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Original Paper

Circulating Tumor DNA as a Sensitive Marker in Patients Undergoing Irreversible Electroporation for Pancreatic Cancer

Mao Lin[a,b] Mohammed Alnaggar[c,d] Shuzhen Liang[a,b] Jibing Chen[a,b] Kecheng Xu[a,b] Shihua Dong[e] Duanming Du[f] Lizhi Niu[a,b,c]

[a]Department of Biological Treatment Center, Fuda Cancer Hospital, Jinan University School, Guangzhou, [b]Fuda Cancer Institute, Guangzhou, [c]Department of Oncology, Fuda Cancer Hospital, Jinan University School, Guangzhou, [d]Biomedical Translational Research Institute and The First Affiliated Hospital, Jinan University, Guangzhou, [e]Laboratory of RNA Epigenetics, Institutes of Biomedical Sciences & Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Shanghai, [f]Department of Interventional Therapy, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China

Key Words

Circulating tumor DNA • KRAS • Pancreatic ductal adenocarcinoma • Overall survival • Droplet digital polymerase chain reaction

Abstract

Background/Aims: Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed at an advanced stage, resulting in extremely poor 5-year survival. Late diagnosis of PDAC is mainly due to lack of a reliable method of early detection. Carbohydrate antigen (CA) 19-9 is often used as a tumor biomarker in PDAC; however, the test lacks sensitivity and specificity. Therefore, new sensitive and minimally invasive diagnostic tools are required to detect pancreatic cancer. ***Methods:*** Here, we investigated circulating tumor DNA (ctDNA) which contained KRAS-mutated as a potential diagnostic tool for PDAC patients who underwent irreversible electroporation (IRE). We used droplet digital polymerase chain reaction (ddPCR) to detect the expression of KRAS-mutated genes in plasma samples of 65 PDAC patients who underwent IRE. ***Results:*** In these 65 cases, ctDNA was detected in 20 (29.2%) samples. The median overall survival (OS) was 11.4 months with ctDNA+ patients and 14.3 months for ctDNA- patients. ctDNA+ patients had a obviously poorer prognosis associated to overall survival ($P < 0.001$). ***Conclusion:*** Our results suggested that the existence of ctDNA was a predictor of survival for PDAC patients. Therefore, ctDNA may be a new sensitive biomarker for monitoring treatment outcome in PDAC.

Mao Lin and Mohammed Alnaggar contributed equally to this work.

Lizhi Niu
and Duanming Du

Department of Biological Treatment Center, Fuda Cancer Hospital
Jinan University School, Guangzhou, (China)
E-Mail 18922210657@163.com / linmaogz@163.com

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant cancers of the digestive system, with incidence invisible and rapid disease progression [1]. Therefore, most PDAC patients are at an advanced stage, owning a bad prognosis [2] with limited opportunity for surgery. One treatment option for PDAC is IRE, which is an emerging, non-thermal, image-guided tumor ablation technique. IRE has been proven to be feasible and safe to locally advanced pancreatic cancer (LAPC), [3-6] and is approved in latest pancreatic cancer (PC) treatment guidelines.

Up to now, the diagnosis of PC mainly relies on imaging modalities [7]; however, early stage PC is difficult to diagnosis, even if the combination imaging techniques are used. A cheap, simple and minimally invasive alternative to imaging is detection of the CA 19-9, but the sensitivity and specificity of the CA 19-9 test is not sufficient for effective early detection of PC. Hence, there is increasing demand to explore the non-invasive biomarkers for early PDAC.

ctDNA has been studied recently [8, 9]. However, the feasibility of detecting ctDNA in PC has not yet been confirmed just for lack of suitable techniques to quantify the target DNA. Droplet digital polymerase chain reaction (ddPCR) is a novel next-generation PCR technique that more sensitivity than qPCR [10]. Accordingly, with the high detection function, ddPCR may be used to quantify ctDNA in PDAC.

Recently, PDAC has been studied at the whole genome level, and some alterations associated with PDAC have been proven [11-13]. There are four genes (TP53, CDKN2A, KRAS and SMAD4) often mutated in PDAC [14]. Among these genes, we assumed that KRAS was likely to be the bestcharacterised tumour-related gene because of following reasons . First, among all human malignancies, PDAC exhibits the highest frequency (75%–100%) of KRAS mutations [15-17]. Second, in PDAC, the most frequent KRAS point mutations are located in two consecutive nucleotides in codon 12 [18]. Third, alterations in this gene appear to occur at an early stage of pancreatic carcinogenesis [19, 20]. Therefore, KRAS-mutated ctDNA represents an important potential biomarker of PDAC.

This study aimed to use ddPCR to detect ctDNA in PDAC patients following IRE, and then determine the association between the ctDNA KRAS status and overall survival.

Materials and Methods

Ethical approval

This clinical research was approved by the Fuda Cancer Hospital ethics committee. According to the Declaration of Helsinki, written informed consent was obtained from each participant.

Patients

Between January 2015 and September 2016, 65 PDCA patients suffered IRE in Fuda Cancer Hospital. At the same time, matched tumor issues and blood samples were collected from 65 PDAC patients. The disease progression was confirmed by imaging at the last follow up. The patients demographic were summarized in Table 1.

Primary tumor samples

In this study, the tumor samples were obtained from biopsy specimens. Only samples diagnosed by histopathologically were admitted. All issue samples were frozen at -80 ºC, and the DNA was extracted using phenol-chloroform-isoamyl alcohol. Approximately 100 ng μl^{-1} extracted DNA solutions were used for ddPCR.

Plasma samples

85 plasma samples were collected, including 20 healthy volunteer samples (11 men and 9 women, ages 28–62 years with no evidence of malignancy). 8 ml whole blood samples were collected before IRE.

Blood collected in EDTA tubes was processed within one hour of sample collection and centrifuged at 820 g for 10 min to separate the plasma from the peripheral blood cells. The plasma was then further centrifuged at 20, 000 g for 10 min to pellet any remaining cells. The plasma was then stored at -80°C until DNA extraction. According to the manufacturer's instructions, cfDNA was extracted from 2 ml plasma, eluted in 100 µl buffer with a QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) [21, 22].

TaqMan assay for specific KRAS amplification

For ddPCR, we used a commercially available Prime PCR for ddPCR KRAS kit

(Bio-Rad, Hercules, CA, USA), which contained sequence-specific forward and reverse primers with dual-labelled FAM- and HEX-labelled fluorescent TaqMan probes intended to conjugate the target and reference regions, respectively [23].

Early reports identified several point mutations in the KRAS oncogene, including Gly12Asp (G12D), Gly12Val (G12V), and Gly12Arg (G12R). Accordingly, these three most frequent mutations in codon 12 of KRAS were amplified in each sample.

Table 1. Patient demographics

Demographics		No. of patients (%)
Median Age, years (range)	57 (40–74)	
Gender	Male	34 (52%)
	Female	31 (48%)
UICC final stage	I/II	5 (8%)
	III/IV	60 (92%)
Adjuvant chemotherapy	Received	57 (88%)
	Gemcitabine	12 (19%)
	S-1	4 (5%)
	Gemcitabine + S-1	41 (64%)
	Not received	8 (12%)

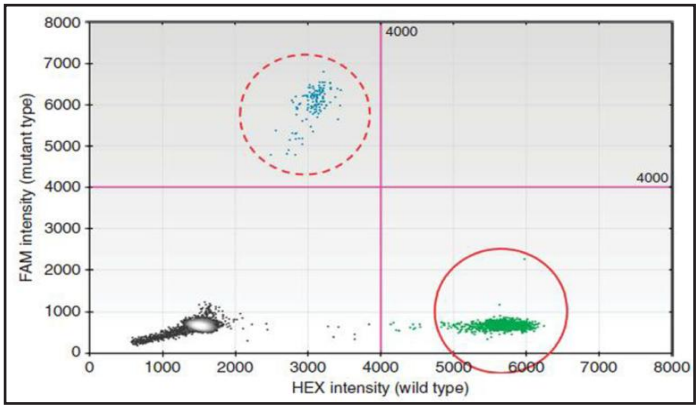


Fig. 1. ddPCR assay for KRAS amplification. FAM (blue) and HEX (green) fluorescence levels were plotted for each droplet. Clusters in the upper and right halves of the plot (dashed circle and solid circle) represent the positive mutant and wild-type KRAS end-point results, respectively.

ddPCR

The KRAS mutation status was analyzed by a Bio-Rad QX100 ddPCR system. Once PCR amplification was performed in each droplet, target DNA molecules can easily be uniformly distributed across thousands of emulsified droplets. After amplification, reactions containing target DNA molecules represented the positive end-point, and conversely, the negative end-point. At last, through Poisson statistics, from the positive end-point reactions fraction, the number of target DNA molecules can be calculated [24].

The total reaction mixture was 22 µl, including: 10 µl 2 × Bio-Rad ddPCR mix, 1 µl each reference and 20 × PCR ddPCR KRAS- prime, and 10 µl template DNA. The mixtures joined into 45 µl droplet generation oil, then fitted into a droplet generator cartridge. They were transferred into a 96-well plate, and subjected to thermo cycling: 95ºC for 8 min; 50 denaturation cycles at 95ºC for 10 s and extension at 55ºC for 1 min; and finally extension at 95ºC for 10 min. After thermo cycling, the droplets were analyzed right away. Next, the number of target DNA was counted by the Bio-Rad QuantaSoft analysis software (Fig. 1). In order to counteract the false-negative results, per plasma sample had eight replicates running.

Statistical analysis

Patient's survival analyses according to the seven elements: gender, age, lymph node metastasis, Union for International Cancer Control (UICC) final stage, KRAS status, CA19-9, and ctDNA presence. Statistical

analyses were performed by the chi-squared test, U test, and Fisher's test. OS was analyzed by Kaplan-Meier method and using the log-rank test to compare. Significant differences were indicated by $P < 0.05$, $P < 0.01$ or $P < 0.001$. All statistical analyses were performed using SPSS 17.

Results

Evaluating ddPCR

To verify the ddPCR capability for detecting ctDNA, we performed an initial study using serial dilutions of a positive control and wild- type plasma samples from healthy controls. We found that our assay could detect a mutation prevalence of 0.01–0.1% (Fig. 2A), which corresponded to 1 mutant copy per background of 10^3-10^4 wild-type copies, and we established a baseline for the positive result of each KRAS mutation (Fig. 2B).

Patient characteristics

In this study, 65 patients were evaluated. 52 (80%) patients had KRAS mutations. The frequencies of the wild-type KRAS, G12V, G12D, and G12R mutations were 20% (13/65), 31% (20/65), 35% (23/65), and 14% (9/65), respectively. There were 20 (31%) of the 65 matched plasma samples at 10.8 copies/ml (range: 3.1–264 copies/ml) existed KRAS-mutated ctDNA. The median of wild-type KRAS fragments is 2810 copies/ml (range: 1234–35230 copies/ml), and the percent of mutant KRAS fragments was 0.05% to 9.5% in the ctDNA+ samples. The frequencies of the G12V, G12D, and G12R were 25% (5/20), 70% (14/20), and 5% (1/20) ctDNA+ samples (Fig. 3A).

The KRAS status consistence of the tissues and plasma samples matched 100% (20/20). In Table 2, the pathological characteristics between the ctDNA+ and ctDNA- groups were listed. There were no significant differences in the demographic characteristics associated with ctDNA+ results. And there was no obviously increase in ctDNA concentration according to tumor stage (Fig. 3B).

Survival analysis

A median follow-up period was 21 months (10 - 32 months). There was no significant difference in OS ($P > 0.05$) between wild-type KRAS tumors patients (n = 13) and mutant KRAS patients (n = 52; Fig. 4A). Aimed at the KRAS mutation subtypes, there were also no differences in median OS (Fig. 4B). On the contrary, the ctDNA presence of plasma samples was significantly in connection with a poor prognosis of OS. The median OS was 11.4 months in ctDNA+ patients, and 14.3 months in ctDNA- patients (Fig. 5A, $P < 0.001$). However, according to the KRAS mutation subtype in ctDNA, no differences were found in OS (Fig. 5B).

Nine independent demographic and clinicopathological variables were included in the univariate OS analysis. In the univariate analysis, four factors (CA19-9, lymph node metastasis, UICC stage, and ctDNA presence) were identified as significant prognostic elements related to OS. Then the four factors were conducted

Table 2. Clinicopathological features between the ctDNA+ and ctDNA- groups

Demographics	ctDNA (No. of patients)		P-value
	Positive	Negative	
Age, years			0.34
< Median (69)	10	23	
≥ Median	12	20	
Gender			0.62
Male	12	22	
Female	10	21	
Lymph node metastasis			0.21
Yes	23	37	
No	1	4	
UICC final stage			0.39
I/II	2	3	
III/IV	21	39	
Adjuvant chemotherapy			0.24
Received	18	39	
Not received	3	5	

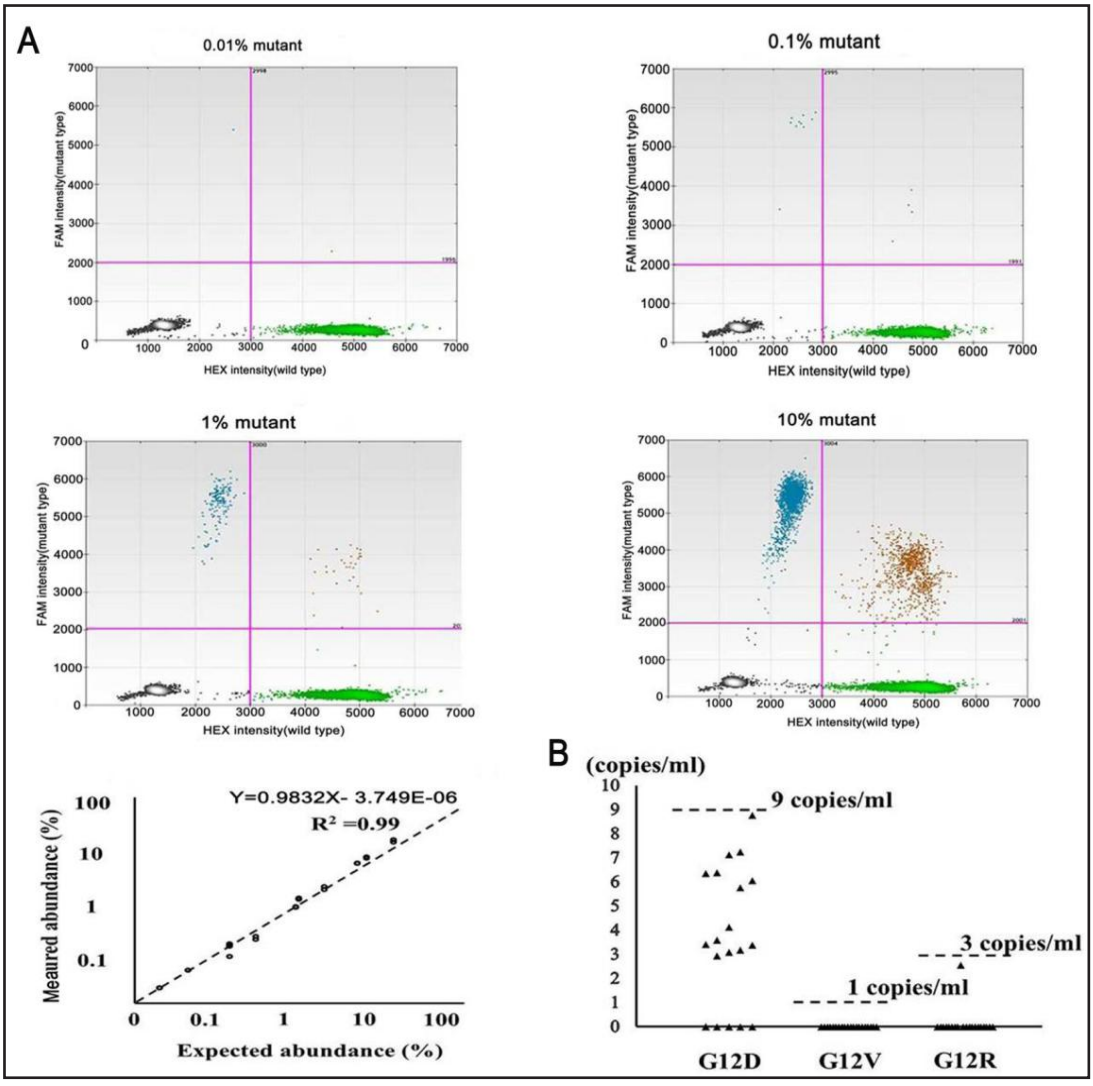
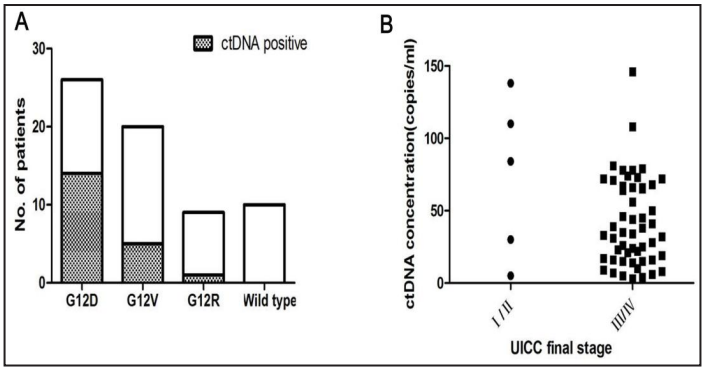


Fig. 2. Limit of detection and threshold baseline for a positive result. (A) Analysis of serial dilutions (0.01%, 0.1%, 1%, and 10%) for detecting mutation prevalence. (B) Establishing the threshold baseline for a positive result for each type of KRAS mutation.

Fig. 3. ctDNA detection in patients (A) Frequency of KRAS mutations in all tumor specimens and plasma samples. (B) ctDNA concentrations in all patients subdivided according to the UICC stage.



in a multivariate Cox proportional hazards regression test: lymph node metastasis (HR = 2.1, 95% CI: 1.2–4.3, $P = 0.025$), UICC final stage (HR = 1.9, 95% CI: 1.2–3.3, $P = 0.014$), and the ctDNA presence (HR = 3.1, 95% CI: 1.6–4.9, $P < 0.001$) were identified as independent

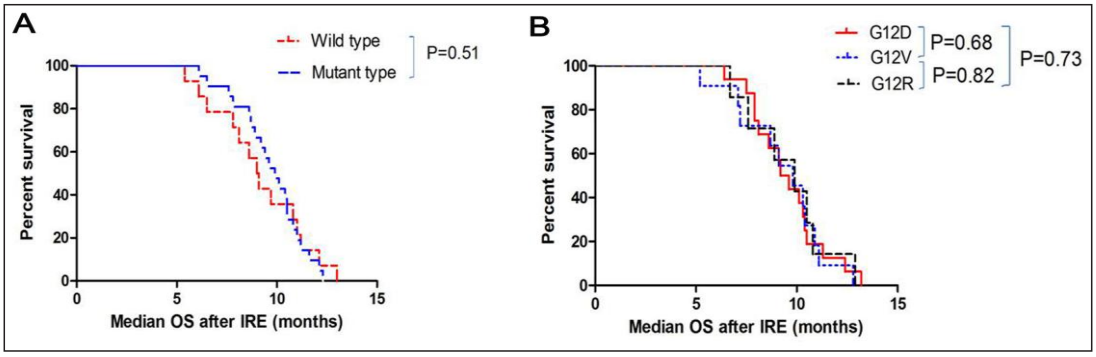


Fig. 4. OS curves according to (A) the KRAS status and (B) KRAS mutation subtypes. (A) There was no significant difference in OS (P = 0.51) between patients with wild-type KRAS tumors (n = 13) and those with mutant KRAS (n = 52). (B) No differences in median OS were observed according to the KRAS mutation subtype.

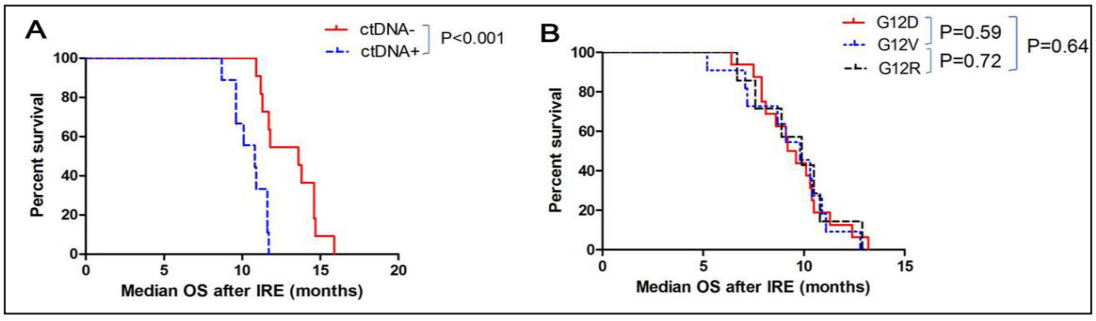


Fig. 5. OS curves according to the (A) presence of ctDNA and (B) KRAS mutation subtypes of ctDNA. (A) The median OS times were 11.4 months in patients who were ctDNA+ and 14.3 months in patients who were ctDNA- (P<0.001). (B) There were no differences in OS according to KRAS mutation subtype in ctDNA.

factors related to poor prognosis. We also found CA19-9 with a high level (HR = 1.1, 95% CI: 0.6–2.1, *P* = 0.54) and had no connection with OS (Table 3).

Discussion

PDAC has a very poor prognosis, mainly due to lack in reliable screening methods. Hence, a predictive and precise marker is urgently demanded. CA19-9 was the most frequent biomarker in PDAC for monitoring treatment and predicting survival in a previous study [25]. Nevertheless, CA19-9 levels are also elevated in some benign diseases, such as liver disease and pancreatitis [26]. There are also some potential pancreatic cancer markers reported in recent years [27-30].

In some previous reports, KRAS status has also been reported in PDAC as a useful prognostic biomarker, which displayed significant differences in OS between wild-type KRAS tumor patients and mutant KRAS tumor patients [17]. It has been reported that KRAS mutation subtypes (such as G12D, G12R) was related to shorter survival periods [31, 32]. These studies suggest the KRAS mutation status has feasibility to use in PDAC as a prognostic biomarker. However, some studies have reported results with controversial [33, 34]. Likewise, our study verified no significant differences in OS between wild-type and mutant KRAS tumors (Fig. 4A and B). Therefore, the clinical feasibility of these biomarkers remains questioningly.

Meanwhile, through univariate and multivariate analyses, our study demonstrated that KRAS-mutated ctDNA was obviously related to poorer survival, and the ctDNA presence

was the only prognostic factor before IRE. Moreover, after IRE, ctDNA+ patients displayed significantly poorer prognosis in OS (Fig. 5A). This result suggested that ctDNA diffused into the systemic circulation in some cases. Indeed, ctDNA+ patients may be related to micrometastasis which cannot be found by imaging, as described by other researchers [35].Therefore, ctDNA+ patients should be considered for other options before IRE, such as moderate chemotherapy.

Table 3. Univariate and multivariate analysis of OS

		Survival		Univariate analysis	Multivariate analysis	
Prognostic factors	No. of patients	Median (months)	1-year (%)	P-value	HR (95% CI)	P-value
Age, years						
< Median (69)	32	12.4	49	0.63		
≥ Median	33	11.5	51			
Gender						
Male	35	11.9	42	0.21		
Female	30	12.6	58			
Lymph node metastasis						
Yes	60	11.2	39	<0.001	2.1(1.2–4.3)	0.025
No	5	35.2	65			
UICC final stage						
I/II	5	22.4	61	<0.001	2.2(1.1–3.3)	0.014
III/IV	60	10.8	23			
KRAS status						
Wild type	13	14.4	67	0.24		
Mutant type	52	11.6	44			
Pre-IRE CA19-9						
< 90 Uml^{-1}	24	13.1	58	0.034	1.1(0.6–2.1)	0.54
≥ 90Uml^{-1}	41	11.7	39			
Presence of ctDNA						
Negative	45	14.7	56	<0.001	3.1(1.6–4.9)	<0.001
Positive	20	10.1	22			

In this study, there was no obvious relation between ctDNA concentrations and UICC stage (Fig. 3B). But it was worth to note that there were no patients survived beyond 1 year who exceeded 20 copies/ml for ctDNA (n = 6, data not shown). For example, we found a unique case of a PDAC patient died within 1 month who had more than 5000 copies/ml. These results suggest that high ctDNA concentration and poorer prognosis are closely related. Likewise, Bette et al [36]. reported a decreased survival rate accompanied by an increase in ctDNA concentration. There was also verified that reduction of ^{mut}KRAS cftDNA in PDCA patients displayed longer median PFS and OS than increase of ^{mut}KRAS cftDNA after given first-line 5-fluorouracil, irinotecan and oxaliplatin or gemcitabine and nab-paclitaxel at day 15 [37].

There are some limitations to our study. For example, we only have a time point of collecting blood before IRE. Comparison between ctDNA concentrations of pre-IRE and post-IRE would be more informative. Following IRE, the changes of ctDNA concentration can display important and interesting information which reflect the PDAC clinical efficacy [38, 39]. Therefore, in future, prospective studies will be needed.

Conclusion

In summary, our study demonstrated a feasibility of KRAS-mutated ctDNA as a precise and prognostic biomarker to PDAC patients who underwent IRE.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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