





Irreversible Electroporation Plus Anti-PD-1 Antibody versus Irreversible Electroporation Alone for Patients with Locally Advanced Pancreatic Cancer

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Background: Irreversible electroporation (IRE) is shown to not only improve the prognosis of patients with locally advanced pancreatic cancer (LAPC) but also activate the immune system. Considering the immune-activating function of IRE, IRE may enhance the effect of immune checkpoint inhibitors in the treatment of LAPC. We aimed to compare the effect and safety of IRE combined with toripalimab versus IRE alone for LAPC.

Methods: We retrospectively collected data from LAPC patients treated with IRE plus toripalimab (240mg, 7 days after IRE) or IRE alone at Sun Yat-sen University Cancer Center. Overall and progression-free survival and treatment-related adverse events were evaluated and compared.

Results: From August 2015 to June 2020, a total of 85 patients were collected and analyzed in this study: 70 in the IRE group and 15 in the IRE plus toripalimab group. The IRE plus toripalimab group showed longer OS [44.33 months (95% CI 17.39–71.27) versus 23.37 months (95% CI 21.20–25.54), $P=0.010$] and PFS [27.5 months (95% CI not reached) versus 10.6 months (95% CI 7.79–13.42), $P=0.036$], compared with IRE group. There were no treatment-related deaths in all patients of this study. Although pancreatic fistula, biliary fistula, abscess, vomiting and gastroparesis were a little more common in IRE plus toripalimab group, no significant differences in the rates of all adverse events between these two groups were observed.

Conclusion: IRE plus toripalimab had acceptable toxic effects and might improve survival in LAPC compared with IRE alone.

Keywords: locally advanced pancreatic cancer, irreversible electroporation, toripalimab, efficacy, prognosis

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal gastrointestinal disease with increasing morbidity, which also has a growing impact on cancer-specific mortality worldwide.¹ Nearly 40% of all PDAC cases are localized to the pancreas and characterized with the involvement of major vascular structures, leading to unresectable disease without metastases detected by radiological examinations, which are also known as locally advanced pancreatic cancer (LAPC).² Despite currently available therapies, the prognosis of LAPC remained unsatisfied with 3% as 5-year survival rate and 14.2 months as median survival.³ The optimal treatment of LAPC

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was still controversial. Based on the development of chemotherapy, more choices for additional treatment, such as conversional surgical resection, were available. However, the rates of downstaging to resection were as low as 4–15%.⁴ Moreover, more than 30% of deaths were due to the progression of local disease,⁵ suggesting the importance of local control approaches.

As an important local destructive method, local ablative therapy was used as a new treatment of LAPC. However, the thermal injury to the adjacent organs and vessels limited the use of most thermal ablative methods, including radiofrequency ablation and microwave ablation.^{6,7} Instead of relying on thermal energy, IRE induces the apoptosis of tumor cells by destroying the cell membrane integrity with short and high-voltage current pulses.⁸ The feature of free from the heat sink effect makes IRE more appropriate than radiofrequency ablation (RFA) in the treatment of LAPC. Additionally, the immune response caused by IRE may be stronger, because the advantage of preservation of vessels is helpful for the transmission of immune molecules or cells. Thus, apart from inducing apoptosis of tumor cells, IRE can also reconstruct the microenvironment within the tumor and induce the immune response.^{9–11} Previous studies had shown that IRE could alleviate immune suppression and induce activation of T cells, indicating that IRE may potentiate the antitumor efficacy of immunotherapy in PDAC.^{9,12}

In recent years, game-changing advances have been achieved in the development of therapies of various cancers, including melanoma, lung and liver cancers with immune checkpoint inhibitors (ICIs).^{13–15} However, little success had been achieved in the use of ICIs in PDAC.¹⁶ The relatively low rates of programmed cell death protein 1 (PD-1) expression, low mutational load and infiltration of T cells, and accumulation of regulatory T-cells (Tregs) could all lead to the failed immunotherapy in PDAC.^{17,18} The immune-suppressive tumor microenvironment (TME) contributed to the poor response to ICIs, indicating the importance of augmenting the ICI-responsiveness with combination therapy. IRE was shown to not only destroy the tumor itself directly, but also activate the immune system. We previously indicated that IRE could induce immunogenic cell death (ICD) and increase the infiltration of effector CD8⁺ T cells.⁹ Additionally, IRE could also enhance the process of antigen presentation by promoting M1 macrophage polarization and maturation of dendritic cells.^{10,19} Based on these findings, a promising approach

to improve the responsiveness of ICIs is to utilize the immunogenic properties of IRE to increase the cytotoxic T cells (CTL) to the “cold” TME, switching its immune status to “hot”. The combination of IRE and PD-1 immune checkpoint blockade could significantly promote survival in Kras-induced pancreatic cancer (KPC) mode by selectively promoting the infiltration of CD8⁺ T cells.²⁰ Based on these promising results, the clinical effect of the combination of IRE and anti-PD-1 therapy in LAPC could be encouraging and should be investigated. However, up to now, no similar studies had focus on this topic. Therefore, we designed this study to evaluate the survival of LAPC patients after IRE combined with anti-PD-1 therapy.

Methods

Patients

This study was approved by the Institutional Review Board of Sun Yat-sen University Cancer Center (SYSUCC). The ethical standards of the 1964 Helsinki Declaration were strictly followed and written informed consent was obtained from patients prior to treatment. Patients diagnosed with LAPC were included in this study. The exclusion criteria included the following: (1) patients who have received surgical resection or RFA; (2) patients with second primary malignant tumor; (3) patients with incomplete follow-up information or loss to follow-up.

Treatment Procedure

Similar to the procedure reported before,^{21,22} a 4-month induction chemotherapy and adjuvant FOLFIRINOX chemotherapy were adopted for all patients. During the procedure of IRE, an electric field around the tumor was created with two to six probes. As for the initial setting of electroporation, an electric field strength of 1500 V/cm and a planned delivery of 90 pulses at a pulse length of 70–90 ms were used. Toripalimab, an anti-PD-1 immune checkpoint blockade, was administered a week after IRE and was repeated every 3 weeks thereafter until progressive disease (PD) was detected.

Data Collection

Routine measurements of pathological and clinical data, including age, gender, tumor size, grade, and site, white blood cell (WBC) count, platelet (PLT) count, serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glutamyl

transpeptidase (GGT), albumin (ALB), total bilirubin (TBIL), indirect bilirubin (IBIL), C-reactive protein (CRP), hepatitis B surface antigen (HBsAg), carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA19-9) were carried out. Survival endpoints, including overall survival (OS) and progression-free survival (PFS), which were defined as the duration from the date of diagnosis to death and tumor progression, respectively, or last follow-up, were analyzed in this study. All patients were followed until May 30, 2021. After IRE treatment, the first follow-up visit was performed 1 month later, and follow-up then occurred every 2–3 months, until death or dropout. Physical examination, serum CA19-9, CEA analysis and at least one imaging examination (abdominal CT or MRI) were performed for each follow-up. Development of new low-density lesions in the region of the IRE (within 1 cm) or in the liver or lungs was considered evidence of local recurrence or distant metastasis, respectively, even in the absence of symptoms. Suspicious nodules in the peritoneum or the omentum, or the presence of newly identified ascites, were defined as peritoneal recurrence. If the findings were equivocal with recurrence, then imaging and CA19-9 were repeated at the second month for confirmation of tumor recurrence or not.

Blood Sample Collection and Analysis

All blood samples were collected as the procedure reported before,²³ which was simply described as duplicate blood sample before (BT) and on the seventh day after IRE (AT). The mononuclear cells (PBMCs) were isolated and analyzed with CD3, CD3CD4, CD3CD8, CD3CD16CD56, and CD4CD25 antibodies by flow cytometry (FACS caliber, 4 color system, BD Bioscience, CA, US).

Similar to the procedure described in our previous study.²³ Quantitative sandwich enzyme immunoassay technique (ELISA kit, R&D system, Minneapolis, MN) was used to measure the concentrations of serum cytokines and humoral immune parameters, including IL-2, IL-6, IL-10, interferon- γ (IFN- γ), tumor-necrosis factor (TNF), C3, C4, IgA, IgM, and IgG in the peripheral blood.

Statistical Analysis

Categorical data were compared with the chi-square test. Survival differences were calculated with Kaplan–Meier method and compared with Log rank test. Cox regression model was used to determine the significant variables of survival in the multivariate analysis. Two tailed P-value was

used and those <0.05 were considered statistically significant. R version 3.4.2 software (The R Foundation for Statistical Computing, Vienna, Austria. <http://www.r-project.org>) was adopted to perform all statistical analyses.

Results

Patient Characteristics

The flow diagram for data selection is shown in Figure 1. Between August 2015 and June 2020, 85 patients met the criteria for inclusion: 15 patients received combination therapy with anti-PD-1 therapy plus IRE (IRE plus toripalimab group), and 70 patients received IRE monotherapy (IRE group). The last follow-up was completed on 30 May 2021. No significant differences were observed in the baseline clinical and pathological characteristics between these two groups. Female occupied predominantly (55.3%) in the whole cohort. The median age for all patients was 57.84 years (range 19–87). Specifically, the median ages for IRE plus toripalimab group and IRE groups were 56.47 years (range 40–66) and 58.13 years (range 19–87), respectively. Additionally, for patients in both treatment groups, similar distributions of tumor site and tumor size were also observed. The median tumor sizes for patients in the IRE plus toripalimab group and IRE groups were 3.5 cm (range 2.4–5.7) and 3.75 cm (range 2.2–5.5), respectively. There were also no significant differences in the proportions of neoadjuvant chemotherapy and their regimens between these two groups (Table 1).

Survival Comparisons Among Three Groups

In this study, disease progression and death were observed in 54 patients and 45 patients, respectively, in the whole cohort. The median OS and median PFS for all patients were 24.63 months [95% confidence interval (CI) 20.91–28.36] and 13.73 months (95% CI 7.91–19.55), respectively. The median OS in the IRE plus toripalimab group was 44.33 months (95% CI 17.39–71.27), compared with 23.37 months (95% CI 21.20–25.54) in the IRE group. The 1-year, 2-year, and 3-year OS rates for IRE plus toripalimab group were 100%, 100%, and 33.3%, respectively, compared with 91.1%, 45.1% and 11.7% for IRE group ($P = 0.010$, Figure 2A). The median PFS for IRE plus toripalimab and IRE groups were 27.5 months (95% CI not reached) and 10.6 months (95% CI 7.79–13.42 months), respectively. The 1-year and 2-year PFS rates for IRE plus toripalimab group were 88.9% and 52.4%,

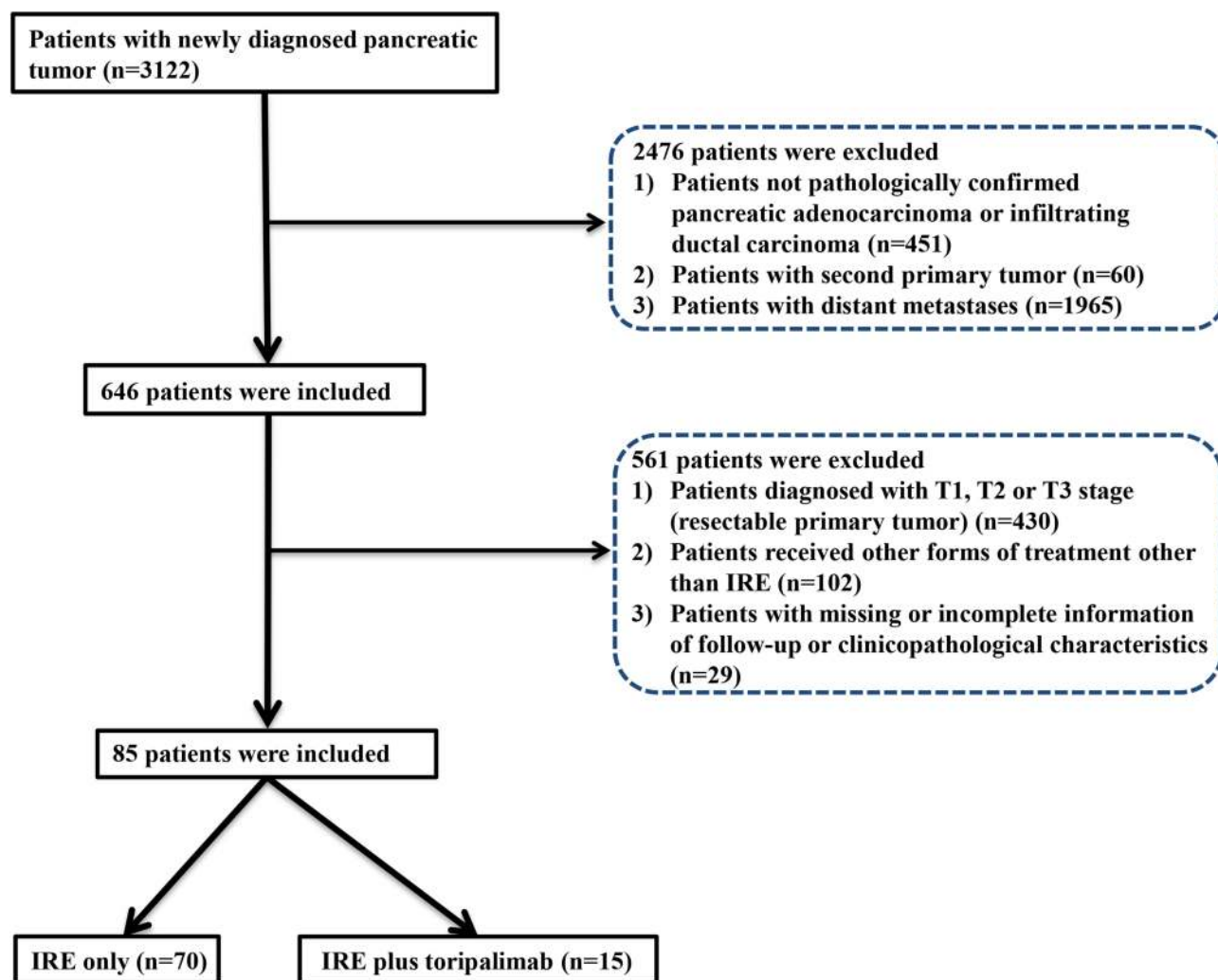


Figure 1 Flowchart of the included patients.

respectively, compared with 46.8% and 17.6% for IRE group ($P = 0.036$, Figure 2B).

Prognostic Factors for Survival

As shown in Table 2, the results of univariate analysis for OS revealed that tumor size, CA125, neoadjuvant chemotherapy regimen, and treatment strategy were associated with OS. Additionally, in the multivariate analysis, it was showed that independent risk factors for OS included tumor size ($>4\text{cm}$ versus $\leq 2\text{cm}$, $\text{HR}=39.760$, 95% CI 1.908–828.050, $P=0.017$), CA125 ($>45\text{U/mL}$ versus $\leq 45\text{U/mL}$, $\text{HR}=2.373$, 95% CI 1.165–4.832, $P=0.017$), and treatment strategy (IRE + PD-1 versus IRE, $\text{HR}=0.274$, 95% CI 0.076–0.985, $P=0.047$). In terms of PFS, tumor grade (poor/moderate differentiation versus well differentiation, $\text{HR}=2.031$, 95% CI 1.125–3.669, $P=0.019$) and treatment strategy (IRE plus

toripalimab versus IRE, $\text{HR}=0.342$, 95% CI 0.119–0.978, $P=0.045$) were identified as significant prognostic factors for PFS (Table 3).

Variation of Circulating Immune Cells

The isolated mononuclear cells were phenotypically characterized by evaluating the absolute number of B cells (identified as $\text{CD}19^+$), T cells (identified as $\text{CD}3^+$), $\text{CD}4^+$ T cell (helpful T cells, identified as $\text{CD}3^+\text{CD}4^+$), $\text{CD}8^+$ T cell (cytotoxic T cell, identified as $\text{CD}3^+\text{CD}8^+$), $\text{CD}4^+$ regulatory T cell ($\text{CD}4^+$ Treg, identified as $\text{CD}4^+\text{CD}25^+\text{FoxP}3^+$), $\text{CD}8^+$ regulatory T cell ($\text{CD}8^+$ Treg, identified as $\text{CD}8^+\text{CD}25^+\text{FoxP}3^+$), and natural killer cell [natural killer cell (NK cell), identified as $\text{CD}3^-\text{CD}16^+\text{CD}56^+$] within seven days before (BT) and after (AT) IRE treatment. It was shown that compared with

Table I Clinicopathological Characteristics of Patients with LAPC Stratified by Treatment

Variable		Treatment		N	P	Variable		Treatment		N	P
		IRE	IRE +Toripalimab					IRE	IRE +Toripalimab		
Age (years)	≤60	41 (58.6%)	10 (66.7%)	51	0.772	CRP (ng/L)	≤3	43 (61.4%)	11 (73.3%)	54	0.556
	>60	29 (41.4%)	5 (33.3%)	34			>3	27 (38.6%)	4 (26.7%)	31	
Gender	Male	30 (42.9%)	8 (53.3%)	38	0.570	CEA (ng/mL)	≤5	47 (67.1%)	7 (46.7%)	54	0.151
	Female	40 (57.1%)	7 (46.7%)	47			>5	23 (32.8%)	8 (53.3%)	31	
WBC (*10 ⁹)	≤10	66 (94.3%)	15 (100%)	81	0.453	CA19-9 (U/mL)	≤35	21 (30.0%)	4 (26.7%)	25	0.533
	>10	4 (5.7%)	0 (0)	4			>35	49 (70.0%)	11 (73.3%)	60	
HGB (g/L)	≤175	17 (24.3%)	1 (6.7%)	18	0.175	CA125	≤35	43 (61.4%)	11 (73.3%)	54	0.482
	>175	53 (75.7%)	14 (93.3%)	67			>35	27 (38.6%)	4 (26.7%)	31	
PLT (*10 ⁹)	≤350	61 (87.1%)	14 (93.1%)	75	0.683	HBsAg	Absence	64 (91.4%)	15 (100%)	79	0.585
	>350	9 (12.9%)	1 (6.7%)	10			Presence	6 (8.6%)	0 (0)	6	
ALT (U/L)	≤50	55 (78.6%)	15 (100%)	70	0.062	Tumor grade	Well	41 (58.6%)	7 (46.7%)	48	0.409
	>50	15 (21.4%)	0 (0)	15			Moderate/ Poor	29 (41.4%)	8 (53.3%)	37	
AST (U/L)	≤40	55 (78.6%)	15 (100%)	70	0.062	Tumor size (cm)	≤2	4 (5.7%)	3 (20.0%)	7	0.184
	>40	15 (21.4%)	0 (0)	15			2~4	36 (51.4%)	7 (46.7%)	43	
ALP (U/L)	≤125	45 (64.3%)	13 (86.7%)	58	0.129		Tumor site	Head	36 (51.4%)	6 (40.0%)	
	>125	25 (35.7%)	2 (13.3%)	27		Body/tail		34 (48.6%)	9 (60.0%)	43	
GGT (U/L)	≤60	40 (57.1%)	11 (73.3%)	51	0.384	Chemotherapy before IRE	Absence	26 (37.1%)	5 (33.3%)	31	0.514
	>60	30 (42.9%)	4 (26.7%)	34			Presence	44 (62.9%)	10 (66.7%)	54	
ALB (g/L)	>40	11 (15.7%)	1 (6.7%)	12	0.683	Chemotherapy type before IRE	S-1	26 (37.2%)	5 (33.3%)	31	0.710
	≤40	59 (84.3%)	14 (93.3%)	73			AG	19 (27.1%)	3 (20.0%)	22	
TBIL (umol/L)	≤20.5	56 (80.0%)	15 (100%)	71	0.066		FOLFIRINOX	25 (35.7%)	7 (46.7%)	32	
	>20.5	14 (20.0%)	0 (0)	14							
IBIL (umol/L)	≤15	60 (85.7%)	15 (100%)	75	0.197						
	>15	10 (14.3%)	0 (0)	10							

Abbreviations: WBC, white blood cell; PLT, platelet; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase; ALB, albumin; TBIL, total bilirubin; IBIL, indirect bilirubin; CRP, C-reactive protein; HBsAg, hepatitis B surface antigen; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; AG, Abraxane-GEM; FOLFIRINOX, leucovorin, fluorouracil, irinotecan, and oxaliplatin.

IRE group, the absolute number of CD4⁺ T cells (P=0.038) and CD8⁺ T cells (P=0.024) steadily increased, while CD8⁺ Treg cells (P=0.023) decreased after treatment in IRE plus toripalimab group. The ratio of CD4⁺ T cells to CD8⁺ T cells was also decreased (P=0.019) in IRE plus toripalimab group (Figure 3).

Variation of Circulating Cytokines and Humoral Immune Parameters

Apart from the variations of immune cells, there were also huge differences on the serum levels of cytokines and immune parameters after treatment. Compared with levels before treatment, marked increases of IL-4, IL-6, TNF, and

IFN-γ were observed after both treatments. Additionally, the increased levels of IL-4, IL-6, TNF, and IFN-γ were much higher in the IRE + PD-1 group than those in the IRE group. The alteration of IL-10 after IRE treatment was not obvious, while it was significant after treatment with IRE combined with toripalimab. Apart from IgM, there were no significant differences in other immunoglobulin, including IgA, IgG, and complements, such as C3, C4, between levels before and after both treatments (Figure 4).

Safety of Treatment

The main treatment-related adverse events are shown in Table 4. No treatment-related deaths were observed in all

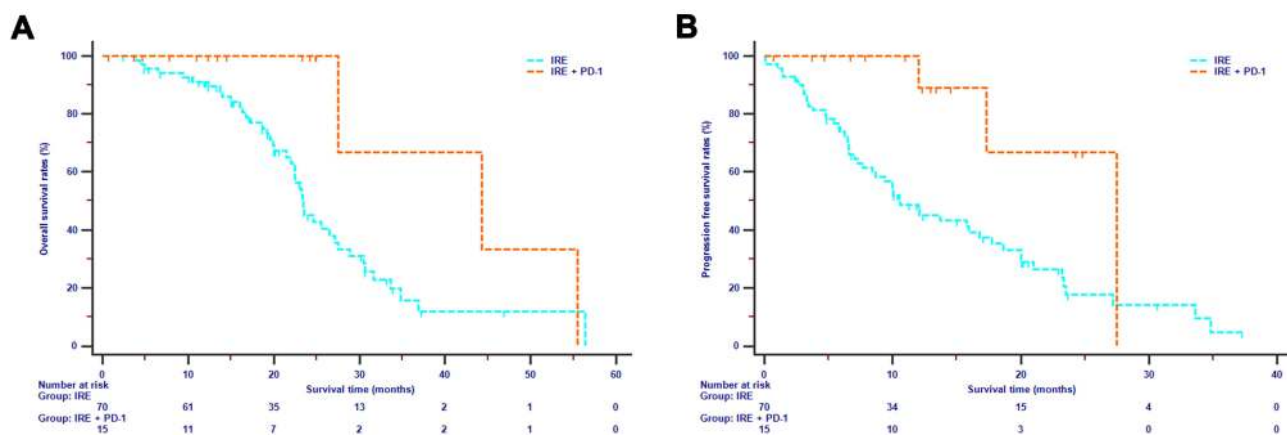


Figure 2 Overall survival (A) and progression free survival (B) analyses stratified by treatments in LAPC patients.

patients of this study. Loss of appetite, which was observed in 27.06% of all patients, happened most frequently, followed by nausea (25.88%), vomiting (22.35%), diarrhea (20.00%), pain (14.12%), and pancreatic fistula

(11.76%). Although pancreatic fistula, biliary fistula, abscess, vomiting and gastroparesis were a little more common in IRE plus toripalimab group, no significant differences were observed in the rates of all adverse events

Table 2 Independent Prognostic Factors for OS

Characteristics		OS					
		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P	HR	95%	P
Age (years)	≤60	Reference			Reference		
	>60	1.072	0.586–1.963	0.821			
Gender	Male	Reference			Reference		
	Female	1.167	0.640–2.127	0.614			
WBC (*10 ⁹)	≤10	Reference			Reference		
	>10	0.979	0.286–3.351	0.973			
HGB (g/L)	≤120	Reference			Reference		
	>120	0.373	0.194–0.719	0.003			
PLT (*10 ⁹)	≤300	Reference			Reference		
	>300	1.579	0.660–3.776	0.305			
ALT (U/L)	≤50	Reference			Reference		
	>50	0.995	0.499–1.983	0.989			
AST (U/L)	≤40	Reference			Reference		
	>40	1.059	0.501–2.241	0.880			
ALP (U/L)	≤125	Reference			Reference		
	>125	1.156	0.608–2.199	0.658			
GGT (U/L)	≤60	Reference			Reference		
	>60	1.000	0.541–1.850	0.999			
ALB (g/L)	>40	Reference			Reference		
	≤40	0.850	0.449–1.609	0.617			

(Continued)

Table 2 (Continued).

Characteristics		OS					
		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P	HR	95%	P
TBIL (umol/L)	≤20.5	Reference			Reference		
	>20.5	1.214	0.622–2.371	0.570			
IBIL(umol/L)	≤15	Reference			Reference		
	>15	1.255	0.551–2.859	0.589			
CRP (ng/L)	≤3	Reference			Reference		
	>3	1.763	0.973–3.197	0.062			
HBSAg	Absence	Reference			Reference		
	Presence	0.749	0.231–2.432	0.631			
CEA (ng/mL)	≤5	Reference			Reference		
	>5	0.913	0.485–1.718	0.777			
CA19-9 (U/mL)	≤35	Reference			Reference		
	>35	1.245	0.639–2.427	0.520			
CA125	≤35	Reference			Reference		
	>35	2.448	1.291–4.644	0.006	2.373	1.165–4.832	0.017
Tumor size	≤2	Reference			Reference		
	2~4	5.697	0.760–42.718	0.091	5.820	20.76–44.58	0.090
	>4	14.403	1.282–161.767	0.031	39.760	1.908–828.050	0.017
Tumor site	Head	Reference			Reference		
	Body/tail	0.789	0.426–1.462	0.452			
Tumor grade	Well	Reference			Reference		
	Moderate/Poor	1.726	0.951–3.133	0.073			
Treatment	IRE	Reference			Reference		
	IRE+toripalimab	0.301	0.092–0.985	0.047	0.274	0.076–0.985	0.047
Chemotherapy before IRE	Absence	Reference			Reference		
	Presence	0.444	0.235–0.840	0.013	0.557	0.171–1.378	0.076
Chemotherapy before IRE	S-I	Reference			Reference		
	AG	0.528	0.250–1.112	0.093	0.674	0.29–1.562	0.357
	FOLFIRINOX	0.382	0.180–0.808	0.012	0.439	0.183–1.051	0.065

Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval; NI, not include, other abbreviations as in Table 1.

(all $P > 0.05$). In terms of immune-related adverse events, one patient in the IRE plus toripalimab group developed a grade 2 rash, which was recovered after the treatment with corticosteroids. No immune-related adverse events were observed in IRE group.

Discussion

The poor survival for LAPC indicated the need in the development of treatment for these patients. As a special and relatively large group of pancreatic cancer, LAPC

showed significantly different biological aggressiveness and histology, compared with metastatic diseases, and also showed limited response to chemotherapy.^{24,25} Although patients may benefit from the extended surgery, the conversional resection rates varied in a great range from 0% to 43%.²² Additionally, relatively high rates of postoperative complications of extended surgery also prevented the treatment of surgery to some degree.²⁶ Considering the fact that progression of local disease, other than distant metastasis, contributed to most of the

Table 3 Independent Prognostic Factors for PFS

Characteristics		PFS					
		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P	HR	95%	P
Age (years)	≤60	Reference			Reference		
	>60	1.055	0.618–1.803	0.843			
Gender	Male	Reference			Reference		
	Female	0.909	0.694–1.190	0.486			
WBC (*10 ⁹)	≤10	Reference			Reference		
	>10	3.153	1.098–9.055	0.033	1.959	0.542–7.081	0.305
HGB (g/L)	≤120	Reference			Reference		
	>120	0.533	0.301–0.945	0.031			
PLT (*10 ⁹)	≤300	Reference			Reference		
	>300	2.309	1.048–5.087	0.038	1.155	0.413–3.227	0.784
ALT (U/L)	≤50	Reference			Reference		
	>50	1.644	0.900–3.003	0.106			
AST (U/L)	≤40	Reference			Reference		
	>40	1.568	0.820–3.001	0.174			
ALP (U/L)	≤125	Reference			Reference		
	>125	1.478	0.833–2.622	0.181			
GGT (U/L)	≤60	Reference			Reference		
	>60	1.275	0.745–2.182	0.376			
ALB (g/L)	>40	Reference			Reference		
	≤40	0.703	0.402–1.230	0.217			
TBIL (umol/L)	≤20.5	Reference			Reference		
	>20.5	1.373	0.760–2.481	0.294			
IBIL(umol/L)	≤15	Reference			Reference		
	>15	1.617	0.761–3.435	0.212			
CRP (ng/L)	≤3	Reference			Reference		
	>3	1.928	1.121–3.318	0.018	1.611	0.898–2.889	0.110
HBSAg	Absence	Reference			Reference		
	Presence	0.555	0.172–1.283	0.322			
CEA (ng/mL)	≤5	Reference			Reference		
	>5	1.130	0.646–1.978	0.668			
CA19-9 (U/mL)	≤35	Reference			Reference		
	>35	1.582	0.878–2.849	0.126			
CA125	≤35	Reference			Reference		
	>35	2.119	1.181–3.802	0.012	1.552	0.836–2.881	0.163
Tumor size	≤2	Reference			Reference		
	2~4	2.917	0.680–12.512	0.150	2.562	0.577–11.372	0.216
	>4	10.445	1.438–75.883	0.020	3.322	0.221–50.029	0.386
Tumor site	Head	Reference			Reference		
	Body/tail	0.678	0.393–1.167	0.161			

(Continued)

Table 3 (Continued).

Characteristics		PFS					
		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P	HR	95%	P
Tumor grade	Well Moderate/Poor	Reference 2.146	1.249–3.687	0.006	Reference 2.031	1.125–3.669	0.019
Treatment	IRE IRE+toripalimab	Reference 0.274	0.099–0.759	0.013	Reference 0.342	0.119–0.978	0.045
Chemotherapy before IRE	Absence Presence	Reference 0.996	0.565–1.754	0.988	Reference		
Chemotherapy before IRE	S-I	Reference			Reference		
	AG	0.989	0.502–1.949	0.975			
	FOLFIRINOX	1.001	0.530–1.890	0.998			

Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval; NI, not include, other abbreviations as in Table 1.

mortalities of LAPC patients, local destructive method is worthy of being tried.²⁷ As a non-thermal local destructive method, IRE induces apoptosis of tumor cells through causing irreversible permeabilization in cell membrane.⁸ Mounting evidences confirmed the safety and effectiveness of IRE in the treatment of LAPC.^{21,28} Additionally, our previous studies showed that compared with the conventional treatments, including chemotherapy and conventional resection, IRE followed by chemotherapy could further extend the survival time of LAPC patients.^{22,29} As a local treatment, radiotherapy plays a role in the localized control of LAPC. Chemotherapy followed by chemoradiotherapy is an option in patients with LAPC,

demonstrating stability than chemotherapy alone. However, current clinical trials comparing chemotherapy with chemoradiotherapy had reported mixed responses.^{30,31} Therefore, there was no consensus concerning the survival benefit of radiotherapy in patients with LAPC based on the current evidence and more clinical trials were needed to validate the role of radiotherapy in the treatment of LAPC. IRE is a novel local control treatment which has been recently applied in the treatment of LAPC. IRE was helpful for chemotherapy delivery to tumor by disrupting the dense stroma of pancreatic cancer.^{20,32} In addition, due to the feature of non-thermal ablation, electric field of extremely high voltage can be

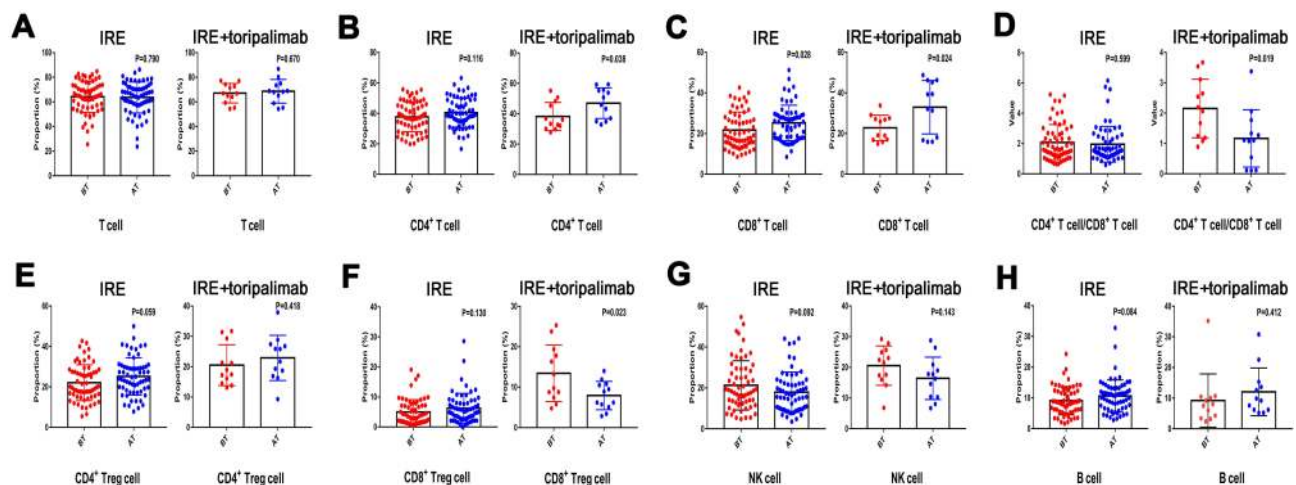


Figure 3 Distribution of serum concentration of T cell (A), CD4+ T cell (B), CD 8+ T cell (C), ratio of CD4+ T cell/CD8+ T cell (D), CD4+ Treg cell (E), CD8+ Treg cell (F), NK cell (H), and B cell (G) before and 7 days after IRE therapy.

Abbreviations: NK cell, natural kill cell; Treg cell, regulatory T cell; IRE, irreversible electroporation.

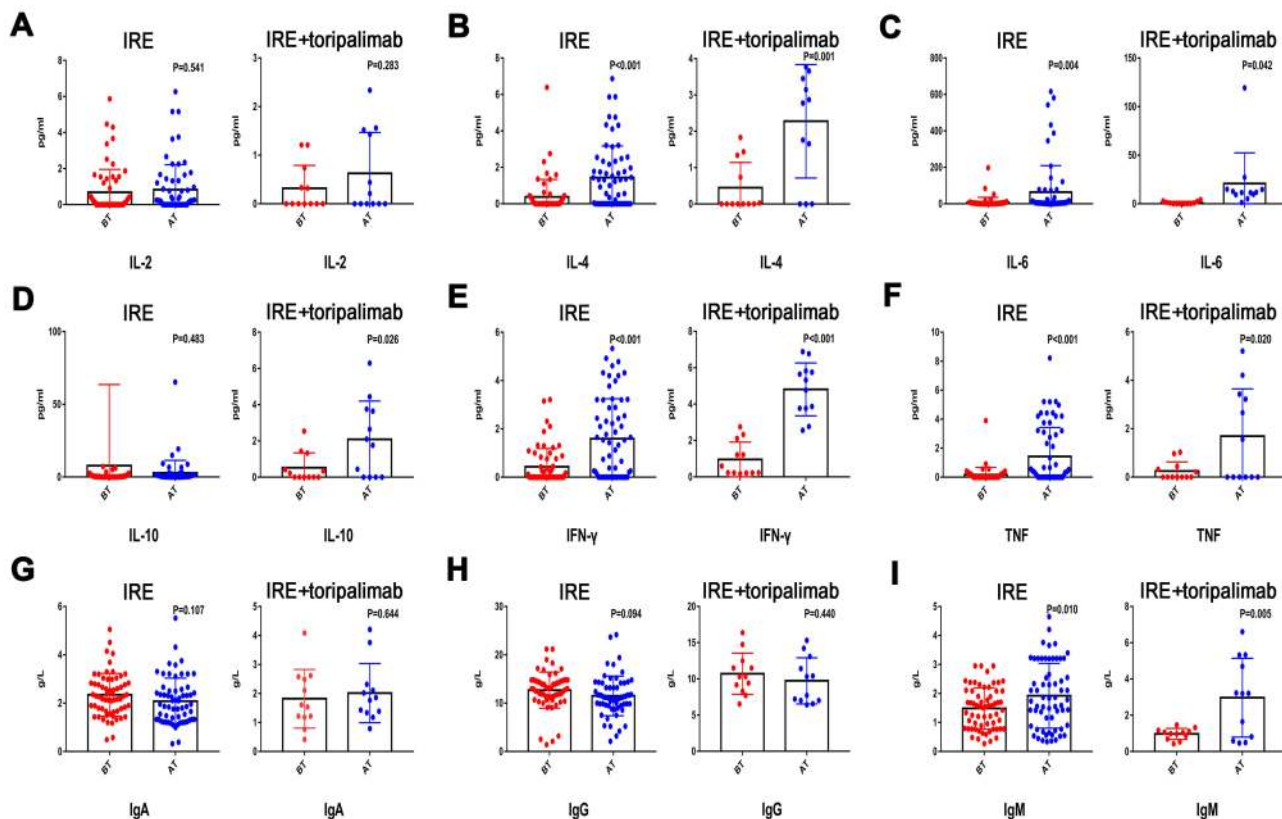


Figure 4 Distribution of serum concentration of IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IFN- γ (E), TNF (F), IgA (G), IgG (H), and IgM (I) before and 7 days after IRE therapy. **Abbreviations:** IL, interleukin; IFN- γ , interferon- γ ; TNF, tumor-necrosis factor; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IRE, irreversible electroporation.

applied through the whole tumor without harming nearby important structure in IRE treatment. In contrast, the duodenum and small intestine were easily harmed by high doses of radiation. Conventional radiotherapy was used most frequently in the treatment of LAPC and one of the reasons that studies failed to demonstrate the superiority of radiotherapy was the insufficient dose of radiation.³³ Therefore, compared with radiotherapy, IRE is more active in inducing tumor destruction and providing survival benefit for patients with LAPC. All these results indicated that IRE played an important role in the treatment of LAPC. The combination therapy of IRE and other treatment may be a promising method for further improving survival of LAPC patients.

In recent years, along with the improvement in the ICIs, immune therapy became more and more popular in the treatment of several malignant tumors.^{15,34} However, the immune-suppressive nature limited the efficacy of immune therapy in PDAC.³⁵ Simultaneously, more and more studies indicated that IRE could further be an immune-stimulating method other than induce tumor cell

death only.^{23,36} We previously found that IRE induced local immunomodulation by promoting M1 macrophage polarization and increasing specific T cell infiltration,^{9,10} indicating that the active immune status stimulated by IRE and the release of tumor antigen might further enhance the efficacy of immune therapy. Additionally, the low expression of PD-1 could contribute to the low response rates of ICIs, which could be partly reversed by the elevated expression of PD-1 in T cells induced by IRE.¹² Using the mouse model, it was shown that systemic antitumor immune response triggered by IRE could be enhanced by stimulating the innate immune system with a TLR7 agonist and the adaptive immune system with anti-PD-1 checkpoint blockade simultaneously.³⁷ Therefore, it was reasonable to speculate the hypothesis that IRE could provide sensibilization to ICIs in LAPC. This hypothesis was supported by the finding of Zhao's study, which reported that IRE enhanced the antitumor efficacy of immunotherapy by reversing resistance to immune checkpoint blockade.²⁰ Notably, the efficacy of this combination therapy was not validated in clinical practice.

Table 4 Comparisons of Complications in Two Treatment Groups

Complication		Treatment		N	P	Complication		Treatment		N	P
		IRE	IRE +Toripalimab					IRE	IRE +Toripalimab		
Hemorrhage	Absence	70	15	85	-	Diarrhea	Absence Presence	56	12	68	0.654
	Presence	0	0					14	3		
Pancreatic fistula	Absence	62	13	75	0.561	Gastroparesis	Absence Presence	68	14	82	0.446
	Presence	8	2					2	1		
Abdominal infection	Absence	66	14	80	0.631	Pancreatitis	Absence Presence	62	13	75	0.561
	Presence	4	1					8	2		
Biliary fistula	Absence	68	14	82	0.446	Abscess	Absence Presence	66	14	80	0.631
	Presence	2	1					4	1		
Vomiting	Absence	56	10	66	0.309	Pain	Absence Presence	59	14	73	0.683
	Presence	14	5					11	1		
Loss of appetite	Absence	52	10	62	0.537	Cardiac arrhythmias	Absence Presence	66	15	81	0.453
	Presence	18	5					4	0		
Nausea	Absence	52	11	63	0.585	Portal vein thrombosis	Absence Presence	67	15	82	0.554
	Presence	18	4					3	0		

Subsequently, we firstly built a new treatment regimen with IRE followed by systemic infusion of toripalimab. In the present study, it was shown that compared with IRE monotherapy, IRE combined with toripalimab significantly prolonged the survival of LAPC patients by enhancing immune function and suppressing tumor growth in this study. The OS of LAPC after IRE combined with toripalimab can be elevated to 44.3 months, indicating this treatment has great potential as a promising therapy for LAPC.

Remarkably, it was observed that there was a steady increase of CD4⁺ T helper cells, CD8⁺ T cytotoxic cells, while a reverse trend for CD8⁺ Treg cells was observed after IRE combined with toripalimab. In addition to the increase of T cells, the release of several cytokines, such as IL-4, IL-6, IL-10, TNF, and IFN- γ , were also elevated more significantly after treatment with IRE combined with toripalimab, compared with IRE alone, suggesting a stronger antitumor immune response. The improved expression of TNF and IFN- γ , which were mostly released by CD8⁺ T cytotoxic cells and induced specific immune killing, indicated that IRE plus anti-PD-1 therapy were more inclined to activate the specific immune system. Additionally, TNF- α not only played an important role in M1 macrophage polarization, but also contributed to the release of IL-4, IL-6, and IL-10 from

M1 macrophage to enhance the antigen-presenting function.³⁸ Similar with other study, higher levels of these immune-activating cytokines contributed to active immune system and maintain longer tumor progression-free status. This might partly contribute to the extended survival in LAPC patients after treatment with IRE combined with toripalimab.

Our study confirmed that the addition of anti-PD-1 therapy could further enhance the efficacy of IRE in the treatment of LAPC. In the present study, neoadjuvant chemotherapy was shown to independently indicate superior survival, indicating neoadjuvant chemotherapy was the basis of the combination therapy. The neoadjuvant chemotherapy, which aimed to eliminate some potential micrometastases, was extremely important for the cancer control. In terms of chemotherapy regimens, it was shown that survival of LAPC patients was not significantly influenced by neoadjuvant chemotherapy regimens. In the present study, only one-third of patients have received chemotherapy with full doses of FOLFIRINOX. This might partly explain the reason for the unobvious survival advantages of full doses of FOLFIRINOX, compared with other chemotherapy regimens. Therefore, it is believed that FOLFIRINOX combined with IRE and anti-PD-1 therapy would further improve the survival of LAPC patients, which will be further validated in the future

prospective studies. Additionally, similar with previous study,³⁹ more adverse effects were not observed from this combination therapy, compared with IRE therapy alone, showing the safety of combination method. In this study, toripalimab was administrated on the seventh day after IRE. An appropriate time interval not only provides a time window for immune activation,⁹ but also is helpful for the recovery from the prior treatment, which may partly contribute to the low rates of adverse effects of this study. In summary, our study indicated that the survival time of LAPC patients was further improved after anti-PD-1 therapy combined with IRE, indicating this combination therapy might be a suitable treatment for LAPC patients.

There were several limitations in this study. First, the retrospective design and the nonrandomized nature made it vulnerable to a variable of potential biases even though the distribution of basic characteristics was balanced between these two groups. The lack of microsatellite instability (MSI) status and the response of neoadjuvant chemotherapy were also limitations. More prospective randomized controlled trials are necessary to verify the results of this study. Second, the numbers of included patients were small. Although no similar studies had been reported before, the efficacy of IRE combined with anti-PD-1 therapy should be validated in more LAPC cohorts. Third, the follow-up time was a little insufficient for OS because the survival endpoint of OS was obtained in only nearly half of all patients. Subsequent results with longer follow-up time were necessary for a more complete overview of this combination therapy.

Conclusion

In conclusion, compared with IRE, IRE combined with toripalimab significantly prolonged survival of LAPC patients. A randomized clinical trial evaluating this combination therapy is therefore warranted.

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Disclosure

The authors declare no conflict of interest.

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