

B7-H7 Suppression Increases the Expression of CTLA-4 and VISTA Genes in Gastric Cancer Cell Line

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Abstract

Gastric cancer (GC) is a multifactorial genetic malignancy that tumor metastasis is one of the principal characteristics of this disease. The B7 family immune checkpoints have important functions in maintaining the immune system's equilibrium that participates in the regulation of invasion, metastasis, and development of tumors. Silencing of gene expression via small interference RNA (siRNA) delivery technology is one of the significant approaches in gene therapy. The main goal of the current study was to determine the effect of B7-H7 suppression by siRNA on the expression of CTLA-4 and VISTA in the MKN-45 GC cell line. For this purpose, MKN-45 cells were transfected with B7-H7-siRNA. Then, transcript levels of CTLA-4 and VISTA genes following the suppression of the B7-H7 gene were investigated using quantitative real-time PCR. This research demonstrated that the transcript levels of CTLA-4 and VISTA were increased after transfection of B7-H7-siRNA compared to the control cells. These experiments revealed that the knockdown of B7-H7 altered the expression of two immune checkpoints in the GC cell line.

Introduction

Gastric cancer (GC) is still considered one of the significant unmet clinical problems, and the mortality rates of this cancer are higher than its incidence.¹ For patients with GC in the later phases of disease who show lower responses for surgery, chemotherapy is used as an effective method for these patients. On the other hand, chemotherapy toxicity and drug resistance to chemotherapy have restricted its efficiency. Hence, alternative therapeutic approaches to improve GC treatment are required.² In this regard, RNA interference (RNAi), an efficient biologic method, has gained extensive attention in treating several pathologic conditions like cancer. The potential and high efficiency of RNAi shows promise in the suppression and regulation of target genes expression. The Gene therapy method has no side effects compared to chemotherapy, and reasonable pricing of this method compared to the other methods can pave the avenue toward integrating gene therapies in cancer medical care.³ One of the major types of RNAi is small interfering RNA (siRNA) that inhibits the expression of carcinogenic genes in a sequence-specific manner.⁴ Over the last few years, in the wake of the

efficient cancer therapy via immune checkpoint inhibitors, members of the B7 family have been well-studied as co-stimulatory or co-inhibitory ligands. They revealed a significant role in regulating T lymphocytes responses. Therefore, the B7 family immune checkpoints as a new therapeutic target or biomarker show an important role in human cancers and might improve the diagnosis and treatment of cancers.⁵ B7-H7, also termed B7-y and human endogenous retro virus-H long terminal repeat associating-2 (HHLA2), is a ligand for transmembrane and immunoglobulin domain-containing 2 (TMIGD2) and belongs to the immune checkpoints of the B7 family.⁶ Co-inhibitory also a co-stimulatory function of B7-H7 has been reported on T cells. The increased expression of B7-H7 on numerous tumor cells promotes tumor immune escape in breast cancer,⁶ lung cancer,⁷ colorectal cancer,⁸ renal cell carcinoma,⁹ bladder cancer,¹⁰ and GC.¹¹ Hence, targeting the B7-H7 can increase the ability of the immune system to eradicate cancer cells.¹² Besides, B7-H7 can be expressed considerably not only on monocytes and macrophages but also on B cells.¹³ It has also been noted that expression of B7-H7 is detected in the GC tissue

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compared to normal gastric tissue and high expression of B7-H7 leads to poor overall survival (OS) in GC patients. Thus, a high level of B7-H7 expression on GC tissue are considered as a significant risk factor for advanced GC.¹¹ Another member of the B7 family, cytotoxic T-lymphocyte antigen 4 (CTLA-4), known as CD152, is an immunogenic checkpoint and a co-inhibitory receptor that on the surface of antigen-presenting cells (APCs) can bind to the B7 family ligands like B7-1 (CD80) and B7-2 (CD86).¹⁴ Expression of CTLA-4 is detected on the neoplastic cells, normal cells, regulatory T cells, activated effector T-cells, and APCs.¹⁵ As a result of the CTLA-4 and its related ligands interaction, co-inhibitory signals inactivate T cells, and the immune system's response to malignant tumors is weakened. So, CTLA-4 has been efficaciously targeted for cancer therapy.¹⁶ Furthermore, V-domain immunoglobulin suppressor of T cell activation (VISTA; also termed B7-H5 and PD-1 homolog (PD-1H)), is identified as an up-and-coming B7 family immune checkpoint and an immunoglobulin membrane protein (type I) that can be a candidate for targeted therapy.¹⁷ It has been revealed that VISTA not only acts as a co-inhibitory receptor expressed on CD4+ T cells but also functions as a co-inhibitory ligand on APCs.^{18,19} Also, hematopoietic tissues and tissues that have large numbers of leukocytes can induce the expression of VISTA. Moreover, expression of VISTA is seen not only in several types of cancer but also in myeloid cells that are correlated with a decreased expression on T cells.^{20,21} Up to now, the deficiency ability of VISTA to increase the immune response as well as the suppression effect of VISTA on the immune system has been described by most studies.²² Previous studies have reported contradictory results regarding the predictive role of CTLA-4 and VISTA genes expression in GC.²³⁻²⁶ Therefore, further research is needed to identify expression patterns both of them in GC. No studies have been performed on the suppression effect of the B7 family on the other members of this family in cancer cell lines. This is the first study that examines associations between suppression of B7-H7 expression and expression of CTLA-4/VISTA in GC cells. Given the overexpression of B7H7 in GC samples and serving as a biomarker for this malignancy¹¹ as well as the tight interaction of B7H7, CTLA-4, and VISTA in cancer progression,²⁷ in the current work, we determined CTLA-4 and VISTA mRNA expression in GC cell line, as well as the effects of B7-H7 silencing via siRNA on the expression of CTLA-4 and VISTA genes, were investigated in GC cell line. A limitation of this study was that the study did not evaluate the expression levels of target genes at the protein level. However, the findings in this study might provide a new understanding of expression patterns among B7 family members in GC.

Materials and Methods

Cell culture

Fetal bovine serum (FBS) and RPMI-1640 growth

medium were obtained from GibcoBRL Company, and gastric cancer cell line (MKN-45) was received from the cell bank at the Pasteur Institute (Tehran, Iran). Also, RPMI-1640 supplemented with 10% FBS was prepared for MKN-45 cell growth, and when cell culture reached around 90% confluence, the cells were separated with 0.25 % trypsin/EDTA solution. Then, the cultured cells were maintained in humidified 5% CO₂ atmosphere at 37°C. All the analyzes were performed at the logarithmic phase of cell growth regarding the doubling time of MKN-45 cells.

B7-H7-siRNA transfection

Prior to cell seeding of MKN-45 GC cells, B7-H7-siRNA was transfected following the manufacturer's instructions using the Gene Pulser electroporation system (Bio-Rad) to the cells and they were cultured in six-well plates in 10% FBS medium at the density of 3×10^5 cells, and the group that was not transfected with B7-H7-siRNA was considered as a control group. The cells were transfected with concentration 80 pmol of B7-H7 siRNA via Gene Pulser Xcell (Bio-Rad, USA) in voltage of 130 v for 12.5 ms afterward incubation period of 48 hours. The sequence of B7-H7 siRNA is reported in Table 1.

RNA extraction and real-time PCR

In order to assessment of CTLA-4 and VISTA transcription, the whole RNA was initially isolated from cells by Trizol reagent (GeneAll Biotechnology, Korea) according to the manufacturer's manuals. To synthesis of cDNA, the reverse transcription reactions were done with oligo (dT) primer and random hexamer primer according to the manufacturer's protocol using 1000 ng of total RNA from each sample. Then, a standard SYBR Green PCR Master Mix (BioFact) was used to carry out qRT-PCR in the Roche LightCycler 96 system (Roche, Germany). It is to note that the PCR conditions for the reaction were as follows: 5 μ L of SYBR green reagent, 3 μ L of nuclease-free distilled water, 1 μ L of each primer, and 1 μ L of cDNA. The complete qRT-PCR for this study was as follows: 95°C for 15 minutes as enzyme activation phase, 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 20 seconds (45 cycles). GAPDH was used as a control gene to assess mRNA expression of genes, and the primer sequences of B7-H7, CTLA-4, and VISTA genes are presented in Table 2. Also, all reactions were analyzed in triplicate, and to evaluate the expression levels of a target gene, the $2^{-\Delta\Delta Ct}$ method was used. Table 2 presents the oligonucleotide sequences.

Statistical analysis

Statistical analysis of results was done via GraphPad Prism 8.0.0 software (San Diego, CA, USA). *P* values below 0.05 were considered to show a statistically significant difference, and all data were expressed as mean \pm SD.

Table 1. Sequence of B7-H7-siRNA

siRNA	Sense	Antisense
B7-H7	GCCAAGAAACAGCUUCCAUAdTdT	UAUGGGAAGCUGUUUCUUGGcdTdT

Table 2. Primer sequences in qRT-PCR

Target Gene	Forward Primer	Reverse Primer
B7-H7	CTGAAGCAAACATGGACAGG	GTTGACATGAGAGTGAAACG
CTLA-4	CATGATGGGGAATGAGTTGACC	TCAGTCCCTGGATAGTGAGGTTT
VISTA	GCGGATGGACAGCAACATT	TTGGAGAGTCAGGGACAGGG
GAPDH	AACATCATCCCTGCCTCTAC	CTGCTTACCACCTTCTTG

Results

Upregulation of CTLA-4 expression following B7-H7-siRNA knockdown

The expression level of CTLA-4 in MKN-45 GC cells before and after transfection with 80 pmol of B7-H7 siRNA was assessed via qRT-PCR assay (Figure 1). Our results revealed that the expression of CTLA-4 mRNA in the MKN-45 GC cell line was low. In addition, when the MKN-45 GC cell line was transfected with B7-H7-siRNA, the cells underwent significantly high expression of CTLA-4 as compared to the control group ($***P < 0.001$).

Upregulation of VISTA expression following B7-H7-siRNA knockdown

In order to evaluate the suppression effect of B7-H7 on VISTA expression, we investigated its expression before and after B7-H7-siRNA knockdown via the qRT-PCR test in the MKN-45 GC cell line. We achieved that the mRNA expression of VISTA significantly ($****P < 0.0001$) was up-regulated in MKN-45 cells after transfected with B7-H7-siRNA in comparison with the control group (Figure 2).

Discussion

Currently, targeted molecular therapy is a developing novel technology that is used for cancer therapy, such as GC. Targeted treatment is considered as one common method of gene therapy. Indeed, the target is a particular gene that is induced or inhibited in different types of cancer.²⁸ In this regard, RNAi technologies like siRNA as a promising gene therapy method provide high target specificity via gene sequences. They can inhibit the expression of genes through matching with targeted genes involved in metastasis, growth, and formation of the various types of tumors.² In the previous decade, targeting immune checkpoint members of the B7 family have been tested and developed in solid tumors due to the relationship between cancer and the immune system. Also, considering the significance of the expression pattern of the B7 family in cancerous tissues, this family may be regarded as a significant indicator to predict the OS in patients with cancer. It is presented that B7 family members, through the interaction with related receptors, can deliver negative and positive signals to inhibit and support T lymphocytes responses, respectively.^{5,29} Eleven members of the B7 family have been identified, and three of them, B7-H7,

CTLA4, and VISTA, are our target genes in this study. Most interactions of these immune checkpoints occur during the regulation of T cell activity especially, CTLA-4 and B7H7 and in some cases VISTA.³⁰ Based on a previous study, B7-H7 expression reduces OS in GC patients because of its high expression in these patients.¹¹ Also, it was reported that expression of B7 family members like B7-H7, VISTA, B7-H3, B7-H4, B7-H6 was considerably increased, but the expression of B7-1, B7-2, PD-L1, and ICOS-L as B7 family members were significantly reduced in patients with GC.³¹ Also, evidence from Yang et al revealed that high levels of CTLA-4 protein and corresponding ligands expression increase angiogenesis of tumor in GC via activation of VEGF-A expression.²³ Besides, Pereira and coworkers demonstrated that expression of CTLA-4 at high levels in GC patients is correlated with tumor-infiltrating CD8+ T lymphocytes and better survival following curative resected in GC.³² According to a study by Hu and colleagues, high levels of VISTA expression are detected in the cytoplasm and nucleus of GC patients, and overexpression of VISTA correlated with poor OS in patients with GC.²⁵ On the other hand, in contrast to the

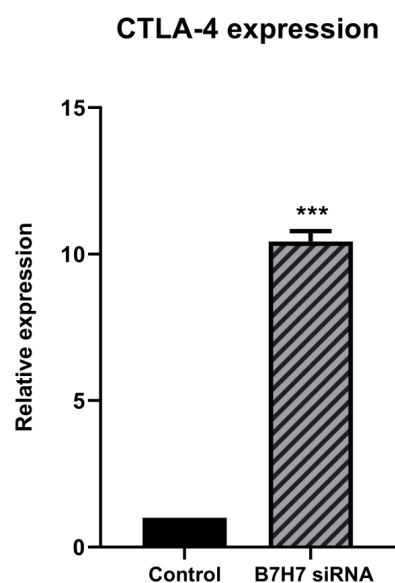


Figure 1. Effect of B7-H7 suppression on the expression of CTLA-4 in MKN-45 GC cell line. To determine the effect of B7-H7-siRNA knockdown on the expression of this gene, we assessed the level of CTLA-4 expression via RT-PCR assay. *** shows a significant increase in CTLA-4 level in MKN-45 GC cells after transfection with B7-H7-siRNA (80 pmol dose) compared to the control group cells.

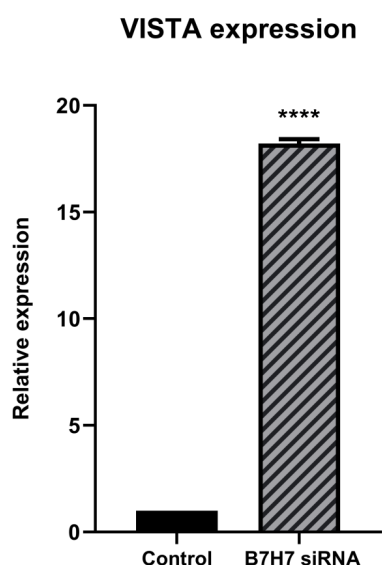


Figure 2. Silencing effect of B7-H7-siRNA on VISTA expression in MKN-45 GC cells. As shown in this figure, the qRT-PCR analysis showed that the knockdown of B7-H7 expression significantly promoted the VISTA mRNA level in MKN-45 GC cells as compared to control cells (**** $P < 0.0001$).

above results, Oliveira et al demonstrated that expression of VISTA in GC cells is remarkably lower than normal gastric tissue.²⁶ The present study sought to determine the expression levels of CTLA-4 and VISTA in GC cells; also, the effect of B7-H7-siRNA transfection on the expression of CTLA-4 and VISTA genes in MKN-45 GC cell line were analyzed. Other studies have been conducted regarding targeting checkpoints and their impacts on the expression of other immune checkpoints. In this regard, the result of a study demonstrated that suppression of the A2a receptor as a significant immune checkpoint via CPI-444 resulted in checkpoint pathways like LAG-3 and PD-1 being downregulated on regulatory T cells (Treg cells) and CD8+T lymphocytes in tumor-bearing mice. Moreover, it has been proved that immunotherapy via anti-PD-1 is improved following the suppression of the A2a receptor by CPI-444. Also, the outcome of the suppression of both PD-1 and A2a receptors using siRNA-loaded nanoparticles in tumor experimental models increased the potential of cancer immunotherapy.^{33,34} Besides, it is revealed that CTLA-4 suppression alongside inhibition of A2a receptor via siRNA-loaded polyethylene glycol-chitosan-alginate nanoparticles cooperatively improves anti-tumor immunity in tumor experimental models.³⁵ In this work, we assessed the B7-H7-siRNA knockdown impact on the expression of both B7 family members, CTLA-4 and VISTA. The results of the qRT-PCR assay showed that expression levels of CTLA-4 and VISTA were significantly increased following transfection of B7-H7-siRNA in the MKN-45 GC cell. The obtained results address us to this supposition that low mRNA levels of two genes belonging to the B7 family (CTLA-4 and VISTA) in the GC cell line might be related to the expression of B7-H7 in this cancer due to induction of CTLA-4 and VISTA expression after B7-H7 silencing in GC cell line. A key strength of the present study was that this is the first study

to identify the suppression effect of B7 family members on the expression of other members of this family like CTLA-4 and VISTA in MKN-45 GC cells. The main weakness of our study was that we examined expression levels of the aforementioned genes only at the mRNA level and not at the protein level.

Conclusion

In conclusion, these results strengthen the supposition that upregulation and downregulation of VISTA and CTLA-4 expression in MKN-45 GC cell line may be affected by expression of the B7-H7 gene. The results of this investigation showed that suppression of B7-H7 gene expression led to upregulation of CTLA-4 and VISTA expression in MKN-45 GC cells. The present study will serve as a base for future studies, and it is suggested that the association of elevated CTLA-4 and VISTA genes expression following the suppression of the B7-H7 gene in other GC cell lines is investigated in future studies.

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Authors contribution

NB: Data collection, literature review, preparing manuscript. MHKA: Co-corresponding author, Data visualizing. YR: Statistical analyses, conception analyses. BB: Corresponding author, preparing manuscript.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research.

Conflict of Interest

The authors certify that there is no potential conflict of interest in relation to this article.

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