Evaluation of targetable biomarkers for chimeric antigen receptor T-cell (CAR-T) in the treatment of pancreatic cancer: a systematic review and meta-analysis of preclinical studies

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Introduction

Pancreatic cancer is one of the main causes of mortality and one of the most lethal malignant tumors worldwide [1]. Patient's treatment includes surgery, radiation, chemotherapy or a combination of these approaches, with an average 20% five-year survival [1, 2]. In spite of recent advances in systemic chemotherapy, the median overall survival (OS) for pancreatic cancer patients remains poor (<1 year) and novel treatment strategies remain an unmet clinical need [3]. Chimeric antigen receptor (CAR) therapy employing autologous engineered T cells is capable of identifying tumor antigens through an HLA-independent mechan- ism [1]. T-cell therapy employs genetic engineering to redirect patient's autologous T cells against malignant cells. The newest significant results in haematological cancer inspired investigation of CAR T-cell therapy in solid tumors such as pancreatic cancer [4]. CAR T-cell therapy was introduced after targeting CD19 for the treatment of lymphomas and leukemia in 1989 result- ing in significant growth in CART therapy and, as a direct consequence, in surface biomarker discovery [5]. Five clinical trials have been conducted until 2012, one of them targeting Mesothelin antigen while the others targeting CD19 antigen. The promising results from these preliminary studies with CAR T-cell and the fol- lowing research efforts led to the discovery of novel druggable biomarkers [6]. However, one major issue of this approach is the lack of specific targets for the treatment of solid tumors. Additionally, targets must be more specific and should not be expressed in healthy tissue [7]. Biomarker discovery is essential for the progress and success of CAR T therapy. Despite recent pro- gress, more research is still needed in the field of bio- marker identification, tumor cytotoxicity, cytokine release syndrome, persistence CAR T-cell in vivo, immunosuppressive tumor microenvironment [6]. Simultaneously with the development of immunother- apy in cancer, new markers are discovered and go through the validation process via clinical trials. Here we have summarized the most import studies, report- ing the preliminary preclinical data that may be useful for the next improvements for CAR T therapies.

Materials and methods

Search strategy

A comprehensive search of online literature databases including Medline, Embase, Cochrane Library, ISI Web of Science, and Google Scholar was carried out until March 2019 using the following medical subject headings (MeSH) words: "chimeric antigen T cell receptor", "CAR T-cell therapy", "CAR cell" and "pancreatic cancer". The search was limited to animal preclinical studies and duplicate publications were removed. Language of publications was restricted to English. Only studies that reported outcomes of inter- est were included whereas letters, commentaries, and reviews were excluded. Two authors independently screened the studies based on titles and abstracts, and finally, if required, the full texts were reviewed. This systematic review has been carried out in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [8].
Selection criteria Only studies that assessed CAR T-cell therapy in pan- creatic cancer animal models were included in our study. The researchers performed at least one com- parison between antigen targeted by CAR T-cell group and untreated group. Preclinical animal studies and peer-reviewed original articles were included in this systematic review.

Study selection and data extraction

Sahlolbei M. and Kheri B. independently screened the studies based on titles, abstracts, full texts and inclu- sion criteria using a checklist and reference lists of pertinent studies. The following extracted information and characteristics of all studies were included: author's last name, year and country of the study population, animal group, animal protocol, animal sample size and gender, target, vector, CAR gener- ation, experimental and control group, model, cell- line, co-stimulatory domain, signaling domain, scFv and animal protocol (Table 1). Two reviewers inde- pendently extracted data from all studies and the third author Dehghane M. resolved differences or discrep- ancies between investigators.

Risk of bias assessment

The methodological quality of included studies was assessed according to the SYstematic Review Center for Laboratory Animal Experimentation (SYRCLE) for animal studies was adopted from the Cochrane Risk of Bias Tool [26]. The items (and domains) in this tool include critical Assessment of selection bias (sequence generation, baseline characteristics, and allocation concealment), performance bias (random housing and blinding), detection bias (random out- come assessment and blinded outcome assessment), attrition bias (completeness of outcome data), report- ing bias (selective reporting), and other biases. For each eligible study, yes, no, and unclear responses were scored as low, high, and unclear risk of bias, respectively. All included studies were assessed inde- pendently by two authors for all 10 SYRCLE items and domains and verified by a Kiani J. Discrepancies were resolved by consensus (Table 2). Statistical analysis All statistical analyses were performed using Stata ver- sion 13.0

software (Stata Corp., College Station, TX, USA). Percentage and 95% CI were calculated by the percentage (ratio), standard deviation, and sample size in each study. Pooled cytotoxicity assay ratio (percent- age) and 95% confidence intervals were calculated by metaprop command using a random-effects model [27] with the inverse-variance weights method based on the transformed values and their variance [28]. We fitted the Freeman–Tuckey variant of the arcsine square root transformation of proportions to avoid variance instability when handling proportions close to one [29]. Random-effects meta-analysis was used taking into account conceptual heterogeneity and due to animal population and models, CAR generation, cell lines, and designs across studies. Forest plot was produced to show the cytotoxicity assay ratio (per- centage) and corresponding 95% CI for visual inspec- tion across studies. Heterogeneity between studies was assessed using the I2 statistics [30] (I2¼0% indicates no observed heterogeneity and I2 2:50% indicates sub- stantial heterogeneity). The Cochran's Q statistic was also used to analyze the statistical significance of the heterogeneity [31]. Sensitivity analysis was performed to find out which study (if any) had the most impact on the heterogeneity test and assess the robustness of summary findings. Visual inspection of funnel plots was performed to assess the Publication bias [32]. Furthermore, Publication bias was formally tested with Egger's regression asymmetry tests for asym- metry of the funnel plots, where $P < 0.10$ was consid- ered evidence of bias. Begg's adjusted rank correlation test and the trim-and-fill method for simulation were used [33, 34]. All statistical tests were two-tailed and the significance level was considered less than 0.05 for analyses.

Results

Using a systematic search of databases, 485 records were identified. Of these, 292 studies including com- mentaries, editorials, study protocols, and irrelevant themes were removed. After screening by title and abstract, 56 studies reporting the therapeutic potential of CAR T-cell for pancreatic cancer were reviewed in depth. 16 preclinical animal studies were eligible for inclusion in the systematic review and 11 studies reported the Cytokine Assay curve used for meta-analysis. Figure 1 shows a flow diagram relating to the selection of pub- lished studies.

Characteristics of studies

Eight (8) subgroups of CAR T-cells were included: CAR T-Mesothelin (CAR T-meso), CAR T-Prostate stem cellantigen (CAR T-PSCA), CAR T-Carcinoembryonic anti-HER2), NKG2 CAR T (family of C-type lectin receptors gen (CAR T-CEA), CAR T-Mucin (CAR T-MUC), CAR on the surface of NK cells CAR T), Cluster of differenti- T-human epidermal growth factor receptor 2 (CAR T-ation 47 CART (CD47- CART) and CAR T-epidermalgrowth factor receptor (CAR T-EGFR). The animal mod- els were induced by pancreatic cancer cell administered intraperitoneally, subcutaneously, or via tail vein injection. The majority of the animals were female, 4-6-week old, NOD mice or NSG mice. The doses of the interventions ranged from 105 to 107 CAR T -cells, which were injected intravenously approximately 15 days (when tumors became palpable) after induction of the pancreatic cancer model. The main characteristics of the 16 studies included in the present systematic review are shown in Table 1. Studies have been conducted to investigate the quality of CAR T-Cell therapy performance in cell cultures and Laboratory Animals. It necessitates modi- fication of T cells evaluated in cell culture by Cytotoxicity assay, proliferation assay, lysis cell assay and so on. The next step is to transfuse the expanded CAR T-cells injected into the mice at an appropriate dose. Finally, mice need to be closely monitored, by Bio Luminescence, Tumor volume, Metastases num- ber, and Survival in the following few days (Data shows in Supplemental Table1).

Quality or risk of bias assessment

All 16 articles in Table 1 were assessed methodologic- ally using the SYRCLE Risk of Bias tool. None of the studies was judged as having a low risk of bias across all domains. The majority of studies reported the same group size, minimizing the risk of selection bias based on animal characteristics. Only a few studies explained the method of random sequence generation and animals for the experimental and control groups were allocated randomly. Most studies were scored as unclear risk.

Main findings

Based on our investigation on the cytotoxic effect of CAR T-cell, it has been shown that this treatment has the potential to kill pancreatic cancer cells. Our sub- group analysis observed that CAR -PSCA was the most efficient in pancreatic cancer animals treatment, at the next rank of performance, dual CAR T-CEA/ MSLN, Meso, Her2/neu are located, respectively. Use of dual CAR T-cell or its combination with adeno- virus or cytokine has been effective in increasing effi- cacy. CAR T immunotherapy significantly increased the cytotoxicity assay in meta-analysis. There was no evidence for significant heterogeneity across studies [P ¼ 0.38 (Q statistics), I2 ¼ 7.14%]. Sensitivity ana- lysis, performed by successively removing a particular study at a time to assess the influence of every single study on pooled cytotoxicity assay, indicated that the meta-analysis model is robust.

There was no evidence of publication bias regard- ing the cytotoxicity assay ratio as a marker in treat- ment of

pancreatic cancer (P ¼ 0.34, for Begg's adjusted rank correlation test and P ¼ 0.29 for Egger's regression asymmetry test) and visual inspection of the funnel plot indicated that publication bias is unlikely (data not reported).

Discussion

Pancreatic Cancer is the fourth cause of death in the United States of America and is predicted to be the second most lethal cancer in the world by 2030 [35, 36]. Simultaneously, initial results from immunothera- peutic studiesin solid tumor and hematologic malig- nancies have led to the development of multiple CAR T therapies in pancreatic cancer [37]. Unlike conven- tional approaches used to manage cancer disease, CAR T-cell therapy is a patient-specific, "living drug" and self-replicating drug approach in the elimination of resistant, metastatic or recurrent cancers [19]. As of the end of October 2019, there were about 350 CAR T-cell trials registered onclinicaltrials.gov, but the vast majority failed. Optimizing the design of CARs with the discovery of new, more specific antigen-targets and the potential combination of CAR T-cell therapy with current approaches may help achieving effective treatment for pancreatic cancer [4]. Driving CAR T-cells against tumors via specific anti- gens in the setting of pancreatic cancer will continue to improve clinical trials. This is a fast evolving field that has benefit from a few targets such as Prostate stem cell antigen (PSCA), antigens mucin1 (MUC1), mesothelin Carcinoma-Embryonic Antigen (CEA) and HER2 neu [3].

PSCA is a glycosylphosphoinositol-anchored cell surface antigen that is overexpressed in several major cancers including prostate, bladder and pancreatic cancer [38]. CAR specifically is capable of killing PSCA expression in pancreatic cancer cell lines [39]. Besides, the preclinical study showed that CAR T -PSCA were able to reduce the size of pancreatic tumors [13, 14]. Therefore, PSCA has been proposed as a biomarker of diagnosis and prognosis, as well as a target of therapy for these cancers [40]. The low expression of PSCA in some normal tissue [39] might be solved by creating dual-target CAR T-cells that can target both MUC-1 and PSCA simultaneously and may reduce the risk of immune escape, but corres- pondingly accept the risk of "on-target off-tumour"toxicity [15, 41]. Since MUC1 is widely expressed in most epithelial-derived solid tumors, including pan- creatic and breast cancer, the MUC28z CAR T-cells will likely have broad applications, which may lead to successful eradication of above-mentioned tumors [20]. CAR T-cell trafficking depends on the expression of receptors for chemokines secreted by the tumor. CAR T-cells endogenously express chemokine recep- tors, but their cognate ligands are often not highly expressed by solid tumors. For improve trafficking to tumors, CAR T-cells can be engineered to express receptors for chemokines naturally secreted by the tumor [42]. A barrier in treating pancreatic cancer is the release of inhibitory cytokines that can suppress the immune system and limit CAR T-cell performance and stability. Pancreatic tumors use immune evasion strategies such as the production of inhibitory cyto- kines, which limit CAR T-cell function and persistence. Thus, to protect CAR T-cells from the immunosup- pressive cytokine IL-4, engendered inverted cytokine receptor in which theIL-4 receptor exodomain was joined to the IL-7 receptor endodomain (4/7 ICR). The expression of this molecule in CAR T-PSCA can invert the inhibitory effects of tumor-derived IL-4 and pro-mote T cell proliferation, resulting in enhanced antitu- mor activity [12]. CAR T-cells designed to inhibit signals produced by the tumor environment for example inhibitory signals such as TGFb, IL4 are expressed alongside CAR T-PSCA (Smart T-cells). This approach is evaluated with the cytolyticactivity, viabil- ity, and expansion of CAR [17].

Mesothelin is a promising target for immune-based therapy, specifically for mesothelioma, pancreatic and ovarian cancers that have high levels of mesothelin expression. Many preclinical and clinical studies that target tumors expressing high mesothelin levels with antibodies, immunotoxins, antibody-drug conjugates and vaccines have shown the potential of mesothelin as a target [43]. Pancreatic cancer is often reported to be metastatic at early stages, which causes poor prog- nosis. One of the up-regulated antigens at the final stage is Mesothelin, normally observed only in fully transformed cells that have acquired metastatic fea- tures [38]. Findings showed that CAR T-meso killed pancreatic cancer cells in vitro and inhibited subcuta- neous tumor growth in vivo. Also, CAR T-meso suc-cessfully repressed lung metastases originating from pancreatic cancer [10, 11].

To address these safety concerns while retaining potent cytotoxicity against cognate tumor cells, several innovative strategies have recently been described to regulate the selectivity and activity of CAR T-cells, including the suicide gene, inhibitory CAR, dual-antigen receptor, and an exogenous molecule used as a switch to control CAR T-cell activity [44].

Another novel approach of treating metastaic pan- creatic cancer is targeting the expression of antigen Her2/neu (ErbB2) human epidermal growth factor receptor 2 (HER2) which shows a 20-60% increased expression in pancreatic cancer [45]. In a study, CAR against Her2/neu was designed to stop the growth and metastasis of pancreatic xenografts cancer in mice. The survival of mice was increased and some of them had no cancer-free after two months [22]. Low expression of HER2 antigen has been observed in lung and other tissues, which may be problematic in CAR T-cell therapy. For this purpose, Deepak Raj et al. have used switchable CAR T-cells to target the antigen HER2. A major advantage of switchable CAR T-cells was their specific activation within the presence of a switch, which represent favorable option for neoplasm antigens with some expression on healthy tissue. These

results corroborated it is efficacy against aggressive and disseminated pancreatic tumors [18, 21].
To enhance the specificity and controllable activity of CAR T-cell therapy, engeenered T cells must distin- guish tumor tissues from normal tissues and prevent tumor escaping from immune surveillance [46]. Dual- receptor CAR (dCAR) can target CEA and mesothelin (MSLN) with a specific cytotoxic effect on cells that express both antigens [47]. Overall, this method can reduce "on-target, off-tumour" and "side effect" tox- icity against solid tumors.
Pancreatic ductal adenocarcinoma is highly aggressive with immunosuppressive tumor microenvironment that limits T cell penetration and induces T cell hypofunction [16]. To overcome this barrier, a possible approach is to employ adenovirus with recombinant cytokines [9, 19]. Simultaneous use of CAR T- meso and oncolytic adeno- virus (expressing TNF-a and IL-2) enhanced the efficacy of CAR T- meso and showed significant tumor regres- sion in mice with pancreatic cancer [48].
CEA have highly expressed on the surface of pan- creatic adenocarcinoma cells. On the other hand physiologically expressed on healthy epithelium of the gastrointestinal tract and the lung so CEA is not tumor dedicated antigen [49]. Anti-CEA T-cells can eliminate CEA pancreatic carcinoma specifically and efficiently without inducing substantial autoimmunity when redirected by CAR. Unfortunately, increased serum level of CEA is found in cancer patients and might lead a major issue in the clinical outcome see- ing soluble CEA can block T-cell recognition by bind- ing to the anti-CEA CAR [50].
Intratumoral hypoxia is a common feature of solid tumors. Recent advances in cancer biology indicate that hypoxia is not only a consequence of unre- strained tumor growth, but also plays an active role in promoting tumor progression, malignancy, and resist- ance to therapy [51]. Hypoxic conditions usually may alter the expression of glycosyltransferases [20]. MUC1 acts as a pivotal regulator of the metabolic sig- naling, facilitates metabolic alterations in hypoxic environments and promotes tumor cells survivale [52]. CAR T targeting the Tn and STn antigens (com- mon tumor aberrant O-glycosylation sites) which is able to specifically recognize multiple types of tumors in vitro [53].
High-throughput screening showed several factors leading to the resistance of tumor cells to treatment. Among those, Indoleamine 2, 3-dioxygenase 1 (IDO1), an enzyme that metabolizes tryptophan to kynurenine acid caused a metabolic advantage to can- cer cells against T cells. IDO1 acts down-regulating T cell metabolism resulting in peripheral immune toler- ance and evasion of cancer from T cells [54]. Another gene, Galectin-9 (Gal-9) is a tandem- repeat type galectin that has multiple biological func- tions such as cell adhesion and aggregation [55]. Cyclooxygenase (COX) 1/2 enzyme have a significant role in the synthesis of prostaglandin E2 (PGE2), a major player in angiogenesis, inflammation, and immunosuppression in cancer [56]. Results of tMUC1 CAR T-cells against treatment-refractory PDCA in combination with inhibitors against IDO1, COX1/2, and Gal-9 were promising. MUC1-CAR T-cells have a synergistic effect with chemotherapy drugs [57].
A promising method is the use of CARs not based on a single-chain antibody variable fragment (for dir- ect antigen recognition) but relying on the activating Fc-receptor CD16 [58]. Currently, CAR T- CD16- are evaluated in combination with monoclonal antibodies such as rituximab. Preliminary clinical trials have been tested combining the CD20-targeting antibody rituximab with a CD16-CAR T-cell product (NCT03189836). Cluster of Differentiation (CD) markers such as CD16, CD47 play an important role in pancreatic immunotherapy. The high-affinity of CD16 158 V variant in combination with CART- CD16 promotes anti-tumor activity. Furthermore, intra-tumoral injection of CD47-CAR T-cells signifi- cantly reduced BxPC3 (Human primary pancreatic adenocarcinoma). Humanized CD47-CART-cells spe- cifically killed CD47-positive cancer cells [24, 59]. Nowadays, the main challenge is the inhibition of tumor-induced immunosuppressive mechanisms and remodeling the tumor microenvironment to be favor- able for delivering efficacious antitumor immunother- apeutics [60]. Additionally, the heterogeneity of tumor antigens in solid tumors usually leads to invalid immune surveillance and consequent relapsed tumor [61]. The paper aims to summarize CAR T therapy studies to increase the level of research- ers's knowledge.

Limitations

There are some limitations to this study. Firstly, all the preclinical trials evaluating CARs have small group sizes, leading to some uncertainties of the result. Secondly, doses of CAR T-cells vary in different experiments, ranging from 5 103 to 1 107 cell/ mouse. Accordingly, some of the comparisons between CARs and control groups may be unreliable.

Future prospective

Preclinical studies play a critical role in applying data to clinical trial practice and their outcome can be esti- mated more methodologically and objectively. Immunotherapy based on chimeric antigen have a positive effect on cancer treatment, along with trad- itional therapies.

Ethical approval

This article does not contain any studies with human par- ticipants or animals performed by any of the authors.

Declaration of Interest

Author Jafar Kiani has received research grants from IRAN Science Medical University. The authors declare that they have no conflict of interest.

Funding

This project was supported financially by research grant number 98-3-37-16198 from Iran University of Medical Sciences.

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