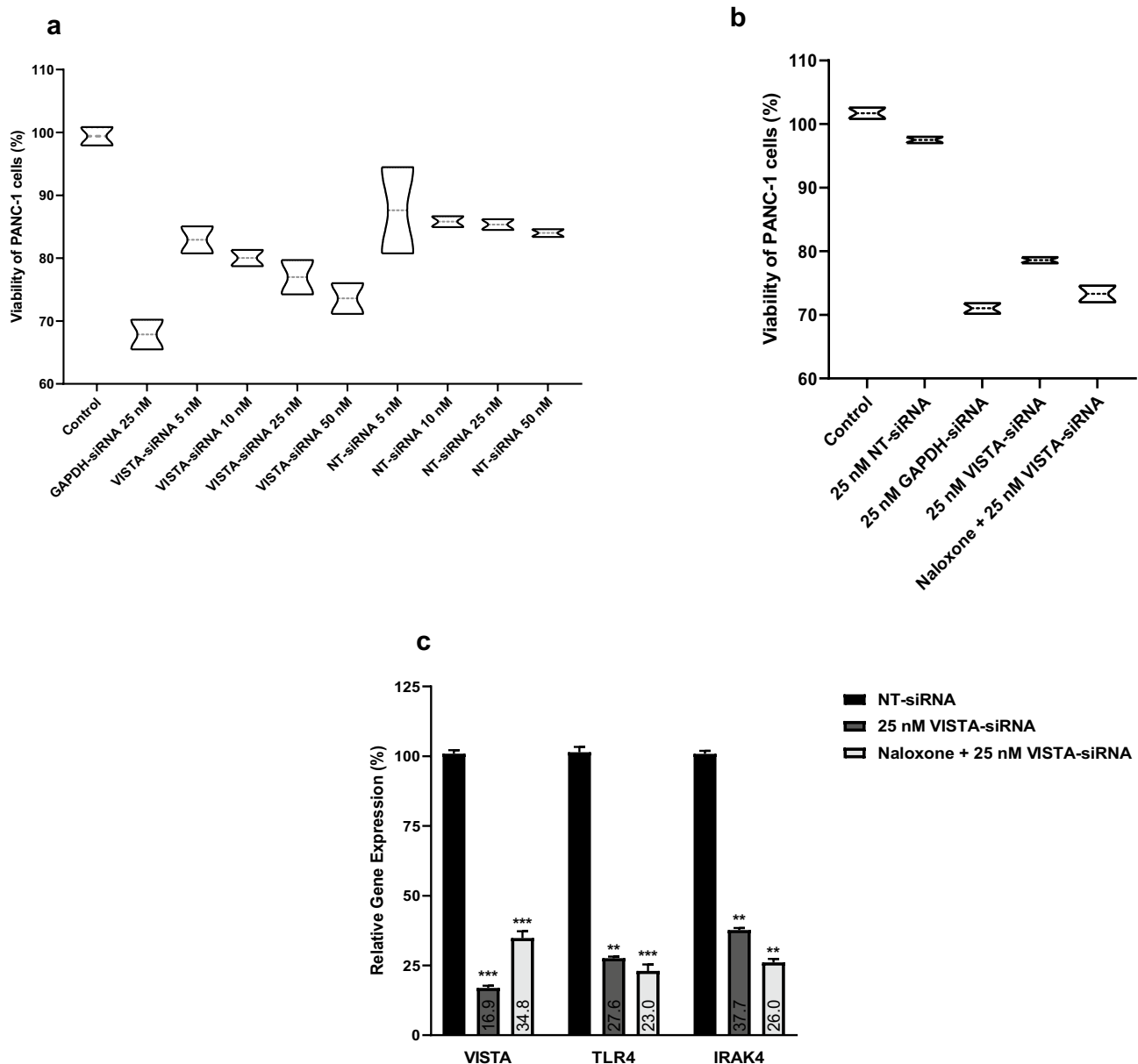


Fig. 3 Enrichment analysis of differential expression genes associated with VISTA expression in pancreatic cancer. **a** Dose dependence of cell viability activities 24 h after transfection with VISTA and NT siRNA into PANC-1 cells ($p < 0.001$). The viability of PANC-1 cells treated with 5 nM, 10 nM, 25 nM, and 50 nM VISTA-siRNA, decreased to 85%, 81%, 79%, and 76%, respectively. The viability of cells treated with 25 GAPDH-siRNA decreased to 70%. **b** naloxone and 25 nM VISTA-siRNA application in combination to PANC-1 cells showed suppression of 65% ($p < 0.001$), 77% ($p < 0.001$) and 74% ($p < 0.01$) in VISTA, TLR4 and IRAK4 gene expressions,

respectively. **c** MTT analysis graph of the effect of 25 nM NT-siRNA ($p < 0.05$), 25 nM GAPDH-siRNA ($p < 0.01$), 25 nM VISTA-siRNA ($p < 0.01$) and combination treatment of naloxone and 25 nM VISTA-siRNA ($p < 0.01$) application to PANC-1 cells on cell viability. The percentage of cell viability decreased to 98%, 71% and 79%, respectively, in cells treated with 24-h NT-siRNA, GAPDH-siRNA, and VISTA-siRNA compared to control cells. In cells treated with 72 h of naloxone and 24 h of VISTA-siRNA, the percentage of cell viability decreased to 77% compared to control cells

Suppression rates of GAPDH gene expression of 25 nM GAPDH-siRNA transfection into PANC-1 cells

24-h 25 nM GAPDH-siRNA was applied to PANC-1 cells and gene expression levels were compared with the control

group. As a result, a significant knockdown was observed in GAPDH gene expression compared to the control group ($p < 0.0001$; Fig. 2a).

VISTA-siRNA transfection applied to PANC-1 cells at different concentrations causes suppression of VISTA, TLR4 and IRAK4 gene expression

VISTA-siRNA and NT-siRNA were transfected into the PANC-1 cells at concentrations of 5, 10, 25, 50 nM. Gene expression results were normalized to the GAPDH gene. The siRNA dose that provided the maximum suppression was determined and used for further studies.

As a result of the application of 5 nM VISTA-siRNA to the PANC-1 cells for 24 h, no significant suppression was observed in VISTA, TLR4 and IRAK4 gene expression compared to the control group.

However, as a result of the application of 10 nM, 25 nM and 50 nM VISTA-siRNA to PANC-1 cells for 24 h, a significant decrease in VISTA (Fig. 2b, c and d), TLR4 (Fig. 2e, f and g) and IRAK4 (Fig. 2h, i and j) gene expression was observed compared to the control group.

Effect of siRNA transfection on PANC-1 cells viability

MTT method was used as a viability analysis test to observe the effect of the VISTA gene on proliferation on the PANC-1 cells and to determine the effective dose on the cells. In the analyzes, different doses of VISTA-siRNA were studied in 5 replicates for each group. Absorbance values were measured at 570 nm with a spectrophotometer 24 h after transfection and calculated with their mean values. The mean absorbance values of the siRNA-transfected cells were proportional to the mean absorbance values of the untransfected control cell group and the percentage of viability of the cells was calculated according to the siRNA doses. As a result, there was a significant decrease in viability in cells transfected with different concentrations of VISTA-siRNA compared to the control group (Fig. 3a).

Naloxone and siRNA combined applications create a synergistic effect on target genes in the PANC-1 cells

PANC-1 cells were treated with 24 h of 25 nM VISTA-siRNA, 25 nM GAPDH-siRNA, and 25 nM NT-siRNA in combination with 72 h of naloxone. At the end of the combination application, the effects on VISTA, TLR4 and IRAK4 gene expressions were examined and statistically significant results were found.

As a result of the combination of 24-h 25 nM VISTA-siRNA and 72 h naloxone administration to PANC-1 cells, a significant knockdown was observed in VISTA, TLR4, and IRAK4 gene expression compared to the control (Fig. 3b).

Effect of naloxone and siRNA on PANC-1 cells in combination applications on cell viability

MTT viability analysis was performed to observe the effect of VISTA and TLR4 genes on cell proliferation by creating a synergistic effect on PANC-1 cells. PANC-1 cells were treated with 24-h siRNA and 72 h of naloxone. In the analyzes, each group was studied with 3 replications. The averages of absorbance values measured at 570 nm wavelength of 3 wells were calculated. The mean absorbance values of the cells treated with naloxone and siRNA were compared to the non-transfected control cells, and the percentage of viability was calculated. As a result, cell viability was significantly reduced when compared to the control group (Figs. 3c).

Discussion

PDAC is one of the most lethal solid malignancies and despite significant improvement in surgical techniques and postoperative results, the overall recurrence rate is approximately 85% and the 5-year survival is less than 30% [3, 12].

Pancreatic cancer develops resistance to chemotherapy, radiotherapy, targeted therapies due to the complex tumor microenvironment, genetic and epigenetic changes [13]. Due to this context, more effective treatment strategies are needed. Immunotherapeutic approaches have emerged for the treatment of advanced malignancies. Recently, attention has been drawn to the development of drugs that target specific receptors on pancreatic cancer tumor cells and their use in combination with chemotherapeutic agents or inhibition of genes overexpressed by siRNA molecules [14, 15].

In this study, naloxone was applied to block TLR4, which is highly expressed in PANC-1 cells, in the first part of the study. Then, the IC50 dose was found and used in the analysis to detect the suppression of TLR4, IRAK4 and VISTA gene expressions by qRT-PCR. As a result, whereas a significant down-regulation was observed in TLR4 gene expression, no significant results were obtained in IRAK 4 and VISTA gene expressions. Human TLRs are expressed in pancreatic cancer tissue and many cancer cell lines, whereas they are low or not expressed in normal pancreatic cells [15, 16]. TLR4 causes tumor progression by communicating with tumor cells, immune cells and tumor microenvironment. In many studies, TLR4 plays an important role in the inhibition of chemotherapy resistance and tumor formation [8]. In a study conducted in 2009, the effect of LPS on the invasive ability of PDAC was examined by targeting TLR4 and Myd88 with siRNA. It was shown that LPS increases

the invasive ability of PDAC and blockade of the NF- κ B pathway reduces the increased invasive ability of LPS [17]. In a study by Hsiao et al. on hepatocellular carcinoma (HepG2) cells, it was observed that IL-6 upregulated LPS-induced TLR4. It was also determined that TLR4 activation increased proliferation, nitric oxide synthase (NOS) expression and NO production in HepG2 cells. In addition, high chemoresistance was developed against antitumor drugs applied to HepG2 cells with very high TLR4 expression [18]. Once upon TLR4 was discovered, naloxone was found to block the biological activities of LPS, a component of the cell walls of gram-negative bacteria. Today, naloxone acts as a TLR4 antagonist by binding to the LPS binding site of the MD2 protein [19].

In the second part of the study, the effect of siRNA silencing of VISTA, which is highly expressed in the PANC-1 cell, on gene expression of TLR4 and IRAK4 was investigated. VISTA-siRNA designed specifically for the VISTA gene and NT-siRNA were transfected into cells at 4 (four) various concentrations by lipofection method. The effect of siRNA on VISTA was examined by qRT-PCR. Based on the qRT-PCR results, it has been observed that 24 nM VISTA-siRNA application for 24 h in PANC-1 provides the most ideal suppression since the combination will be applied in the optimizations and subsequent studies. The percentages of silencing in the cell were found to be statistically significant compared to the control group, and excessive silencing occurred when siRNAs were successfully transfected. qRT-PCR was performed to show the suppression of TLR4 and IRAK4 gene expressions in cells treated with VISTA-siRNA. After 24 h of siRNA treatment at 25 nM dose, suppression of IRAK4 and TLR4 gene expressions was found to be significant.

VISTA, which is one of the immune checkpoints and can act as both a ligand and a receptor, is known to suppress T cell proliferation and cytokine release. VISTA, which suppresses the T cell-associated response for immune avoidance and survival in many human cancers such as prostate cancer, non-small cell lung cancer (NSCLC), and colorectal carcinoma, is also highly expressed in CD68 + macrophages in pancreatic cancer [19–21]. Preclinical studies have shown that VISTA blockade releases T-cell effector functions and suppresses tumor growth in colon cancer models by synergizing with other immune checkpoint inhibitors such as anti-programmed cell death-1 ligand1 (anti-PD-L1) [22].

Preclinical studies have shown that VISTA blockade releases T-cell effector functions and suppresses tumor growth in colon cancer models by synergizing with other immune checkpoint inhibitors such as anti-PD-L1 [22].

Patients with ovarian and endometrial cancer were treated with immune checkpoint inhibitors such as PD-1 blockade, but the results were not satisfactory for both ovarian (11.5–15% overall) and endometrial cancers (about 13%

overall) [20]. Therefore, the development of new treatment strategies, such as combined therapies, is anticipated successfully to treat patients who are resistant to anti-PD-1 therapy [22, 23].

As a result, both proteins were found to be associated with the NF- κ B signaling pathway. However, a little knowledge about the expression and clinical significance of VISTA in pancreatic cancer is known. Potential relations between VISTA and TLR4 expression have been yet discovered. Targeting the connections in the downstream signaling pathways of VISTA and TLR4 in pancreatic cancer will be beneficial for clinical studies on immune checkpoint inhibitors and oncogenic pathway inhibitors.

Since TLR4 is thought to have a synergistic effect in VISTA regulation, VISTA-siRNA and naloxone were given together to PANC-1 cells. Cells were treated with initially an IC50 dose of naloxone alone for 48 h and then treated subsequently with naloxone- VISTA-siRNA combination for 24 h. VISTA, IRAK4 and TLR4 gene expressions were compared by qRT-PCR according to NT-siRNA applied cells. As a result of the study, a significant knockdown was detected in VISTA, TLR4 and IRAK4 gene expression levels when compared to the control group.

Greater suppression of VISTA gene expression was observed when treated with 25 nM VISTA-siRNA alone compared to the combined treatment of naloxone and VISTA-siRNA. Our hypothesis is; this is due to the fact of naloxone and VISTA-siRNA may decrease the efficacy by negatively affecting each other in VISTA gene expression. Combination treatment of VISTA-siRNA with agents used in TLR4 inhibition into PANC-1 cells was found to significantly downregulate TLR4 and IRAK4 gene expressions which are located in its downstream pathway compared to VISTA-siRNA treatment alone. In addition, according to the results of MTT analysis, knockdown of VISTA and TLR4 caused a significant decrease in cell viability. Based on these results, the relationship between VISTA, TLR4 and IRAK4 molecule which are responsible for pancreatic cancer progression was determined at both gene expression and cell viability levels. The obtained data of the study show that TLR4 and IRAK4 molecules which is involved in the TLR4 downstream pathway, are associated with poor prognosis in the development and proliferation of pancreatic cancer. Furthermore, TLR4 signaling pathway is associated with negative immune checkpoint VISTA in cancer development.

As a result, it is thought that the existence of the relationship between the immune checkpoint VISTA and TLR4 will contribute to the development of anti-tumor studies via accepting these molecules as a therapeutic target and understanding better the signaling pathways and ligands which are regulated by VISTA.

Conclusion

In our study, we initially used gene expression data using public databases to highlight the importance of the immune checkpoint and TLR4 in pancreatic cancer patients. In addition, we have shown the critical importance of TLR4 signaling pathway and immune checkpoint VISTA in PDAC's growth and proliferation regarding our analysis. It was concluded that the use of inhibitors designed for these proteins may be possible to halt the progression of cancer cells. Since TLR4 and VISTA signaling pathways are highly expressed in pancreatic cancer and are associated with different signaling pathways, potential connections with other signaling pathways need to be determined we conclude that immune checkpoint inhibitors may be an important marker in determining response in cancer therapy.

Acknowledgements We acknowledge the GEPIA2 and UALCAN databases for free use.

Authors' contribution Emine Nedime Korucu conceptualized the Project. Emine Nedime Korucu, Nadir Kocak and Kubra Sena Bas Topcu designed experiments. Emine Nedime Korucu, Kubra Sena Bas Topcu and Tugce Duran performed experiments. Esma Menevse analyzed data. Kubra Sena Bas Topcu wrote the original manuscript draft. Esma Menevse wrote a revised manuscript draft. Emine Nedime Korucu supervised and administered the project.

Funding This study was funded by Necmettin Erbakan University Scientific Research Projects Coordination Unit, Project number 191315002.

Data availability All data generated or analyzed during this study are included in this published article. Raw data is available under reasonable request.

Declarations

Consent for publication All authors consent to the publication of this study.

Research involving human participants and/or animals Not applicable.

Ethical approval This study were approved by the Pharmaceutical and Non-Medical Research Ethical Committee of Necmettin Erbakan University, Meram Faculty of Medicine (Ethics number: 2019/2070).

Informed consent Not applicable.

Conflict of interests The authors declare no conflict of interest.

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