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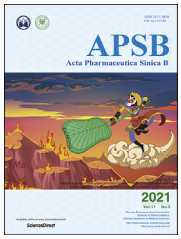

REVIEW

Immune checkpoints in targeted-immunotherapy of pancreatic cancer: New hope for clinical development

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Abstract Immunotherapy has been recently considered as a promising alternative for cancer treatment. Indeed, targeting of immune checkpoint (ICP) strategies have shown significant success in human malignancies. However, despite remarkable success of cancer immunotherapy in pancreatic cancer (PCa), many of the developed immunotherapy methods show poor therapeutic outcomes in PCa with no or few effective treatment options thus far. In this process, immunosuppression in the tumor microenvironment (TME) is found to be the main obstacle to the effectiveness of antitumor immune response induced by an immunotherapy method. In this paper, the latest findings on the ICPs, which mediate immunosuppression in the TME have been reviewed. In addition, different approaches for targeting ICPs in the TME

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of PCa have been discussed. This review has also synopsized the cutting-edge advances in the latest studies to clinical applications of ICP-targeted therapy in PCa.

1. Introduction

In recent years, researchers have highlighted the importance of pancreatic cancer (PCa) in its severity and generalizability. In the past few decades, the concept of PCa has been faced with many arguments in many aspects, however the general view toward the PCa has been kept consistent, and PCa is still one of the most fatal cancers in the world. Accordingly, PCa is defined as the disease in which malignant cancer cells appear in the tissues of the pancreas[1]. Pancreatic tumors are classified into exocrine or neuroendocrine based on the cell from which they originate. This classification is critical as it provides distinct functional characteristics and treatment strategies between these two types. In the United States, PCa is the 9th and 10th most frequently diagnosed cancer in females and males, respectively[2]. Approximately 93% of PCa patients are exocrine tumors. According to the American Cancer Society, PCa patients account for approximately 3% of all adult cancer cases in the United States, with only about 22% of exocrine PCa patients still living one year after surgery. Above 56,000 Americans are expected to be diagnosed with PCa in 2019, with an average of above 150 diagnoses per day[3]. Recently, significant developments have been made in cognizing the molecular biology, diagnosis, staging, and treatment of PCa in patients[4]. As a turning point, cancer immunotherapy has emerged through monoclonal antibodies (mAbs) that obstructs inhibitory receptors on immune-effector cells or their ligands on tumor cells and antigen-presenting cells (APCs) alleged 'immune checkpoints' (ICPs). The main ICPs that are expressed on immune cells are programmed death 1 (PD-1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3). The ligands of PD-1 (PD-L1 and PD-L2), CTLA-4 (CD80 and CD86), and LAG-3 (MHC-II) could bind to ICPs and trigger immunosuppression in the tumor microenvironment (TME)[5]. This review is an attempt to focus on recent advances as well as an outlook on targeting ICPs for attenuation or elimination of PCa cells.

ICPs are regulatory molecules that maintain immune homeostasis; however, they are overexpressed to suppress the antitumoral immune response in the TME[6]. The immune system plays a critical role in the elimination of tumor cells and fight against cancer. It was demonstrated that the immune system unremittingly checks cells recognize any inappropriate and foreign antigens such as tumor antigens. This process of inspection of the immune system over the cells is called immune surveillance[7]. One of the strategies used by tumors such as pancreatic ductal adenocarcinoma (PDA) is to bypass the immune surveillance by the misusage of ICPs to escape immune recognition[8]. It is in view of this particular phenomenon that immunotherapy against ICPs is expounded as a powerful strategy in the field of anti-cancer therapy[9], in which mAbs against PD-L1 and CTLA-4 were established to be effective[9–11]. An immunosuppressive hallmark of elevated expression of the ICP is the reduction of T cells activity[12]. B7-H1 or PD-L1 (CD274), as well as B7-DC and PD-L2 (CD273), are ligands of PD-1 (CD279). PD-L1, PD-L2, and PD-1 are transmembrane glycoprotein type I belong to the immunoglobulin (Ig) superfamily B7 and CD28, respectively[13]. The interaction between PD-1 on T cells and PD-L1 on APCs of the TME and PCa cells promotes the suppression and exhaustion of the T cells. Exhausted T cells have a significant role in leading to a defective T cell reaction which weakens the tumor-specific responses[14]. In PDA, CTLA-4, PD-1, and PD-L1 are three major inhibitory checkpoints which are expressed in the TME[15]. CTLA-4 (CD152) is a member of the Ig superfamily that binds to CD28 on T cells and transmits an inhibitory signal to T cells[16]. CTLA-4 has been first demonstrated as an inhibitory ICP molecule, which can suppress T cells, as well as autoreactive T cells, ordinary in lymph nodes[16–18]. Despite the mode of action of CTLA-4, PD-1 influences T cells with its inhibitory role at late stages of a T cell activity where PD-1 ligands are expressed. The expression of PD-L1 on tumor cells and also myeloid-derived suppressor cells (MDSC) in the TME could inhibit the activation of T cells[5]. A study showed that PD-L1 expression in PDA cells enhanced cancer progression[19]. In addition to the direct suppressive effects of the PD-1/PD-L1 axis on effector T cells, studies have shown the role of this pathway in the induction of regulatory T cells (T_{reg})[20]. *In vitro* studies have indicated that the presence of PD-L1 induces T_{reg} activity[21,22]. Furthermore, PD-1 inhibitors could hamper the induction of transforming growth factor-β (TGF-β) and retinoic acid (RA)-induced T_{reg}[23]. It was shown the elevated proportion of T_{reg} for T $CD8^+$ cells led to poor prognosis in cancers, suggesting that the induction of T_{reg} is one of the major approaches used by tumor cells to escape immune surveillance[24–26].

In PCa, the TME and the immune system show a vital role in tumor growth. PCa features an extremely immunosuppressive microenvironment, described by a dense desmoplastic stroma that inhibits blood flow to the area, prevents delivery of drug, and stops the antitumor immune reaction[27]. This supports cancer development and metastasis through protecting pancreatic tumors from immune surveillance. Moreover, the hypoxic milieu, acidic extracellular pH, and high interstitial fluid pressure in the TME also have a role in augmenting tumorigenesis[28]. Furthermore, the PDA microenvironment is generally composed of T_{reg}, MQs and MDSCs, which stop the anti-tumoral function of effector $CD4^+$ and $CD8^+$ T cells. T_{reg} has been shown to play critical role in PDA tumor development[29]. Tumor-associated macrophages (TAMs) are also responsible for inflammation, development and metastasis of PCa[30]. Upregulation of negative T cell co-stimulatory molecules is another reason of stimulation of PDA immunosuppression[31]. PD-L1 and PD-L2 are overexpressed in PDA patients and correlate with decreased tumor-infiltrating leukocytes (TILs) and a poor prognosis. Therefore, PD-L1 downregulation prevents cell proliferation in PCa[32,33]. In addition, an augmented expression of

inhibitory molecules on inactivated T cells has been presented as another strategy to stimulate immunosuppression of PCa[34].

High $CD8^+$ TIL correlates with a better prognosis due to their cytotoxic functions in several types of solid tumors. The acquisition of effector T cells ($CD4^+$ and $CD8^+$) in human PCa can improve overall survival (OS). Likewise, $CD8^+$ effector T cells reduce whereas suppressive T_{reg} contain a higher CD4 T cell level, which leads to a low number of TIL and a high number of immunosuppressive cells[35]. PCa is poorly immune-responsive, mainly due to its complex and suppressive TME. Therefore, PCa requires combinational therapy to provoke an immune response, for instance by employing vaccines to enhance the accumulation of lymphoid aggregates[36]. Thus, TME, on account of high levels of immunosuppression and poor immunogenicity, provides a unique challenge in PCa immunotherapy[36]. The significant milestones for the expression profile of immune cell and ICPs in the TME are pointed out in Fig. 1.

2. Immune checkpoint pathways

2.1. CTLA-4 pathway

Activation of T cells is a complex process and requires more than one activating signal. Binding T cell receptor (TCR) to major histocompatibility complex (MHC) is vital for T cell activation, and other required costimulatory signals. In order to activate the stimulatory signal in T cell, B7-2 (CD86) or B7-1 (CD80) molecules bind to CD28 molecules on the APCs. Adequate concentrations of CD28 for binding to B7 1/2 (CD80/CD86) leads to generation of T cell with improved endurance, differentiation in the enhanced cytokines structure like interleukin-2 (IL-2), adjustment of durability of cell for genes and improved energy metabolism. CTLA-4 delivers a negative regulatory signal to the T cell through binding to B7 1/2 molecules. CTLA-4, a CD28 homologue, shows higher binding affinity to B7[37,38]; however, the binding of CTLA-4 to B7 withholds a stimulating signal through CD28 reverse. The competition binding can block the stimulatory signal generated by CD28−B7 binding[38–40]. Furthermore, the corresponding quantity of CD28−B7 *versus* CTLA-4−B7 restricts

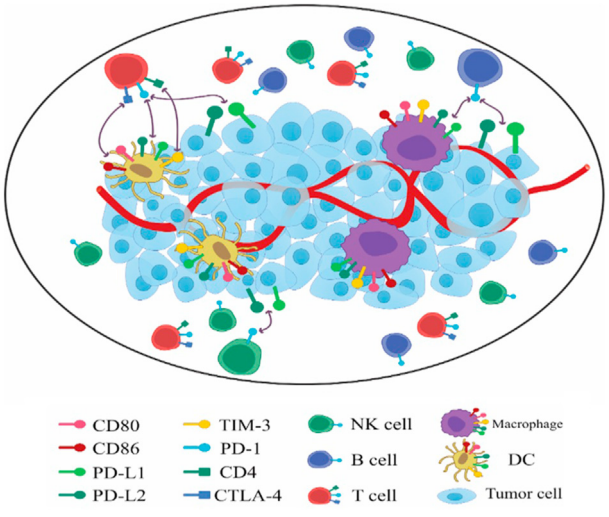

Figure 1 The expression profile of immune cells and ICPs in the TME.

the activation or energy of a T cell[16]. In addition, some data suggest that CTLA-4−B7 can truly provide inhibitory signals which prevent CD28−B7 and TCR:MHC binding stimulating signals[41,42]. CTLA-4 location within the cell is under a controlled mechanism and initially located in the intracellular segment of resting naive T cells[40,41,43]. Consequently, the stimulatory signals, including both TCR and CD28−B7 binding, trigger upregulation of CTLA-4 through CTLA-4-included vesicles exocytosis on the cell surface[44], which explains how TCR signals extract high translocation of CTLA-4 in a categorized feedback loop to the cell surface. Whereas, inhibition of IL-2 production as well as stimulation of the cell cycle progression has limited CTLA-4−B7 binding to fully activate of T cells[45]. T_{reg}s regulate the effector T cells' activity and are indispensable to maintain peripheral tolerance[46,47]. T_{reg}s constitutively express CTLA-4 unlike effector T cell, thus, this is deemed crucial for their suppressive capabilities[46]. In the animals, the absence of CTLA-4 on T_{reg} impaired their suppressive ability[46,48]. The downregulation of CD80 and CD86 on APCs is one of T_{reg}'s mechanisms to regulate the function of effector T cells[48,49].

2.2. PD-1 pathway

PD-1 as a part of the B7/CD28 costimulatory family regulates T cell activation by binding to their ligands, PD-L1 and PD-L2. Unlike CTLA-4, binding to PD-1 blocks the generation of stimulating cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-2, which lead to decrease in the activity of T cells[50]. Where T cell activation coincides with TCR−PD-1 binding, the phosphorylation of intermediate TCR signals is inhibited by PD-1 generated signals, which with terminating TCR signals result in decrease T cell activation[41,51]. Expression of PD-1 indicates "exhausted" T cells that practice extreme rates of stimulation or reduced $CD4^+$ T cell support[52]. Dysfunction of T cell which is happening in the suboptimal administration of tumors and some diseases, identifies this stage of fatigue that occurs when chronic infections and cancer occur. Both CTLA-4 and PD-1 receptors have proven negative effects on T cell activation; however, the timing of downregulation, efficiency of negative signaling pathway and the anatomical regions of inhibition are different[17,50]. While CTLA-4 utilizes T cell activation in the priming state, PD-1 operates at the point of the effector, predominantly within peripheral tissues[50]. For CTLA-4, the combination of PD-1 ligands varies too. The B7 ligands for CTLA-4 are expressed by special APCs that usually stay in the lymph nodes or spleen, although PD-1 and PD-L2 are expressed in T cell and, APC and more widely cancer cells, respectively[17,39,53,54]. PD-L1 is expressed on leukocytes, non-hematopoietic cells, non-lymphoid tissues as well as in parenchymal cells through tumorigenic signals or inflammatory cytokines[55]. Expression of PD-L1 is differently identified in several tumor types and is incorporated with the increase and decrease of the amount of TILs and prognosis, respectively[56–58]. The expression of PD-L2 is considerable in DCs and monocytes and can be triggered depending on the microenvironment by a broad range of particular immune and non-immune cells[59]. Binding affinity of PD-1 for PD-L2 compared to PD-L1 showed unique tendency, which can be efficient for the differential immune response involvement of the ligands[60]. The interactions of PD-1 with PD-L1 and PD-L2 are developed to make tolerance within infiltrated tissue around due to PD-1 ligands are expressed in peripheral tissues. One type of contradictory roles of PD-L1 and

PD-L2 on natural killer (NK) T cells activation includes improved Th2 activity through restriction of PD-L2 binding points, although CD80 binding to PD-L1 was provided to hinder T cell responses[61–63]. The multiple biologic effects lead to differences in the toxicity and activity for PD-1-directed antibodies which prevent to bind both ligands as compared to interaction of PD-1 with PD-L1. Despite T_{reg} expression on both PD-1 and CTLA-4, the purpose of PD-1 expression remains dubious. PD-L1 not only provides T_{reg} with the exchange of naive $CD4^+$ T cells, but also prevents T cell responses by enhancing T_{reg} induction and maintenance[22]. Similar to these results, the blockade of PD-1 is capable of inverting the T_{reg}-mediated defeat of the effector T cells *in vivo*[64]. The immune response is reduced by PD-1 binding to its ligands on T cells, which are busied on the effector T cell responses. This equal restriction of T cell activation, which is linked to CTLA-4 blockade, may describe immune related adverse events through the potentially inferior occurrence of PD-1, equivalent to a CTLA-4 blockade[52,65]. Comparisons between CTLA-4 and PD-1 demonstrate that they are both B7 receptor family components[66], expressed by activated T cells[44,50], expression affected by the strength and/or continuity of TCR signaling[50,52,67], decreased T cell proliferation, glucose metabolism, cytokine manufacturing, durability and also arranging an extended T cell proliferation[17,39,50].

However, there are such differences include CTLA-4 expressed through T cells, whereas PD-1 expressed through T cells and other immune cells and further CTLA-4 first restricts T cell responses, originally on lymphoid tissues, whereas PD-1 limits T cell response anywhere usually. In peripheral tissues, PD-1 action clashes with more T cell signaling mechanisms than CTLA-4 ligands expressed by professional antigen-presenting neurons. PD-L1/2 expressed by APCs and other immune cells mostly, although the ligands can be expressed on non-immune cells such tumor cells. On the other hand, CTLA-4 inducing T_{reg} functioning, thus, the function of PD-1 on T_{reg} is uncertain[17,39,48,50].

3. Potential predictive biomarkers

3.1. CTLA-4

The structural similarity of CTLA-4 to CD28, let this inhibitory checkpoint the ability to interact with CD80 and CD86 but in a stronger affinity than CD28[68]. CTLA-4 can mediate the suppression of T cells through several mechanisms. First, CTLA-4 plays an efficient role to reduce CD28 co-stimulation[16,69], and its interaction with CD80/86 of APCs can decrease APCs stimulatory role in the T cells activation[70]. Second, expression of CTLA-4 on T_{reg} which gives them the ability to inhibit T cells function[71]. Third, inhibition of TCR genes and CD28-induced genes[72,73]. Fourth, the expression of CTLA-4 in dendritic cells (DCs) that inhibits T cells[18].

In the case of PDA, it was shown that anti-CTLA-4 approach did not provide an optimal clinical benefit and anti-tumor function neither in human nor in cancer models[74,75]. However, a study stated that there was an induction of the infiltration of $CD4^+$ T cells in the TME, as a result of anti-CTLA-4 approach, which has no remarkable changes in the migration of $CD8^+$ T cells[75]. This is in total contrast with the approach of anti-CTLA-4 therapy for melanoma patients, which showed a significant increase in infiltration of $CD8^+$ T cells[76]. Thus, it can be suggested that CTLA-4 implicates the recruitment of $CD4^+$ T cell in PDA patients. Like these findings, Bengsch et al.[75] exhibited that anti-CTLA-4 therapy promotes T cell infiltration into the TME as well as T cell activation. However, this approach was not clinically useful in PDA patients. The interesting observation was the influence of CD80 inhibition in the induction of T cell recruitment into the TME. Consequently, the interaction of CTLA-4 and costimulatory molecules (CD80/86) can regulate the migration of T cells to the TME[75]. Basso et al.[77] demonstrated that despite expression of PD-L1 and CTLA-4 in splenic DCs, no PDA patients showed increased levels of both PD-L1 and CTLA-4 expressions. They observed that, for the first time, the S100A8/A9, also known as calprotectin, could downregulate CTLA-4. Intriguingly, CTLA-4 negative DCs diminished T cell proliferation. DCs were also shown to augment the inhibitory role of T_{reg} in the inhibition of allogeneic T cell responses through their CD80[78]. This is while CD86 showed opposing function on the T_{reg} activity[78].

3.2. PD-1

Activated monocytes, DC, NK, T and B cells express another important ICP called PD-1[79]. Like to CTLA-4, the responsibility of PD-1 is to regulate T cell responses[80]. Domain-containing phosphatase-1 and 2 (SHP-1&2) affiliated with immunoreceptor tyrosine-based switch motif (ITSM) of PD-1 for stimulation human T cell, whereas ligation of PD-1 inhibit T cell activation[81,82]. It was shown that CTLA-4 acts as an inhibitory molecule in the primary phases of activation of T cells[83] while the interaction of PD-1 and its ligands mainly happens on activated effector T cells in the periphery[5]. In the TME of PDA, the PD-1 expression has predominantly happened in TILs, which leads to immune escape by tumor cells[13,84]. However, recent research presented PD-1 expression in PDA tumor cells[85]. It was demonstrated that the amount of PD-1, which was expressed on $CD8^+$ T cells elevated in tumor tissue where this expression was significantly correlated with the clinical stage of PDA patients. Indeed, higher expression of PD-1 in PDA tumor tissue was considered as a poor prognostic factor[86]. However, another investigation disclosed elevated stromal PD-1+TILs as a decisive prognostic factor. In fact, they suggested a correlation between intraepithelial and peripheral compartment of PD-1+TILs infiltration with better progression-free survival (PFS) and distant metastases-free survival (DMFS)[87]. Nevertheless, investigations on a diversity of cancers such as renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), and melanoma demonstrated an enhanced PD-1 expression, which was affiliated with disease with poor prognosis[88–90]. Thus, there is a controversy about the role of PD-1 as a satisfactory target for the targeted ICP therapy[87]. It was demonstrated that PDA patients with elevated PD-1 expression and dense stroma were associated with better OS[91]. Interestingly, the stroma of PDA, is observed to have a role in the upsurge of immune cell migration and accumulation, which led to a boosted inflammatory response[92]. The expression of PD-1 in peripheral blood stays stable even after surgical treatment and this stable PD-1 level of peripheral $CD8^+$ T cells was shown to be associated with higher PDA recurrence[86].

3.3. PD-L1

As conferred, PD-L1 is one of the inhibitory molecules expressed in solid tumors, DCs and MQs of the TME[93,94]. It was shown that PD-L1 from T $\gamma\delta$ cells of PDA could inhibit tumor-specific cytotoxic T lymphocytes and Th1 cells[95]. Interesting

data revealed that not all but some the PD-L1 producing cells have the ability to suppress T cells *via* binding to PD-1[14]. In general, the B7 family consists of PD-L1 (B7-H1) and PD-L2 (B7-H2)[93]. Although there is still a need for further studies to understand the differences between the practical function of PD-L1 and PD-L2[14], it was shown that PD-L1 is the primary molecule that is presented in solid tumors[13]. PD-L1 can bind to PD-1, CD80 of T cells, and APCs[63]. This binding leads to apoptosis, energy exhaustion, and finally inhibition of effector T cells[13,96,97]. In the case of PDA, PD-L1 was demonstrated to have the ability to upregulate T_{reg} infiltration and to induce immune suppression[20]. The expression of PD-L1 in PDA was shown to be associated with tumor growth, drug resistance, and finally to high tumor invasion[98]. Another study in human PDA showed that PD-L1 and PD-L2 were expressed in cancerous pancreatic tissue. There was also a negative correlation between PD-L1 expression and $CD8^+$ T cells. Furthermore, 20 out of 51 PDA patients who were PD-L1 positive showed poor prognosis[99]. The inhibition of blocking colony-stimulating factor 1 (CSF-1) and its receptor resulted in an increase in $CD4^+/CD8^+$ T cells infiltration and the expression of PD-L1[100]. Similarly, high expression of HLA class I with PD-L1−PDA was indicated to be associated with high $CD8^+$ T cells infiltration and good prognosis. It was suggested that the presence of HLA-I probability possess the potential to promote immunostimulatory condition, whereas PD-L1 could induce the immunosuppressive one. Thus, the immunological response of PDA pertains to the balance of the TME between the two discussed conditions[101]. Intriguingly, there was an association between the expression of PD-L1, low tumor differentiation, and diminished advanced tumor stage[102]. However, PD-L1 expression correlates with the existence of immunosuppressive cells[101].

It was shown that the remarkable function of PD-L1 happened in the early stages of the disease[99], while it was previously shown that both PD-L1 and PD-L2 had their strong effect in the advanced stages of esophageal cancer. This is while a correlation exists between the expression of PD-L1 at high levels in PCa and the metastasis of lymph node[103]. In another study, researchers reported that there might be an association between miss match repair (MMR) system deficient-cancer and the anti-PD-L1 treatment. This is a result of the fact that this deficiency promotes the production of multiple neo-antigens, which are further targeted by the augmented immune system[91].

4. Targeting immune checkpoints

Tumor cells can grow fast and spread in part by targeting the immune system of the host. During the past decade, immunotherapy has emerged as an effective and standard method of treating different cancers[104]. Even though, different treatment strategies, such as surgery and conventional chemotherapies, have prolonged patient survival and they do not inhibit restorative. Consequently, new treatment strategies are indispensable[105,106]. Over the past decade, cancer immunotherapy has paved the path from a promising preclinical use to a clinical reality[107]. Manipulation of ICPs is one of the most current promising strategies for cancer therapy[35], therefore clinical studies confirm that inhibition of ICPs disarranges adverse immune regulations and induces immune system as well as antitumor activities[108]. For this purpose, mAbs target inhibitory ICPs and show significant results in different cancers[109]. Since they are accepted by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), ICP blockade drugs have been developed for application to treat different cancers[109−114]. Accordingly, it could also be a highly effective therapy against PCa[15]. In this part, the latest advances and future challenges of ICP inhibitors application in PCa are being summarized. Pivotal ICPs in PCa and their interplay with their specific ligands have been shown in Fig. 2.

4.1. *CTLA-4 immune checkpoint blockade*

Recently, CTLA-4, the first clinically targeted ICP receptor, has been studied extensively in cancer immunotherapy[11,39,115]. According to studies conducted in the late 90s, the opposing effect of CD28 and CTLA-4 had demonstrated on T cell activation *in vitro*, whereas blocking CD28 and CTLA-4 have declined and intensified anti-tumor responses, respectively[116]. Indeed, monotherapy-based mice treated with anti-CTLA-4 antibodies led to the complete tumor elimination and durable immunity. Subsequently, mechanistic studies showed that this anti-tumor function was related to the augmented ratio of both CD4 and CD8 effector cells to $FoxP3^+$ (forkhead box P3) T_{reg}[117]. Ipilimumab (Ipi) and tremelimumab (Tre), two human anti-CTLA-4-antibodies, have been applied clinically in PCa.

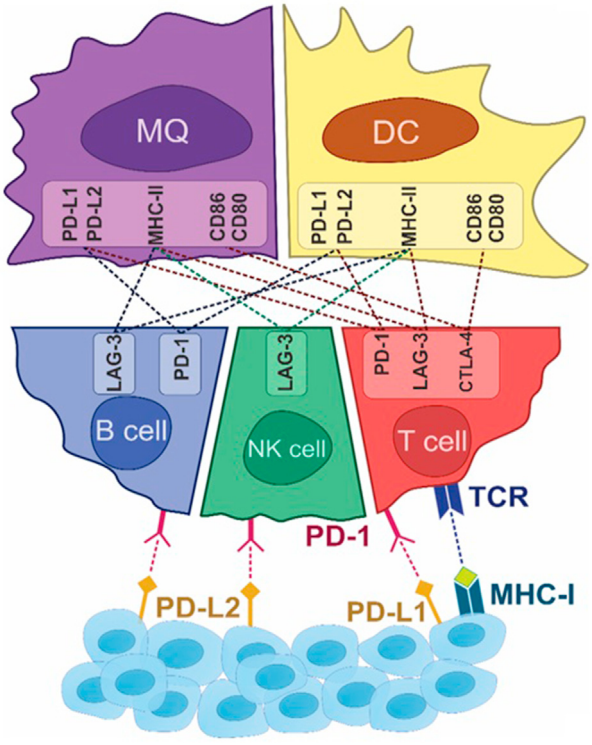

Figure 2 Pivotal ICPs in PCa and their interaction with specific ligand. The interaction of negative ICPs, including PD-1, CTLA-4, LAG-3, with their specific ligand were shown in detail. Interplay of PD-L2 on the TME with B cells and NK cells and PD-L1 and CTLA-4 on the TME with T cell through their specific ligand activates the immunosuppression and causes tumor immune evasion. Overexpressed LAG-3 on all of B cells, NK cells and T cell deactivates DC and MQ functions which result in tumor-induced immunosuppression.

4.1.1. *Ipilimumab*

Ipi, a fully humanized IgG1 kappa mAb, was the first drug developed against CTLA-4[118]. In 2011, Ipi was approved by the FDA to treat melanoma and is now under clinical evaluation in various tumors[119]. Royal et al.[120] investigated the effect of Ipi in the regression of advanced pancreatic adenocarcinoma (APA) during the phase II study. Twenty-seven people with APA received 3.0 mg intravenous Ipi, following which response evaluation criteria in solid tumors (RECIST) and toxicity were measured. This study found that the above mentioned dose-scheme of Ipi is not effective in PCa and suggested the use of higher dosages of Ipi at earlier stages of the disease, possibly with combinatorial agents[120]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) of cell-based vaccines have been discovered to provide a synergistic activity to anti-CTLA-4 antibodies. Therefore, in a phase Ib of a study, Ipi has effectively been combined with GM-CSF cell-based vaccines GVAX (a cancer vaccine containing whole tumor cells which modified to secrete the immune stimulatory agents). In this study, thirty patients with PCa were listed and Ipi (arm 1) and Ipi + GVAX (arm 2) were examined[121]. Following the administration, fifth patients indicated stable disease, and seven patients indicated CA19-9 reduction. An enhancement in OS was detected that was related to the clinical activity in the combination arm. In conclusion, this study showed that checkpoint blockade plus GVAX is a potential candidate for checkpoint immunotherapy, and requires assessment in larger studies[121]. Kamath et al.[122] tested a phase Ib clinical trial of CTLA-4 inhibition with Ipi in combination with gemcitabine (Gem) in patients with advanced PCa. This study established a maximum tolerated dose for Gem and Ipi with a good overall safety profile. Although the observed response rate was similar to Gem alone, the durability of responses suggests a component of immune activation that may warrant further investigation[122]. Moreover, Parikh et al.[123] conducted a phase II study of Ipi and Niv with radiation in metastatic PCa. Dual blockade of CTLA-4 and PD-1 with radiation is feasible and reveals hopeful action in these patients.

4.1.2. *Tremelimumab*

Tre is an anti-CTLA-4 mAb, which is assessed in melanoma, colon cancer, gastric cancer, and mesothelioma in clinical trials[124] possesses a greater affinity toward CTLA-4 than CD28. *Ex vivo* studies of patient samples with expanded and metastatic melanoma disclosed that application of Tre decreases tumor development *via* considerable stimulation of cytotoxic T cell activity[125]. A phase I study in PCa patients, analyzed the tolerability, safety, and maximum tolerated dose of Tre plus Gem. This combination indicated a manageable safety and tolerability profile and prolonged OS[126]. A recent study investigated durvalumab (Dur) with or without Tre for patients with metastatic PDA. Treatment was well tolerated, and the efficiency of Dur plus Tre therapy and Dur monotherapy presented a population of patients with mPDAC who had poor prognoses and quickly developing disease[127]. Moreover, a phase II study is testing Dur alone or Dur plus Tre in metastatic PCa[128]. Furthermore, a study of hypofractionated radiotherapy in combination with Dur and Tre in metastatic PCa patients is underway[129].

4.2. *PD-1/PD-L1 immune checkpoint blockade*

The second ICP receptor is PD-1, which appeared as a marker for successful cancer immunotherapy[39]. It interacts with the PD-L1 and PD-L2 ligands and stops T cell induction[130]. PD-1/PD-L1 axis enables tumor evasion from the immune responses, and this interaction blockade with anti-PD-1 as well as anti-PD-L1 can augment the tumor immunity. Moreover, the proliferation of $CD8^+$ T cell and production of cytokine can be induced by this blockade[131]. Numerous PCa-related anti-PD-1 and anti-PD-L1 antibodies have been assessed in clinical trials. Nivolumab (Niv), pembrolizumab (Pem), and pidilizumab (Pid) are the antibodies that block PD-1. On the other hand, Dur and BMS-936559 are PD-L1 targeted antibodies[132].

4.2.1. *Pembrolizumab*

Pem (MK-3475) is a mAb of the IgG4κ isotype considered to stop PD-1 and PD-L1/2 interaction[133]. Recently, a phase I/IIa study is analyzing the effect of colony-stimulating factor 1 receptor (CSF1R) inhibitor (pexidartinib/PLX3397) and Pem combination in PDA[134]. NCT02305186, another phase Ib/II study, assesses safety and immunological effect of chemoradiation therapy (CRT) plus Pem and CRT monotherapy in PCa patients[135]. Also, NCT02362048 is ongoing to investigate the Bruton tyrosine kinase (BTK) inhibitor, acalabrutinib, alone or with Pem for metastatic PCa[136]. Furthermore, Mahalingam et al.[137] reported that reolysin (pelareorep), in combination with chemotherapy and Pem, can be clinically successful and demonstrated convenient safety profiles and anti-tumor function in metastatic PCa patients. Furthermore, a study investigated Pem in combination with the oncolytic virus pelareorep and chemotherapy in patients with advanced PCa. Pelareorep in combination with chemotherapy and Pem in these patients was well-tolerated and presented durable efficacy. Further examination of pelareorep and anti-PD-1 therapy is ongoing in follow-up studies[138]. A phase II study of PEGPH20 in combination with Pem for patients with hyaluronan (HA)-high refractory metastatic PCa is ongoing. This research will examine the efficacy, safety and translational biomarkers of PEGPH20 plus Pem in these patients[139]. Moreover, Halama et al.[140] tested pharmacodynamic impact and safety of monotherapy with CXCL12 inhibitor NOX-A12 plus Pem in PCa patients. These patients with impaired immune systems and a high tumor load had several unsuccessful prior lines of treatment. NOX-A12 plus Pem showed induction of immune response, stabilize disease in 25% of patients, and prolonged time on treatment *versus* prior therapy for 35% of patients[140].

4.2.2. *Nivolumab*

Niv is another therapeutic IgG4 antibody against PD-1 for improving anti-tumor responses. *In vitro* assays showed that Niv enhances T cell induction and cytokine production. Similar to Pem, Niv stops PD-1 and its ligands interaction[141]. To evaluate the effect of CY/GVAX and CRS-207 alone or in combination with Niv, a phase II study is being directed in PDA patients who have unsuccessful chemotherapy for metastatic disease[142]. Correspondingly, an ongoing study by Firdaus et al.[143] is studying nab-paclitaxel and Niv plus Gem in advanced PCa patients. Presently, Niv is being tested for safety and efficacy in phases I and II trials for resectable (NCT02451982) and metastatic (NCT02423954) PDA[144]. Wang-Gillam et al.[145] are testing the effect of cabiralizumab (Cab) given with Niv with and without chemotherapy in PCa patients. Also, a recent research is evaluating SD-101 (toll-like receptor 9 agonist), Niv, and radiation therapy in treating patients with chemotherapy-refractory metastatic PCa[146].

4.2.3. *Pidilizumab*

In recent years, Pid as another anti-PD-1 ICP inhibitor has received consideration. It is a humanized IgG4κ mAb. In PDA, two clinical trials, were administered imperfectly, and stopped thenceforth[147,148].

4.2.4. *Durvalumab*

Dur is also another mAb of the IgG4κ isotype which targets PD-L1, stops the binding of PD-L1 to the PD-1 and CD80 molecules. In a recent study, Duffy et al.[149] evaluated the impact of combination therapy of radiation together with Tre and/or Dur in PCa patients. This study showed that radiation could intensify the impact of ICP stopping in these patients. Dur with or without Tre in metastatic PCa patients was performed in phase II study[129]. Additionally, a phase Ib/II study assesses the safety and efficacy of Dur plus ibrutinib (BTK inhibitor) in PCa patients[150]. Currently, a phase Ib study examined the combination of galunisertib (Gal) and Dur in recurrent or refractory metastatic PCa. The combination of Gal plus Dur had a suitable tolerability and safety profile. The effect of this combination in second and third line PCa patients warrants additional attention[151]. The study of Dur and stereotactic radiotherapy in locally advanced PCa was safe, well tolerated and appears to be clinically active with high rates of margin-negative resection[152]. Cassier et al.[153] performed a phase I dose escalation research to assess the safety and clinical function of a combined treatment relating an anti-CSF1R (pexidartinib) with Dur in patients with advanced/metastatic PCa. Toxicity was consistent with the expected profiles of the individual drugs and no unpredicted findings were observed with the combination.

4.2.5. *Status of dMMR/MSI and PD1/PD-L1 and CTLA-4 antibodies*

The mismatch repair (MMR) system plays an important role in repair of DNA sequence during replication. Deficiency in the MMR system (dMMR) or lack of performance of one of the MMR proteins, including MLH1, MSH2, MSH6 and PMS2, lead to an accumulation of somatic mutations, resulting in microsatellite instability (MSI) and a higher neoantigen load that enhances proinflammatory cytokines and activation of T cells[154]. Given the recent tissue-agnostic approval of Pem, MSI testing is now recommended by the National Comprehensive Cancer Network (NCCN) for locally advanced or metastatic PDA. A recent study of MMR status in PDA using next generation sequencing (NGS) revealed that dMMR occurs at a low frequency of 0.8% in PDA, and all these cases also have Lynch syndrome[155]. Furthermore, Yamamoto et al.[156] and Macherla et al.[157] examined the prognostic impact of MSI in PCa cases and found that MSI positives patients had longer survival time than negative ones.

PD-L1 overexpression and dMMR/MSI status could indeed be useful predictive biomarkers for the response to immunotherapy. For this purpose, Kim et al.[158] analyzed both PD-L1 and MLH1/MSH2 expressions and showed a remarkable association between PD-L1 expression and MLH1/MSH2 loss. Moreover, Salem et al.[159] studied the correlation between tumor mutational load (TML), dMMR, and PD-L1 and found a lower frequency of TML-high in PDA, and a positive PD-L1 expression in MSI-H and MSS PDA cases at about 11.1% and 9%, respectively[157,159]. The majority of pancreatic adenocarcinoma patients with either MSI-H or MSI-L showed low TML level. Future studies are needed to indicate the suitability of PD-L1, MSI, and TML as appropriate predictive markers of response to immunotherapy in PDAC[157,160]. Pem was recently evaluated in clinical trials in heavily pretreated patients with MSI-H tumors[161,162]. Interestingly, these studies demonstrated the benefit ICB in patients with MSI-H tumors regardless of their PD-L1 status. Furthermore, Niv, as a PD-1 inhibitor, which is approved for progressed MSI-H/dMMR metastatic colorectal cancer (CRC) following first-line treatment, indicated that dMMR plays a robust predictive of response to ICB in comparison to PD-L1[157,163]. Furthermore, the activated V-domain immunoglobulin suppressor of T cell activation (VISTA) which is expressed on the MQs pathway, decreases T cell responses in the tumor at a greater rate compared to PD-L1 blockade. Therefore, PD-1/PD-L1 blockade might breakdown PDA treatment due to suppression of the immune response through an untreated VISTA pathway. Enhancement of T cell infiltration, using anti-CTLA-4 mAb with anti-VISTA antibody to target MQs as combination therapy, holds a promising new strategy for PDA treatment[164,165].

4.2.6. *BMS-936559*

BMS-936559, a humanized IgG4 and PD-L1-specific mAb, can prevent the interaction of PD-L1 with both PD-1 and CD80. In NSCLC, RCC and melanoma patients, durable tumor regression and long-term disease stabilization were observed by PD-L1 blockade; however, in PCa, no objective response was detected[9,166].

4.3. *LAG-3 immune checkpoint blockade*

LAG-3 or CD223, a homolog of CD4, was cloned over 25 years ago[167]. The negative regulatory role for LAG3−MHC-II interaction is the most prominent characteristic of LAG-3, and this fact represents LAG-3 as a potential treatment target[168]. In 2006, targeted immunotherapy through LAG with a soluble LAG-3Ig fusion protein (IMP321) was introduced. While, IMP321 was used for three clinical trials in RCC, metastatic breast carcinoma, and melanoma previously with average success. It is still being tested in other novel clinical trials, where it may reveal additional therapeutic advantages[169−171]. LAG-3 was shown to synergize with PD-1 in downregulating T cell activities and stimulating immune evasion by cancer cells. Even though, targeting of LAG-3 alone showed little effect, and blockade of LAG-3 has been revealed to synergize with PD-L1 and augment its anti-tumor effect[172]. These trials are investigating the application of combination anti-LAG-3 and anti-PD-1 *versus* anti-LAG-3 alone in diverse solid tumors[173]. These results have received certain interest in perspective, hence LAG-3 will be considered as the promising targeted immunotherapy and predictive biomarker[174]. Table 1[9,120−123,127,129,134−139,142−146,149−153,166,175,176] summarizes the clinical trials of targeted ICPs in PCa.

5. *In vitro* studies of immune checkpoint blockade

The expression of PD-L1 protein is typically limited to MQ lineage in human and it has the capacity to be induced in B cells, tumor cells such as PCa, as well as other hematological cells, and non-lymphatic tissues[13,177]. Furthermore, PD-L2 expression is inducible for MQs and DCs[178]. A study was conducted in Panc-02 cells, where they were directly injected into the pancreas. It was shown that blocking antibodies against B7−H1 could suppress tumor growth[179]. Studies in the MiaPaCa-2 and Su86.80 cell lines showed that the anti-inflammatory cytokines

Table 1 Clinical trials of targeted ICP in Pca.

Target	Drug	Phase	Status	Clinical Trials identifier	Ref.
CTLA-4	Ipilimumab	II	Completed	NCT00112580	120
	Ipilimumab ± GVAX	I	Completed	NCT00836407	121
	Ipilimumab + GVAX	II	Recruiting	NCT01896869	175
	Ipilimumab + gemcitabine	Ib	Completed	NCT01473940	122
	Ipilimumab + nivolumab with radiation	II	Recruiting	NCT03104439	123
	Tremelimumab (CP-675,206) + gemcitabine	Ib	Completed	NCT00556023	128
	Tremelimumab + durvalumab	II	Completed	NCT02558894	127
PD-1	Pembrolizumab + REOLYSIN + chemotherapy	II	Recruiting	NCT02620423	137
	Pembrolizumab + ACP-196	II	Active but not recruiting	NCT02362048	136
	Pembrolizumab (MK3475)	I/II	Recruiting	NCT02305186	135
	Pembrolizumab + PLX3397	I	Recruiting	NCT02452424	134
	Pembrolizumab + oncolytic virus pelareorep	Ib	Recruiting	NCT02620423	138
	Pembrolizumab + PEGPH20	II	Recruiting	NCT03634332	139
	Pembrolizumab + NOX-A12	II	Completed	NCT03168139	176
	Nivolumab + GVAX + cyclophosphamide	I/II	Active but not recruiting	NCT02451982	144
	Nivolumab + Nab-paclitaxel + gemcitabine	I	Recruiting	NCT02309177	143
	Nivolumab + GVAX + CRS-207	II	Recruiting	NCT02243371	142
	Nivolumab + cabiralizumab + chemotherapy	II	Active but not recruiting	NCT03336216	145
	Nivolumab + SD-101	I	Recruiting	NCT04050085	146
PD-L1	BMS-936559	I	Completed	NCT00729664	9,166
	Durvalumab + ibrutinib mesylate	Ib/II	Recruiting	NCT02403271	150
	Durvalumab + galunisertib	1 b	Completed	NCT02734160	151
	Durvalumab + stereotactic radiotherapy	I/II	Recruiting	NCT03245541	152
	Durvalumab + pexidartinib	I	Completed	NCT02777710	153
PD-L1, CTLA-4	Tremelimumab + MEDI4736	I	Recruiting	NCT02311361	149
	Durvalumab + tremelimumab	II	Recruiting	NCT02558894	129

such as IL-10 resulted in a modest decrease in mRNA level of PD-L1 in PCa cells. In contrast, treatment with IFN-γ upregulated mRNA expression of PD-L1[20]. Following hemi-spleen implantation of tumor Panc-02 cells, which are treated with cyclophosphamide (Cy) and GVAX or αPD-1/αPD-L1 therapy, it showed elevated amounts of IFN-γ secreted from $CD8^+$ T cells and tumor-specific $CD8^+$ T cells in the TME. $CD8^+$ T cells isolated from spleen and TILs with tumor Panc-02 cells as antigenic targets were treated with anti-PD-1, Cy + GVAX + IgG, and Cy + GVAX + anti-PD-1, showing enhanced amounts of IFN-γ secreted from $CD8^+$ T cells in incremental order compared to the untreated cells[180]. A syngeneic orthotopic tumor Panc-02 cells poorly responded for blockage of PD-L1 and CTLA-4 as well as Gem, while CD40 agonist antibody (αCD40) treatment significantly delayed tumor growth and increased survival. It also drove maturation of myeloid cells and expansion of memory T cell in spleen and upregulated the PD-L1 mRNA expression in Pan-02 tumors. The combination of αCD40 with αPD-L1 resulted in a significant upsurge of OS rate compared to either agent of the alone treatment trials[181]. Many of the cancer studies have observed tumor cells *via* types I and type II IFN signaling pathways upregulated PD-L1 expression in human and murine PCa cell lines. In many studies, nivolumab on PANC-1 cell[182], ruxolitinib (JAK−STAT pathway inhibitor) on PANC-02-H7 cell[183] and 5-fluorouracil, Gem or paclitaxel (upregulate cell surface PD-L1 expression) on AsPC-1, MIA PaCa-2 and Pan-02 cells[184] have a significant influence in downregulation of PD-1/PD-L1 protein levels on the surface of tumor cells. The combined effect of these drugs and IFNs indicated that PD-L1 is activated through the JAK−STAT1 pathway by both type I and II IFNs. It also uncovered that JAK−STAT pathway inhibition increases the effectiveness of anti-PD-1 immunotherapy to suppress the growth of PCa[185]. Both type I and II interferon cytokines engage JAK−STAT signaling pathways. The expression of PD-L1 is a primary limiting factor for CTL activities in gastric cancers and is significantly upregulated by IFN-γ exposure[184,186,187]. Taken together, these findings suggest that PD-1/PD-L1 might be a critical target for controlling the growth of PCa.

CTLA-4 is homologous to the T-cell costimulatory protein called CD28 and is constitutively expressed in T_{reg} but is only upregulated after activation in conventional T cells. It appears to be particularly notable in cancers and acts as an "off" switch on the surface of APCs once bounded to CD80 (B7-1) or CD86 (B7-2)[16,188,189].

LAG-3 which belongs to immunoglobulin (Ig) superfamily is expressed on activated T cells, NK cells, B cells, and plasmacytoid DCs. Although LAG-3 acts as target of different drug development programs, it has diverse biological effects on T cell functions[167,190–193]. The binding of LAG-3 to MHC class II (its ligand) has a higher affinity than CD4 (immune cell surface glycoprotein)[194]. LAG-3 helps to maintain the tolerogenicity of $CD8^+$ T cells and CD8 exhaustion during chronic infection, contributing to the maturation as well as activation of DCs, and like CTLA-4 and PD-1, it negatively regulates proliferation, activation, and homeostasis of T cells[12,161,162,167,195], therefore plays a vital role in the suppression of T_{reg} function[168]. Co-inhibitory molecules, including IL-12 and TGF-β, which play the role of induction and suppression of LAG-3 and PD-1, respectively, block ICPs to reverse pathogenic T_{reg}. In addition, the effects of co-inhibitors on NK cells indicate different expression in response to cytokine stimulations of IL-15 at least, demonstrating the regulatory role of the co-inhibitors on human NK cells. Furthermore, IL-15 can promote NK cell-mediated killing in pancreatic stellate cells (PSC)[196,197].

6. siRNA as immune checkpoints inhibitors

siRNAs, a group of regulatory therapeutic factors, were rabidly designed to combat various diseases, including cancer, neurodegenerative disorders, and infectious diseases[198,199]. The application of siRNA in PCa to target immune checkpoints was studied in some investigations. A recent study used a PD-L1-specific siRNA conjugated to a magnetic nanocarrier (MN-siPDL1) in combination with gemcitabine as a therapeutic method in murine PCa models. The strategy reduced the tumor growth and elevated the survival ratio significantly. At 2 weeks from the beginning of the treatment, a 90% tumor volume reduction was attained. This is while 100% of the control group animals were observed with a tumor by 6 weeks after starting the treatment[200].

Although the failure of anticancer vaccines in PCa patients, a multilateral effort to develop new vaccines demonstrates an efficient efficacy in case survival. Several strategies have been developed to improve the efficacy and safety of tumor vaccines. These include single immunotherapy approaches such as breaking immunosuppression within the tumor microenvironment and overcoming tolerance to TAAs, as well as combinatorial immunotherapy approaches such as the combination of anticancer vaccines with ICP inhibitors and chemotherapy, radiation therapy or even surgery[201,202]. Recent findings suggest a multipronged approach for therapeutic efficacy, including various types of agents such as vaccines or oncolytic viruses and additional agents to prime the immune microenvironment, followed by ICP inhibitors[127]. Despite the lack of an effective tumor vaccine, a dozen more clinical trials are ongoing. For instance, use of heterologous prime-boost followed by a low dose of Cy/GM-CSF gene-transfected tumor cell (GVAX) and live-attenuated *Listeria* monocytogenes-expressing mesothelin (CRS-207) has extended patient survival with minimal toxicity through enhancement of innate and adaptive immunity[203].

7. Conclusions and perspectives

The adverse effects caused by immunotherapy in the order of their importance include neutropenia, nausea and vomiting, alopecia, fatigue, and thrombocytopenia[204]. Despite of the adverse effects, high levels of immunosuppression in the TME and poor immunogenicity provide significant challenges to prosperous immunotherapy of human PCa. On the other hand, ICPs have been discovered to perform an axial role in the establishment of tumor-induced immunosuppression in the TME. Therefore, targeting of ICPs is suggested to be a hopeful strategy for improving the therapeutic efficacy of PCa immunotherapy. CTLA-4 and PD1/PD-L1 are the most well-known ICPs that have been targeted for overcoming immunosuppression in the TME and induction of effective anticancer immune responses in PCa. The results of clinical studies in patients showed the safety and superior therapeutic efficacy of combination therapy with anti-CTLA-4 and anti-PD1 or anti-PD-L1 mAbs in PCa. Despite the encouraging results, several clinical studies showed poor response to checkpoint blockade with anti-CTLA-4 and anti-PD-1 or anti-PD-L1 immunotherapies in PCa patients due to resistance to CTLA-4 and PD-1/PD-L1-targeted therapies. The lack of significant clinical response to PD-1 or CTLA-4-targeted therapy in PCa is possibly due to the presence of unknown and new emerging ICPs that may be involved in tumor-induced immunosuppression and our incomplete understanding of the function of immune cells and molecules in the TME. Our continued progress toward understanding the immunobiology and targeting of ICPs in this type of human malignancy, as discussed in the present article, promises a new hope in the treatment of PCa in the future.

Author contributions

Seyed Hossein Kiaie and Behzad Baradaran designed the work. Seyed Hossein Kiaie, Mohammad Javad Sanaei, Masoud Heshmati, Zahra Asadzadeh, and Saleh Hadidi collected data and wrote the manuscript. Seyed Hossein Kiaie and Mohammad Javad Sanaei designed and regenerated the conceptual pictures. Seyed Hossein Kiaie, Iman Azimi and Reza Jafari checked and revised the article. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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