

Review

Circulating Tumor Cells and Circulating Tumor DNA Provide New Insights into Pancreatic Cancer

Yang Gao, Yayun Zhu, Zhou Yuan✉

Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, People's Republic of China

✉ Corresponding author: Zhou Yuan, zhouyuan669@163.com

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Abstract

Pancreatic cancer has a rather dismal prognosis mainly due to high malignance of tumor biology. Up to now, the relevant researches on pancreatic cancer lag behind seriously partly due to the obstacles for tissue biopsy, which handicaps the understanding of molecular and genetic features of pancreatic cancer. In the last two decades, liquid biopsy, including circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), is promising to provide new insights into the biological and clinical characteristics of malignant tumors. Both CTCs and ctDNA provide an opportunity for studying tumor heterogeneity, drug resistance, and metastatic mechanism for pancreatic cancer. Furthermore, they can also play important roles in detecting early-stage tumors, providing prognostic information, monitoring tumor progression and guiding treatment regimens. In this review, we will introduce the latest findings on biological features and clinical applications of both CTCs and ctDNA in pancreatic cancer. In a word, CTCs and ctDNA are promising to promote precision medicine in pancreatic cancer.

Key words: Circulating tumor cells; Circulating tumor DNA; Pancreatic cancer; Precision medicine; Metastasis; Drug resistance; Tumor heterogeneity

Introduction

Pancreatic cancer is one of the most devastating malignant tumors with a 5-year survival rate of approximately 5% and increasing incidence rate, which is the seventh leading cause of cancer related death in both men and women worldwide [1-3]. In 2015, about 48,960 new cases are expected to occur and about 40,560 people are expected to die from pancreatic cancer in USA [4]. In China, the incidence of pancreatic cancer has reached 14-17 per 100,000 people in some area [5]. What's worse, the annual mortality of pancreatic cancer almost equals to the morbidity. The poor prognosis of pancreatic cancer is mainly associated with delayed diagnosis, deep anatomic location and non-specific symptoms. At present, surgical resection is the only potentially curative treatment for pancreatic cancer. Unfortunately, only 15%-20% of patients are candidates for pancreatectomy at the time of diagnosis [2]. Tissue biopsy is the golden standard for

the diagnosis of pancreatic cancer for those patients without surgery or before neoadjuvant therapy administration. However, there are many obstacles for tissue biopsy, including potential surgical complications, tumor dissemination, and false negative results [6, 7]. In addition, sufficient material from primary tumors in pancreatic cancer is scarce as the majority of patients present with advanced disease and only biopsy material is available and thus CTC and ctDNA can help fill this gap in order to perform the genomic analysis.

Recently, liquid biopsy, as a less invasive approach, is becoming the research hotspot and attracts much attention owing to remarkable advantages. The broad conception of liquid biopsy includes circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating microRNAs, circulating proteins, extracellular vesicles and so on [8, 9]. Particularly, CTCs and ctDNA are crucial

components in the realm of liquid biopsy. CTCs and ctDNA have several prominent characteristics for cancer managements: (1) assessing risk factor and achieving early diagnosis; (2) monitoring treatment response and drug resistance dynamically; (3) providing prognostic information by evaluating relapse and metastatic risk; (4) opening a window for studying tumor heterogeneity and evolution procedure; (5) helping to understand the tumorigenesis, recurrence and metastasis [10, 11]. Recently, a great deal of attention has been focused on pancreatic cancer and some remarkable findings on biological underpinnings have been made via CTCs and ctDNA [9, 12, 13]. In this review, we will summary the relevant studies on CTCs and ctDNA and their potential applications in managements of pancreatic cancer.

Biological characteristics of CTCs and ctDNA

CTCs are shedding from both primary and secondary tumors into bloodstream [14]. CTCs are generally more likely to be detected in advanced tumors due to higher tumor burden. Meanwhile, CTCs could also appear unexpectedly early in the disease process, after primary radical treatment, and even when clinically detectable tumor or recurrence doesn't appear [15, 16]. Because the half-time of CTCs

is quite short (1-2.4 hours), they could reflect the current status of both primary tumors and secondary deposits accurately and sensitively [17]. In particular, a subset of CTCs have the phenotypes of cancer stem cells (CSCs), which may initiate tumor formation and drug resistance [18]. The mutual transformation of CTCs and CSCs are linked by epithelial-to-mesenchymal transition (EMT) process (Figure 1) [19]. CTCs bear great potential for early diagnosis, treatment monitoring, and predicting prognosis for various cancer types. With the development of detection technology of CTCs, some sophisticated and exquisite devices have been developed for efficient enrichment and identification of CTCs, especially for viable CTCs, which paves the way for further exploration of tumor heterogeneity, tumor metastasis, and drug resistance [20, 21].

Similar to CTCs, ctDNA provides another approach for monitoring tumor genome as a less invasive approach and it has unique features compared to CTCs. ctDNA is generally considered to be released from necrotic, apoptotic cells in primary tumors, secondary deposits and CTCs [9, 22, 23]. As a fraction of cell free DNA (cfDNA) with genetic mutations (range from 0.01 % and more than 90%, usually 1.0%), ctDNA represents an average of DNA released by all tumor cells, so it has the potential to reflect the entire tumor burden [24, 25]. For pancreatic

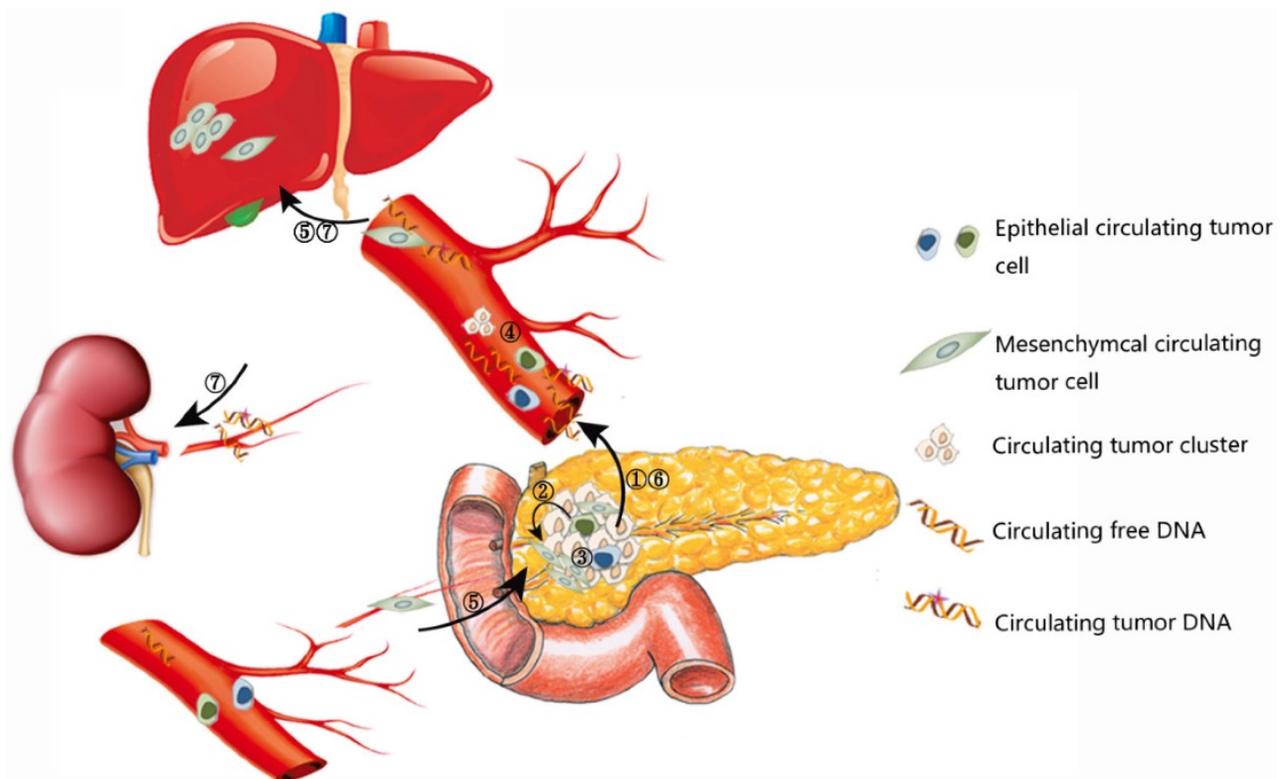


Figure 1. Release of CTCs and ctDNA into the circulation.

cancer, global genomic sequencing of 24 patients revealed an average of 63 genic alterations associated which defined 12 core cellular signaling pathways [26] and the average number of mutated genes in pancreatic cancer ranged from 26 to 42 [27, 28]. In regard to specific mutation type, a recent proof-of-concept study including 99 patients demonstrated that approximately 90% genetic variations were point mutations and small indels, the rest were mainly structural variants [29]. Consistent with the previous studies, the most prevalent mutated genes proved to be KRAS, TP53, SMAD4, CDKN2A and ARID1A. Besides, novel candidate drivers of pancreatic carcinogenesis (KDM6A and PREX2) were also identified [26, 28]. A series of genetic variations lead to carcinogenesis and development of pancreatic cancer. When the mutated DNA was released to bloodstream in a passive or active way, they could be detected by different methods and the detected mutated DNA could well reflect the tumor genomic landscape [25, 30], so the underlying genetic changes revealed by novel sequencing technology will accelerate the development of liquid biopsy.

CTC detection platforms for pancreatic cancer

Many clinical and preclinical studies on pancreatic CTC have been performed via various devices (summarized in **Table 1**). Notably, the classic EpCAM-dependent CellSearch system rendered limited detection rate for pancreatic cancer (11% in

localized advanced pancreatic cancer and 19% in metastatic pancreatic cancer) [31, 32]. The relative low CTC number may result from three reasons. (1) CTCs get trapped in liver as blood flows through portal vein into systematic circulation [33]. (2) The blood flow decreases by 60% in malignant pancreatic tumors compared with normal pancreatic tissues, so fewer tumor cells had the chance to invade into the bloodstream [34]. (3) The process of EMT decreased expression of epithelial markers, such as E-cadherin and EpCAM, making them undetectable by epithelial marker-dependent approaches [15, 35]. Several modified devices have been developed for better detection of pancreatic CTCs. Immuno-FISH platform is a negative-enrichment method for CTC detection and our preliminary results showed that the sensitivity could reach 100% by combining CTC and CA19-9 [36]. The PCR-based strategy has also been reported to detect pancreatic CTCs, but the platform may produce false-positive results [37, 38]. The size-based filtration devices could potentially overcome some limitations in other platforms and has achieved satisfactory results in pancreatic cancer [39, 40]. This approach provides an exciting potential strategy for studying the mechanism of metastases, and predicting clinical outcome by separating both epithelial and mesenchymal CTCs, culturing viable and virginal CTCs [40, 41]. Furthermore, CTC captured by size-based platform can be validated by looking for tumor specific mutations such as a KRAS mutation which occurs in up to 95% of primary tumors [42, 43].

Table 1. Summary of clinical studies on CTCs in pancreatic cancer

Reference	Positive criteria	Positive rate	Mean±SD	No. of patients	Median OS with vs without	Technique
Z'Graggen, et al, 2001 [57].	AE1/AE3-positive	26%	NR	27/105	NS (P=0.35)	Immunocytochemical assay
Allard, et al, 2004 [31].	≥2 CTCs/7.5 ml	19%	2±6/7.5 ml	4/21	NR	CellSearch system
Nagrath, et al, 2007 [45].	≥5 CTCs/ml	100%	196±228/ml	15/15	NR	CTC chip
Kurihara, et al, 2008 [58].	≥1 CTC/7.5 ml	57%	22.8±35.0/7.5 ml	8/14	52.5 vs 308.3 days (P<0.01)	CellSearch system
Zhou, et al, 2011 [59].	EpCAM-positive	100%	NR	25/25	NR	RT-PCR
Khoja, et al, 2012 [60].	≥1 CTC/7.5 ml	39%	6/7.5 ml	21/54	164 vs 127 days (P=0.19) NS (P=0.36)	CellSearch system
Bidard, et al, 2013 [32].	≥1 CTC/7.5 ml	89%	26/7.5 ml	24/27		ISET
	≥1 CTC/7.5 ml	11%	2.7±4.6/7.5 ml	11/79	11 vs 13 months (P=0.01) NR	CellSearch system
Sheng, et al, 2014 [61].	≥1 CTC/ml	50%	8 and 44/7.5 ml	2/4		GEM chip
Bobek, et al, 2014 [41].	Cytomorphological features	94%	2.8±1.8/ml	17/18	NR	MetaCell technology
Cauley, et al, 2015 [62].	Positive-stained	66.7%	NR	16/24	NR	
Zhang, et al, 2015 [63].	≥2 CTCs/ 3.5ml	49%	NR	51/105	NS (P=0.69)	ScreenCell device
Kulemann, et al, 2016 [39].	Positive-stained and KRAS mutation	68.18%	7.4±13.9/3.5 ml	15/22	NR (P=0.0458)	Immuno-FISH
Katherine, et al, 2016 [40]	≥1 CTC/ml	86%	NR	18/21	16 vs 10 months (P=NS)	ScreenCell device
		78%	30/ml	39/50	13.7 vs not reached (P=0.008)	ISET

Abbreviations: SD, standard deviation; OS, overall survival; NR, no reports of CTC number or overall survival; NS, no significant difference, ISET, isolation by size of epithelial tumor cells; GEM chip: geometrically enhanced mixing chip.

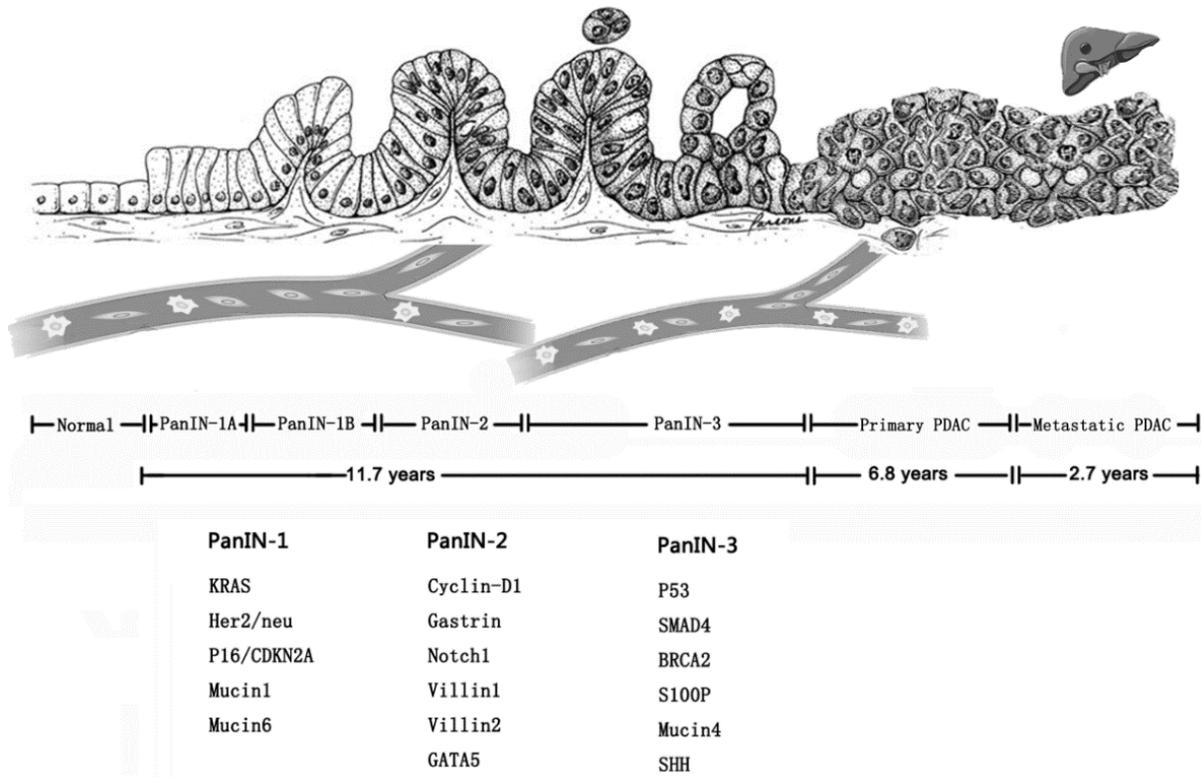


Figure 2. Genetic changes in multistep progress model in pancreatic cancer.

Microfluidic devices, including CTC-chip, HB-chip, CTC-iChip, have shown great promise for CTC enumeration and function analysis [44-46]. In the preclinical studies, the detection rate of various cancer types by the microfluidic devices could reach as high as 90% [44-46], but the detection rates of CTCs in later researches were lower in both localized and metastatic tumors than previous reported results [47]. Despite the unsatisfactory fact, about 98% of CTCs captured by these microfluidic devices maintained viable since the blood specimen needn't excessive pretreatments [45]. Therefore, these viable and intact CTCs could be utilized for genome analysis, expression analysis, protein analysis and functional analysis [48]. In particular, the relevant researches are remarkable in tumor dissemination [13, 15, 27], drug resistance [49, 50], and function analysis in CTC-derived explants [51] on several cancer types, including pancreatic cancer [13, 15].

Pancreatic CTCs in clinical research

Early diagnosis of pancreatic cancer

Most pancreatic cancer can't be radically removed because of delayed diagnosis, thus, it is crucial to find specific and efficient biological marker of pancreatic cancer for early diagnosis. Pancreatic cancer is driven by a subgroup of underlying genetic mutations, including KRAS, CDKN2A, SMAD4 and

TP53 [26, 52]. By analyzing genetic evolution of pancreatic cancer, one model showed that the total disease course of pancreatic cancer was almost 20 years, and if so, there would be enough time to carry out intervening measures to improve the clinical outcomes (Figure 2) [53]. At the early stage of tumor formation, even before tumor formation, CTCs could be detected in the peripheral blood. PanIN, intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasm (MCN) can be evolved into pancreatic cancer, therefore, they are usually regarded as premalignant lesions [2, 54]. In the pancreatic intraepithelial neoplasia (PanIN) mouse models, although micro- or macrometastasis didn't occur, liver seeding could be detected and the single cells were located in the blood vessels with distinguished marker [15]. In another study, Andrew Rhim et al. confirmed that cancer cells could enter circulation before tumor detection by studying patients with IPMN and MCN [55]. The early dissection of pancreatic cancer necessitates sensitive and accurate technique to detect and determine the tumor biology of pancreatic cancer. Although it has been reported CTCs could realize early diagnosis for lung cancer developed from chronic obstructive pulmonary disease (COPD), similar results in pancreatic cancer hasn't been reported [56]. Therefore, devices for CTC capture with high sensitivity and

specificity and some prospective studies are required to validate the clinical significance of early detection of CTCs in pancreatic cancer.

Treatment monitoring

Since half-life of CTCs is quite short, CTCs could monitor the cancer progress dynamically in real-time [17]. The accurate knowledge of disease evolution will be of utmost importance for treatment decision. For pancreatic cancer, neoadjuvant therapy could not only downstage the primary tumor to improve the resectability, but also test the response to treatment regimen to avoid the delayed treatment or chemotherapy after surgery [76]. In general, the conventional imaging examinations usually lag behind the evolution of tumor biology. However, CTCs could provide the accurate scenario of tumor progress and the best time for surgery can be determined by noting the drop of CTCs [77]. In addition, the dynamical changes of CTC enumeration were closely associated with the radiographic tumor response and CTCs could well reflect the genetic information of primary tumor [78]. This phenomenon has been validated in non-small lung cancer and whether it could be applied to other cancer types remains to be confirmed [79]. However, the SWOG S0500 study demonstrated that treatment modification according to the CTC enumeration didn't produce prolonged overall survival or progress free survival [80]. This indicated that necessity of treatment modification and how to swift treatment regimen should be taken into consideration for better clinical efficiency.

Prognostic information

CTCs can be regarded as the seeds for distant dissemination of various cancers. It's reported that only about 2.5% of CTCs would result in micrometastasis and as few as 0.01% would finally develop into macrometastasis which lead to disease recurrence and mortality [81, 82]. Therefore, progress free survival would be direct indicator of the function of these CTCs [83]. Some studies found relationship between CTC enumeration and prognostic information of pancreatic cancer [31, 32, 63, 84], while some didn't [57, 60, 62]. A recent meta-analysis including 623 pancreatic cancer patients revealed that the patients with positive-CTC had worse progress free survival (HR=1.23, 95 %; CI=0.88-2.08, P<0.001) and overall survival (HR=1.89, 95 % CI=1.25-4.00, P<0.001) [85]. The discrepancies on the prognosis information rise an issue whether these isolated cells have malignant biological characteristics or whether they are just a tip of the iceberg [10]. For example, a small pilot study validated that EpCAM⁺ CTCs

indicated poor outcome among cancer patients, whereas the EpCAM⁻ CTCs was not associated with poor overall survival, so a series of analysis should be carried out to study the tumorigenicity of these isolated cells at various levels to provide in-depth explanations of prognostic information [86, 87].

Pancreatic CTCs in basic research

Interpreting tumor heterogeneity

Tumor heterogeneity is a main barrier to conquer cancer. The interpatient tumor heterogeneity has been well studied while inpatient tumor heterogeneity needs more attention which is responsible for drug resistance [10, 88]. Mutational analysis and genomic rearrangements reveal genomic instability, the genetic evolution of pancreatic cancers, temporal and spatial heterogeneity in both primary and secondary pancreatic cancer [89]. Tumor heterogeneity can be generally divided into four classifications: intratumoral heterogeneity, intermetastatic heterogeneity, intrametastatic heterogeneity, and interpatient heterogeneity [90]. Since CTCs act as a bridge between primary tumor and secondary tumors and they are the seeds of metastasis and tumor self-seeding, the underlying mechanism on tumorigenesis, metastasis, and drug resistance can be obtained by analyzing CTCs, thus avoiding the complexity of tumor heterogeneity, which will be conducive to finding new therapeutic targets and promote targeted therapy in return [91, 92].

Deciphering drug-resistance

CTCs also function as a powerful weapon to decipher the acquired drug resistance mechanisms and guide rational use of medicines. Miyamoto et al. reported the research findings of RNA-Seq of single prostate CTCs isolated by CTC-iChip [50]. The analysis of CTCs from patients undertaking androgen receptor inhibitor and untreated cases revealed the activation of noncanonical Wnt signaling, which was involved in multiple downstream regulators of cell survival, proliferation, motility and the maintenance of stem cell populations [93]. By deep analysis of CTCs, more pathogenic mechanism and potential therapeutic targets could be revealed. The application of next-generation sequencing (NGS) for CTCs will provide tumor information in real-time and guide following therapeutic regimen earlier, meeting the targeted therapy for cancer [94]. In another concept-of-proof study, breast CTCs were captured by CTC-iChip and then one or more cell lines were successfully generated from 6 patients of 36 patients [49]. Notably, the captured CTCs shared cytological characteristics with matched primary tumors and

xenograft tumor in immunosuppressed non-obese diabetic scid gamma (NSG) female mice model and similar results were found in small cell lung cancer and colorectal cancer patients by CellSearch system [51, 95]. Therefore, genotyping and functional testing for drug susceptibility in CTCs could accurately reflect these features in primary tumors. For example, optimal treatments for breast cancer patients with ER-positive and ESR1 mutation is unknown, HSP90 inhibitor STA9090 alone could demonstrate cytotoxicity and a low dose of STA9090 indeed suppressed the ER level, which provided an opportunity to offer optimal therapies for cancer patients during the disease course [49].

Explaining the metastatic mechanism

CTCs could help to explain the metastatic mechanism of pancreatic cancer. Single-molecule RNA sequencing of captured pancreatic CTC using ^{Hb}CTC-Chip method proved that noncanonical Wnt signaling pathway may contribute metastatic dissemination in human pancreatic cancer [13]. In this study, Wnt2 mRNA was frequently observed in CTCs and metastatic ascites cells while seldom expressed in the primary tumor tissue. Besides, Wnt2 in pancreatic cancer cells had the ability to suppress anoikis, enhance anchorage-independent sphere formation, and increase metastatic propensity *in vivo*. Then therapy targeted Wnt signaling, such as TGF- β activated kinase 1 (TAK1), could inhibit pancreatic cancer metastasis [96]. In another study, identified pancreatic CTCs using epitope-independent microfluidic capture were analyzed by single-cell RNA sequencing, and extracellular matrix genes, which were responsible for cell migration and invasiveness were found to be highly expressed [12]. This discovery cast light on the metastatic mechanism and the design the proper agents to prevent distant dissemination.

CTCs are found in the circulation as either single CTCs or CTC clusters. As a form of CTCs, CTC clusters were relatively infrequent compared to single CTCs (2.6% versus 97.6%), but the metastatic capability of CTC clusters was as much as 50 times of single CTCs in breast cancer [27]. Besides, CTCs also travel with other cells in circulation such as macrophages and neutrophils that help protect the CTCs whilst in the blood stream. This is one reason why they are not detected very efficiently with some of the CTC detection systems on the market [97]. The presence of CTC clusters predicated a poor clinical outcome in many cancers including lung, breast, or colorectal cancers [27, 98, 99]. CTC clusters were also observed in the blood stream of pancreatic cancer patients, the in-depth implications remained to be

discovered [13]. Single-cell RNA sequencing revealed that expression of plakoglobin, which played a pivotal role in the regulation of cell-cell adhesion and Wnt signaling pathway increased about 200 times in CTC clusters compared with single CTCs [27, 100]. In conclusion, researches in molecular characterization of CTCs and CTC clusters yield novel and profound insights into metastatic mechanism and then targeted drugs can be designed to intervene in corresponding signaling paths.

ctDNA in clinical application

The clinical application of ctDNA was initially studied in 1998 using quite conventional mutant allele-specific amplification method and in recent five years more and more studies were carried out with sophisticated sequencing technology, such as digital PCR, next-generation sequencing [101]. The clinical applications of ctDNA in pancreatic cancer are quite intriguing and important, but the results are also quite mixed, which need further verification and reconsideration. The relevant studies were summarized in **Table 2**.

Early diagnosis of tumors

Analysis of SEER data suggests resectable pancreatic cancer has a dramatic survival advantage compared to unresectable pancreatic cancer (media survival: 36 months vs 7 months) [102], so early detection for higher resectability is very crucial for better clinical outcomes. Pancreatic cancer can be considered as an accumulative process of various genetic aberrations, and the mutated genes in the bloodstream will provide a clue of carcinogenesis of pancreatic cancer. Therefore, the less invasive and actionable ctDNA has great potential for pancreatic tumor screening among high-risk population (ie, a family history of pancreatic cancer, elder than 50 years, new-onset diabetes, smoking) [103, 104].

It has been reported that ctDNA could be detected in about 50% of early-stage pancreatic cancer by digital PCR approaches [22, 73]. Nevertheless, the whole exome sequencing identified an average of 26 mutations (range 1-116) in the tumor tissue in the early pancreatic cancer, so mutations could also be detected in the circulation theoretically because the genetic aberrations will be released in bloodstream [28]. Therefore, if more genetic mutations could be detected, the positivity of ctDNA may increase. To solve this issue, a conceptual "ctDNA-Chip" could be fabricated to assay more genes at a time and the mathematical modeling could be applied to evaluate the risk factor. When ctDNA is used as a diagnostic tool, several problems should be taken into consideration. Firstly, false-positive is a common

problem of genetic diagnosis because many mutations appeared in both malignant and benign lesions and it's difficult to distinguish them solely by a single mutation [105, 106]. Secondly, the origin of ctDNA is difficult to determine because many mutations are shared by different tumors, such as KRAS, TP53 [107, 108]. In order to solve these problems, pancreatic cancer-specific gene markers should be discovered and the potential relationship of different genetic mutation should be revealed. Thirdly, these types of biomarkers, either CTC or ctDNA should be used in conjunction with imaging, as alone they are not 100% reliable. The problem of the overlap of genetic mutations in different cancer types is difficult to overcome. This said, the detection of a tumor associated mutations in KRAS or TP53, for example, in cfDNA may prompt a clinician to perform an imaging scan with the ability to detect a cancer in different anatomical locations not just in the pancreas which is also of clinical benefit.

Treatment Monitoring

Genetic variations in ctDNA could reflect the tumor tissues with considerable accuracy and feasibility [109, 110]. Since the genetic variations have many forms, ctDNA could be used to track tumor progress with higher specificity [111]. Besides, the half-time of ctDNA is only estimated to be about 2 hours in the body, so ctDNA could act as a flexible method to monitor the tumor progress dynamically [112]. Frank Diehl et al. have demonstrated that ctDNA in advanced colorectal cancer patients who received complete resection of all evident tumor tissue experienced a 99.0% of median decrease two to ten days after surgery [112]. In contrast, the patients undertaking incomplete resection showed slighter decreased or even increased level of ctDNA. Interestingly, the undetectable level of ctDNA 10 days after surgery in 4 patients predicted no recurrence, so similar to negative margin, "negative ctDNA" is also a key indicator for long-term survival [112]. Similar conclusions were drawn from relevant researches on

Table 2. Summary of clinical studies on ctDNA in pancreatic cancer

Reference	Origin	Technique	Number of PC patients	Results	Main findings
Terumasa Yamada et al., 1998 [64]	Plasma	Mutant allele-specific amplification	21 I:2, II:2; III:2; IV:13	60% of patients with K-ras mutations in tissue showed identical mutations in plasma	ctDNA may be useful for evaluating tumor burden and treatment efficiency
Antoni Castells et al., 1999 [65]	Plasma	Single-Strand Conformation Polymorphism	44 I:4, II:11; III:5; IV:23	Mutant K-ras was found in 27% of plasma samples	Mutant-type KRAS was associated with shorter survival time
Feng Dianxu et al., 2002 [66]	Plasma/ tissue	Direct sequencing	41 I:2, II:6; III:5; IV:26;NA:2	ctDNA was detected in 70.7% of PCs	Plasma KRAS mutation analysis combined with serum CA19-9 determination could detect the majority of cases of pancreatic carcinoma
Uemura Takanori et al., 2004 [67]	Plasma/ tissue	Mismatch ligation assay	28 I:2, II:8; III:7; IV:11	ctDNA was detected in 35% of PCs	Genetic alterations present in the tumors of pancreatic cancer patients can be detected in their plasma
Rodolfo Marchese et al., 2006 [68]	Plasma/ tissue	Direct sequencing	30 I:3, II:22; III:3; IV:2	Media 333 ng/mL (125-525 ng/mL)	K-ras mutations were detected in 70% of neoplastic tissue samples, but no mutated DNA resulted in circulating DNA samples
Jan Da 'britz et al., 2009 [69]	Plasma	Real-time PCR	56	KRAS mutations could be detected in 36% of PCs	The combination with CA 19-9 and KRAS mutation could improve the sensitivity or the diagnosis of PC
H. Chen et al., 2010 [70]	Plasma	Direct sequencing	91	KRAS mutation rate: 33%	KRAS mutation was correlated with clinical outcome in unresectable pancreatic cancers
Betgegowda C, et al., 2014 [22]	Plasma	Digital PCR	155	ctDNA was detectable in >75% of PC patients	ctDNA is an applicable biomarker that for a variety of clinical and research purposes
Oliver A.Zill, et al., 2015 [30]	Plasma /tissue	NGS	18	Diagnostic accuracy of cfDNA sequencing was 97.7%	cfDNA sequencing is feasible, accurate, and sensitive in identifying tumor-derived mutations and could guide targeted therapy.
Julie Earl et al., 2015 [71]	Plasma	ddPCR	31	KRAS mutation rate: 26%	KRAS mutant cfDNA was correlated strongly with overall survival
Erina Takai et al., 2015 [72]	Plasma	ddPCR and NGS	I-III:95 IV:163	Media: I-III, 17.59 ng/2ml; IV, 21.65 ng/2ml	Potentially targetable somatic mutations were identified in 29.2% of patients examined by targeted deep sequencing of cfDNA.
Mark Sausen et al., 2015 [73]	Plasma	ddPCR	II :51	Mutation rate: 43%	Detection of ctDNA after resection predicts clinical relapse and poor outcome, with recurrence by ctDNA detected 6.5 months earlier than with CT imaging
Hideaki Kinugasa et al., 2015 [74]	Serum	ddPCR	131	KRAS mutation rate: 54.5%-62.6%	KRAS mutations in ctDNA other than in tissue were associated with worse survival, especially in cases with a G12V mutation.
Tjensvoll K et al., 2016 [75]	Plasma	PNA-clamp PCR	locally advanced:2 metastatic:14	KRAS mutation rate: 71%	The pre-therapy ctDNA was a predictor of both progression-free and overall survival. Changes in ctDNA levels corresponded both with radiological follow-up data and CA19-9 levels.

Abbreviations: PC, pancreatic cancer; PNA-clamp PCR, peptide-nucleic acid clamp polymerase chain reaction; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; NGS next-generation sequencing.

early breast cancer patients undertaking curative resection. It was estimated that the detectable ctDNA at a single postsurgical time point predicated metastatic relapse with a hazard ratio of 25.1 [113]. What's more, ctDNA predicted the subsequent relapse with more accuracy compared to primary tumor, which was associated with genetic diversity in the development of micrometastatic disease before relapse [113]. For pancreatic cancer, detection of ctDNA after resection was a poor indicator for clinical relapse, and ctDNA detected clinical recurrence 6.5 months earlier than CT imaging [73]. As ctDNA and to a certain extent CTCs are markers of disease dissemination and relapse, they are important novel biomarkers for dynamic monitoring for these patients [114].

Providing prognostic information

The prognosis of pancreatic cancer is mainly determined by clinical presentations, tumor stage, histological characteristics, and the prognostic significance of ctDNA in pancreatic cancer is quite controversial [6, 115]. Generally speaking, the clinical utility of ctDNA for prognosis assessing is limited in the resected cases because the alteration of subsequent treatment options are variable and the genetic evolution is random and unpredictable [53, 116]. Nevertheless, some potential genetic aberrations appearing in early-stage pancreatic cancers have been found to be associated with survival, which may play a decisive role in the disease progress and more work are warranted [28, 73]. In late-stage pancreatic cancer patients, ctDNA would also be of great help and it would provide thorough information for better management. Several pilot clinical researches have explored the potential prognostic function of ctDNA and the research objects of ctDNA were mainly popular point mutations [65, 112, 117].

Since KRAS gene mutation presents in about 90%-95% of pancreatic cancer and it is considered to be an early event in the tumorigenesis process since more than 90% of PanINs harbor KRAS mutations [118, 119]. 98% of KRAS mutations in PDAC occurs at position G12 and predominant substitution found at this position is G12D (51%), followed by G12V (30%) and G12R (12%) [118]. Therefore, KRAS attracts much attention and it proves to be a significant prognostic factor for survival [71, 120]. It has been demonstrated that ctDNA could be detected in about 50% and 90% of early-stage and late-stage pancreatic cancer patients, respectively, which is an essential condition as an excellent prognostic marker [22]. A pilot study enrolling 45 pancreatic ductal carcinoma patients at different disease stages showed KRAS mutation in the plasma correlated with a significantly worse overall

survival (60 days for KRAS mutation positive vs 772 days for KRAS mutation negative) [71]. In this study, KRAS mutation was detected in 26% (8/31) of patients of all stages by droplet digital PCR and the majority mutation position was G12D (6/8), however, the patients with KRAS mutation was still relatively small and the specific determinant point mutation was difficult to identify. Hideaki Kinugasa et al. reported a higher sensitivity of KRAS mutation in serum (62.6%) by droplet digital PCR and they also find KRAS mutation in serum rather than tissue predicted a worse prognosis in both development set and validation set [120]. Another large-scale research also didn't find the prognostic significance of various G12 mutations in the tumor tissues [121]. These results may reflect the actual pathological progress in which the potent cells with aggressiveness and proliferation release more nucleic acid and this characteristics will help to identify the wrecker nucleic acid, thus resolving the tumor heterogeneity to some extent [120]. Furthermore, G12V mutation in serum was found to be correlated to a significantly shorter survival compared with G12D/G12R/wild-type [120]. The results were concordant with previous basic research findings that G12V mutation contributed to high invasive potential oncogenic activity [122, 123]. With the development of next-generation sequencing, more and more relevant genetic aberrations have been identified by clinical researches such as aschromatin-regulating genes MLL, MLL2, MLL3, ARID1A [73], SLIT receptor ROBO2, amplification at SEMA3A and PLXNA1 [28]. These clinical findings based on ctDNA help reveal valuable targets and then substantial pathologic mechanisms can be revealed by further researches of these targets of most interest, which finally provides proof-of-concept evidence for novel agents.

Managements of chemotherapy and targeted therapy

At present, chemotherapy drugs are usually uniformly administered despite the chemotherapeutic sensitivity. However, some patients will never relapse even without chemotherapy and some patients will relapse soon even with a certain chemotherapy regimen [124]. This situation calls for an accurate evaluation tool that could predict the individualized treatment response, thus avoiding overtreatment or futile treatment. With the advent of targeted therapy, the clinical indications and effect evaluation have been key issues for rational application of targeted drugs. ctDNA exhibits excellent characteristics to resolve the above issues in the era of targeted therapy. On the one hand, ctDNA could clarify the molecular marker of tumor tissue with satisfactory sensitivity

and specificity, which could help to select optimal treatment [30, 110]. On the other hand, low level of ctDNA indicated a favorable prognosis [112, 125]. Therefore, it's feasible and practicable to administer treatment regimen according to genetic status by ctDNA. Several studies have demonstrated the potential of ctDNA in the cancer management [126]. In a recent clinical study, Oliver A. Zill et al. has demonstrated an intriguing case that EGFR deletion was detected in ctDNA 7 months earlier than tissue biopsy and the subsequent capecitabine and erlotinib lead to radiographic response and CA 19-9 normalization [30]. This phenomenon indicated that ctDNA could be used to guide targeted therapy, thus avoiding overtreatment and realizing precision medicine. Another example is BRAF mutation, which appears in about 2.2% of pancreatic tumors, and the targeted agent vemurafenib has been approved for metastatic melanoma with V600G amino-acid substitution in BRAF gene, so this subset of patients harboring such mutation may be susceptible to vemurafenib, which could be an alternative method for this lethal disease [127]. Nevertheless, several issues should be solved due to multiple genetic mutations and crosstalk of signaling pathways: development of multiple targeted drugs, identification of prognostic gene mutations, and selection of patients who will gain utmost benefit from specific targeted agents [128].

Targeted therapy has become standard therapy regimen for some tumors in the past 20 years, such as breast cancer, colorectal cancer, lung cancer, melanoma and so on [129, 130]. For pancreatic cancer,

only erlotinib, an epidermal growth factor receptor inhibitor, is approved by FDA for clinical use [131]. However, the overall survival of gemcitabine plus erlotinib is 0.33 month longer than gemcitabine alone (median 6.24 months vs 5.91 months), so erlotinib hasn't been widely accepted in the management of pancreatic cancer due to the modest survival benefit and cost-effect margin [132, 133]. A potential reason for the unsatisfactory efficacy of targeted therapy in pancreatic cancer was lack identification of genomic profiling due to the inadequate biopsy for molecular characterization [30]. It has been reported that a KRAS-wild type in formalin-fixed and paraffin-embedded (FFPE) tumor samples correlated to a better overall survival (OS) under treatment regimen including erlotinib (median OS, 7.9 months in KRAS wild-type group and 5.7 months in KRAS mutation group; HR=1.68, P=0.005) [131]. The identified genetic status would be conducive to improving clinical outcomes. Besides, since more and more targeting signaling pathways in the epithelial compartments, targets in the stromal compartments and other potential targets have been discovered, the correspondent targeted agents, such as tipifarnib and salirasib targeted KRAS mutations, bevacizumab and sorafenib targeted to VEGF mutations, erlotinib and cetuximab targeted to EGFR mutations have been developed and some agents have resulted in decreased growth of pancreatic tumor in preclinical studies (**Figure 3**) [134-136]. Therefore, it's never more crucial to identify the genomic information of the individual pancreatic cancer.

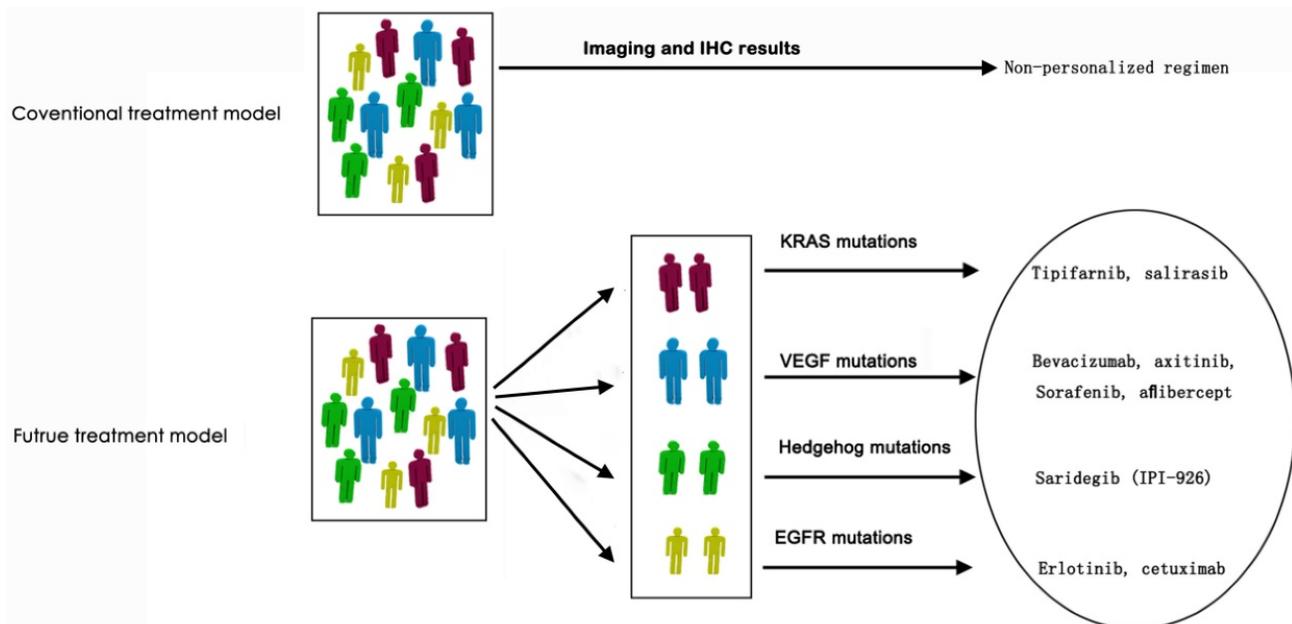


Figure 3: Envisaged revolution of treatment model for pancreatic cancer in the era of precision medicine.

Conclusion

Pancreatic cancer is still a devastating disease, so the tumor biological features and clinical managements of pancreatic cancer require further intensive researches. CTCs and ctDNA are essential components of liquid biopsy and are promising to discover the hidden secrets of pancreatic cancer. Since CTCs and ctDNA are two independent entities, they are complementary in the early diagnosis, selecting treatment regimen, monitoring disease progression and evaluating prognosis [22, 126]. However, we should also keep the existing disadvantages of ctDNA in mind. Firstly, ctDNA detection was still quite low although KRAS mutations were quite high in pancreatic cancer tissue. The mechanism of ctDNA release and degeneration was still poorly understood [101]. Secondly, the ctDNA detection process hasn't been totally standardized at present. Only when standardized specimen preparation, detection technology and data analysis were carried out, could the ctDNA facilitate routine clinical decision. Thirdly, since there are only few targeted drugs for pancreatic cancer, we have no corresponding treatment regimen when ctDNA predicted early relapse. Early detection of disease may not prolong survival time or improve life quality. In contrast, this may bring extra psychological pressure [22]. Although there are some disadvantages, ctDNA could still play as a powerful weapon in clinical trials about prognosis, acquired drug resistance and treatment response, which would promote diagnosis and treatment on pancreatic cancer. CTCs, as viable and intact cells, are very tantalizing approach to perform biological studies, such as invasion, metastasis, and drug resistance both in vitro and in vivo.

In conclusion, CTCs are preferentially used for tumor biological studies and ctDNA is feasible for clinical research with great potential in translations medicine and precision medicine. The development of liquid biopsy is sure to provide essential information for clinical managements and prolong clinical outcomes in pancreatic cancer eventually.

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Competing Interests

The authors have declared that no competing interest exists.

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