Original Article

The Combination of PD-L1 and CTLA-4 Suppression Significantly Decreased the Expression Levels of Cancer Stem Cell Factors in the Pancreatic Cancer Cell Line

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Abstract

Background: It is thought that a limited number of aberrant cancer stem cells (CSCs), which encourage carcinogenesis, tumor metastasis, and treatment resistance, are the cause of pancreatic cancer, a disease with a high mortality rate. Increasing evidence shows that CSCs use immunosuppressive properties to avoid immune system identification. In Pancreatic cancer, the therapeutic consequences of the relationship between the expression of immune checkpoints such as programmed death receptor ligand-1 (PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the presence of CSCs are poorly known. As a result, in the present investigation, we examined how the expression levels of CSCs were affected by PD-L1 and CTLA-4 knockdown using specific siRNA.

Methods: Independently and together, PD-L1 and CTLA-4 siRNA were transfected into MIA PaCa-2 cells. RNA extraction and cDNA synthesis were then performed. Finally, using qRT-PCR, the gene expression levels of CSCs markers, including Nanog, CD133, CD44, and Oct-4, in transfected groups were measured.

Results: In the MIA PaCa-2 cell line, siRNA-mediated inhibition of PD-L1 and CTLA-4 decreased the expression of CSC factors. Moreover, combined suppression of these two stemness-related immune checkpoints significantly decreased Nanog, CD133, CD44, and Oct-4 expression compared to inhibition of PD-L1 and CTLA-4 separately.

Conclusion: Considering that the combination of PD-L1 and CTLA-4 suppression using siRNA significantly decreased the expression levels of CSCs factors, this approach may be regarded as an effective therapy for this cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the deadliest malignancies, is the fourth leading cause of cancer-related death globally, with an overall five-year survival rate of 8% for all stages considered. PDAC accounts for 90% of all pancreatic cancers.[1] Late diagnosis and therapy resistance, which cause local tumor recurrence and distant metastasis, are the major causes of the poor prognosis.[2] Moreover, patient variances add to the difficulty of PDAC therapies. Therefore, the molecular pathogenesis of PDAC must thus be better understood immediately. The outlook for pancreatic cancer in its latter stages remains poor, despite recent improvements in other cancer therapies.[3] Hence, finding new chemotherapeutic medicines to target, such as cancer stem cells (CSCs), is extremely desirable. CSCs are cancer cells that display characteristics of typical stem cells, such as multipotency and self-renewal. Scientists have recently proposed that CSCs produce tumors by sharing these two traits with stem cells.[4,5] The idea behind CSCs is that they may stay in tumors for a long time, causing relapse and metastasis by developing new cancers. In other words, CSCs could be very important for tumor development, growth, and metastasis.[5,6] Various human cancers, including pancreatic cancer, have been reported to include CSCs.[7,8] However, the presence of CSC surface markers like Nanog, CD133, CD44, and Oct-4 in recent studies has shown their involvement in solid tumors.[9] The host immune response significantly shapes the TME, and immune escape has been identified as a crucial cancer characteristic. Hence, strong evidence suggests that immune tolerance in the TME also contributes to the development of tumors.[10]

For CSCs to stimulate the formation of tumor-promoting immune cells, which in turn controls CSC maintenance and differentiation and, as a result,

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maintains tumor initiation, growth, and metastasis, CSCs must interact with the immune system in a way that triggers immune-suppressive processes.[11,12] The immune checkpoint inhibitor axis is displayed by programmed-death ligand 1 (PD-L1)[13] and B7 family produced in tumor cells. These molecules link to the activated T cells' PD-1 and CTLA-4 receptors[14] and send those T cells an inhibitory signal that stops the immune system from eliminating the tumor.[15,16] Immune checkpoints with high levels of expression, including PD-L1 and CTLA-4,[17,18] which prevent the antigen from being presented to immune T cells and hence create an immune suppressive environment, are primarily responsible for CSCs' capacity to evade the immune system.[19] Consequently, CSCs are more susceptible to immune-based therapy when PD-L1 and CTLA-4 levels are high.[14,20] We set out to find the importance of the cell surface markers CD133, CD44, Nanog, and Oct-4 on the CSCs of pancreatic cancer cells since there has been insufficient research on CSCs in pancreatic cancer to date. In the present work, we investigated how immune checkpoint downregulations influenced the expression of CSC genes in the MIA PaCa-2 pancreatic cancer cell line.

Materials and Methods
Cell culture
In order to carry out research on human pancreatic cancer, the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) has provided the MIA PaCa-2 cell line. The cells were cultured in RPMI-1640 media with 1% antibiotics and 10% fetal bovine serum added to it (In a humidified incubator containing 5% carbon dioxide, the cells were maintained at 37 degrees Celsius throughout the experiment). The log phase of development was then used for all subsequent tests.

siRNA transfection
Forty, 60, 80, and 100 pmol of PD-L1 and CTLA-4 siRNA (Santa Cruz, USA) were transfected into MIA PaCa-2 cells using the Gene Pulser electroporation equipment (TC = 12.5 ms and voltage = 160 V, square wave) (Bio-Rad). After transfection, the appropriate number of cultured cells was placed on different cell culture plates depending on the relevant test. After electroporation, cells were placed in a 6-well plate with RPMI-1640 media with 10% fetal bovine serum (FBS) and incubated at 37°C for 24, 48, and 72 hours. The relative expression of CSCs was then evaluated using quantitative reverse transcription

polymerase chain reaction (qRT-PCR).

RNA extraction and qRT-PCR
4 siRNA, mRNA ex In MIA PaCa-2 cells treated with PD-L1 and CTLA- pression of CSCs was identified using qRT-PCR. Total RNA extraction was performed using the manufacturer's procedure, and the RiboEX reagent (GeneAll Biotechnology, Seoul, Korea) was utilized. BioFACT cDNA synthesis kit (Korea) produced complementary DNA (cDNA). CSC-relative gene expression in transfected cells was measured by comparing it to the expression of GAPDH as a reference gene. The Real-Time PCR Gene Expression Assay was utilized for the following genes: Nanog, CD133, CD44, Oct-4, and GAPDH. Table 1 contains primer sequences.

Statistical analysis
GraphPad Prism version 8.3 was used to analyze the data (GraphPad Prism; San Diego, CA). The data on measurements were represented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare groups using parametric data.

Results
Efficient siRNA transfection of PD-L1 and CTLA-4 into MIA PaCa-2 cells
In order to find the ideal conditions for siRNA suppression, we utilized qRT-PCR to assess the mRNA expression levels of PD-L1 and CTLA-4 after transfecting MIA PaCa-2 cells with a chosen range of PD-L1 and CTLA-4 siRNA dosages at various time points. As an outcome, a transfection dosage of 60 nmol of siRNA over 48 hours was determined to be optimal.

PD-L1 and CTLA-4 siRNAs combination decreased CSC markers expression levels in MIA PaCa-2 cell line
We performed a qRT PCR assay to assess the impacts of PD-L1 and CTLA-4 siRNA transfection and their combined effects on the expression of CSCs factors in the MIA PaCa-2 cell line. According to Figure 1, transfection of cells with only PD-L1 and CTLA-4 siRNA led to a substantial ($P < 0.05$, $P < 0.01$) reduction in CSC markers (Nanog, CD133, CD44, Oct-4) in these cells when compared to the control group. However, when these two siRNAs were combined, the expression of CSC markers was reduced much more significantly ($P < 0.01$, $P < 0.0001$) than in the control group and with PD-L1 and CTLA-4 alone (Figure 1).

Table 1. The primer pairs used in this study

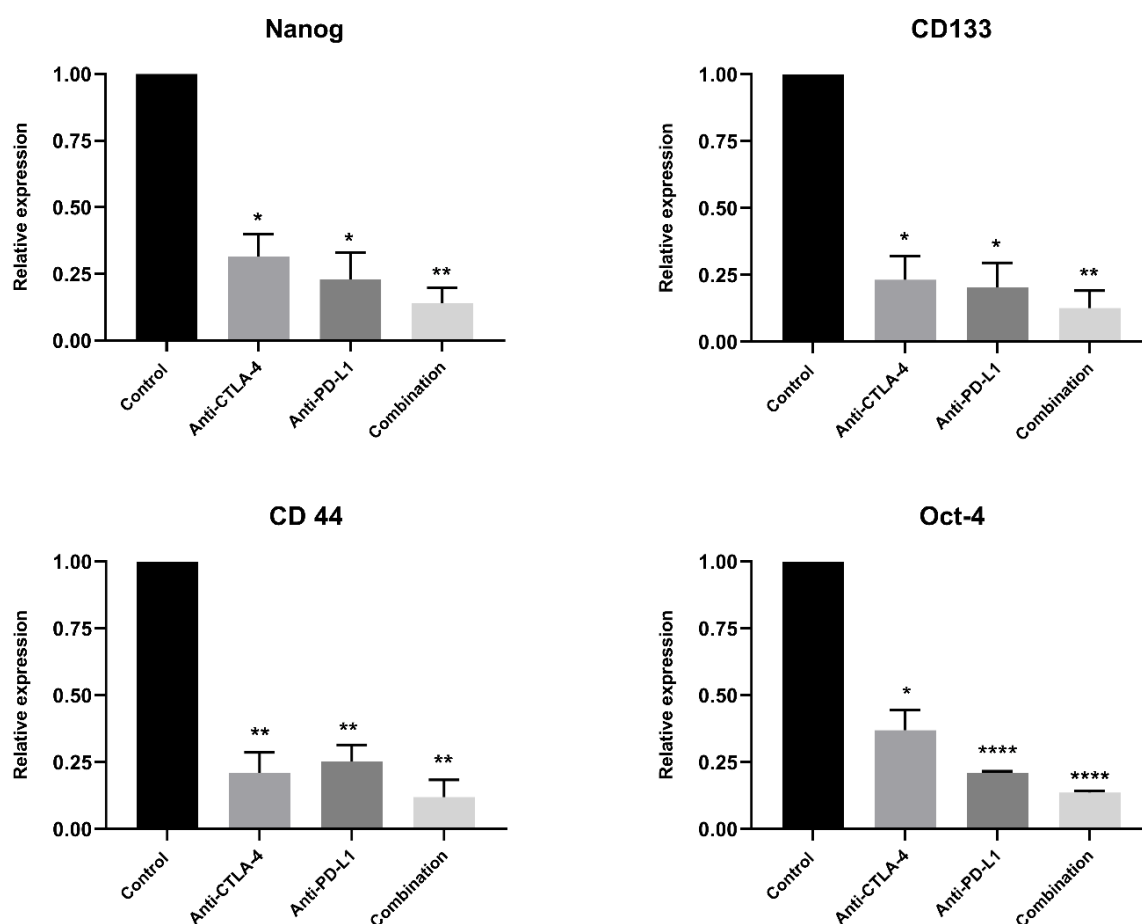| Genes	Forward	Reverse
CD133	5'- GACCGACTGAGACCCAACATC-3'	5'- GGCTAGTTTTCACGCTGGTCA -3'
CD44	5'-CTGCCGCTTTGCAGGTGTA-3'	5'- CATTGTGGGCAAGGTGCTATT -3'
Nanog	5'-AGAGGTCTCGTATTTGCTGC-3'	5'-ACACTCGGTGAAATCAGGGTA-3'
Oct-4	5'-GGGCTCTTTGTCCACTTTGT-3'	5'-GGCATGCATACACACAAACAC-3'
GAPDH	5'-AAGGTGAAGGTCGGAGTCAAC-3'	5'-GGGGTCATTGATGGCAACAA-3'

Figure 1. The effects of PD-L1 and CTLA-4 siRNA on CSCs expression in the MIA PaCa-2 cell line. This figure shows that combined inhibition of PD-L1 and CTLA-4 decreased Nanog, CD133, CD44, and Oct-4 expression. The results are expressed as the means ± SD of triplicate runs. $*P<0.05$, $**P<0.01$, and $****P<0.0001$

Discussion

Despite the therapeutic benefits of conventional radiotherapy and chemotherapy on malignancies, clinical data indicate that CSCs resist these treatments. It has been believed that CSCs, as a source of heterogeneity, are accountable for regional recurrence, tumor progression, and resistant cells to present therapy. Hence, it is important to create targeted therapies that are specialized and efficient for CSCs.[21] Considering the heterogeneity of tumors, immunotherapy could be a reasonable solution that must reach a new level. Until the unclear relationship between cancer stemness and immune evasion was established, manipulating the immune system to eradicate the cause of tumor heterogeneity remained at the hypothetical stage. Evaluation of gene-expression-based metrics revealed a negative relationship between the existence of a stem cell-like phenotype and anticancer immunity in 21 solid tumors, such as pancreatic tumors, suggesting inhibitory signals transduced from CSCs to the immune microenvironment.[22] A number of recent investigations have shown that the expression of immune checkpoints was greater in cancer cells that expressed high amounts of CSC markers such as CD44 and CD133 than in non-CSCs derived from PANC-1 and MIA PaCa-2 cells.[23] Therefore, in this investigation, we evaluated various prospective therapeutic CSC targets, including Nanog,

CD133, CD44, and Oct-4, and their relationship with PD-L1 and CTLA-4 immune checkpoints. In addition, we examined the impact of combination treatment of these immune checkpoints' knockdown on CSCs in the MIA PaCa-2 cell line. In the current work, we inhibited PD-L1 and CTLA-4 immune checkpoints separately and in combination using RNA interference (siRNA) as a viable gene therapy strategy and evaluated it as a promising combination therapy for the suppression of pancreatic cancer cells. Using qRT-PCR, we determined the impact of immune checkpoints combination knockdown on CSCs in MIA PaCa-2 cells. The findings demonstrated that knocking down PD-L1 and CTLA-4 decreased the expression levels of Nanog, CD133, CD44, and Oct-4 CSCs investigated in this work. In line with our findings and according to one study, suppressing CEACAM5, an immune checkpoint associated with stemness in pancreatic cancer, may improve the effectiveness of highly precise immunotherapy that targets the tumor heterogeneity brought on by CSCs. One study found that blocking CEACAM5 as a stemness-related inhibitory immune checkpoint in pancreatic cancer may enhance the efficacy of precision immunotherapy targeting tumor heterogeneity caused by CSCs.[24,25] The key outstanding question is: What precisely happens to CSCs when we combine anti-CTLA-4 and anti-PD-1 therapy? The answer

is that PD-L1 suppression in combination with CTLA-4 significantly decreases the expression of CSCs compared to suppression of these immune checkpoints alone, as shown by the results of our research. One study suggested that administering PD-L1 and CTLA-4 with CSC-DC vaccination to mouse models of melanoma cancer could reverse T cell functions and generate more enhanced T cell activation, proliferative ability, and CTLs that target CSCs.[26] Thus, we could conclude that combination suppression of immune checkpoints has an anticancer impact by reducing the expression of investigated CSCs in pancreatic cancer cell lines, and it can be regarded as an effective future cancer immunotherapy strategy. This research has a number of limitations. Primarily, we only conducted the tests on the pancreatic cancer cell line MIA PaCa-2. Second, we only investigated the impact of immune checkpoint blockade on a restricted number of CSC factors, and we were unable to study the effects of different immune checkpoint blockades on CSCs.

Conclusion

Access to PD-L1 and CTLA-4 gene modification has become increasingly common as a pancreatic cancer treatment due to growing concerns over the efficacy of immune checkpoint inhibitor therapies. CTLA-4 and PD-L1 are inhibitory immune checkpoints that are stemness-related in pancreatic cancer. This research shows that the transfection of PD-L1 and CTLA-4 siRNA may significantly lower CSCs' expression levels, perhaps resulting in a decrease in the factors used in this research, such as Nanog, CD133, CD44, and Oct-4. A novel and effective method for precision immunotherapy that targets tumor heterogeneity will be made available by these combination therapies.

Authors' Contribution
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Validation: Behzad Baradaran.
Visualization: Mahdi Jafarlou.
Writing–original draft: Nazila Alizadeh.
Writing–review & editing: Behzad Baradaran.

Competing Interests
The authors certify that there is no potential conflict of interest concerning this article. It was noteworthy to mention that Behzad Baradaran as a chief editor of the ImmunoAnalysis journal was not involved in any editorial decisions related to the publication of this article, and all author details were blinded to the article's peer reviewers as per the journal's double-blind peer review policy.

Data Availability Statement
The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Ethical Approval
All procedures were conducted in compliance with the ethical principles of the Tabriz University of Medical Science, Tabriz, Iran, and approved by the regional ethical committee for medical research (Ethical code: IR.TBZMED.REC.1400.1114).

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