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Original Articles

Pancreatic circulating tumor cell detection by targeted single-cell next-generation sequencing

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ABSTRACT

Background and aims: Single-cell next-generation sequencing (scNGS) technology has been widely used in genomic profiling, which relies on whole-genome amplification (WGA). However, WGA introduces errors and is especially less accurate when applied to single nucleotide variant (SNV) analysis. Targeted scNGS for SNV without WGA has not been described. We aimed to develop a method to detect circulating tumor cells (CTCs) with DNA SNVs.

Methods: We tested this targeted scNGS method with three driver mutant genes (*KRAS/TP53/SMAD4*) on one pancreatic cancer cell line AsPC-1 and then applied it to patients with metastatic PDAC for the validation.

Results: All single-cell of AsPC-1 and spiked-in AsPC-1 cells in healthy donor blood, which were isolated by the filtration with size or by flow cytometry, were detected by targeted scNGS method. All blood samples from six patients with metastatic PDAC, for the validation of target scNGS method, showed CTCs with SNVs of *KRAS/TP53/SMAD4* and the positive confirmation of immunofluorescent stainings with Pan-CK/Vimentin/CD45. Four patients with early stage disease, one patient with benign pancreatic cyst and a healthy control sample all showed concordant results between targeted scNGS and CTC enumeration.

Conclusions: The novel technique of targeted scNGS for SNV analysis, without pre-amplification, is a promising method for identifying and characterizing circulating tumor cells.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a 5-year survival rate of less than 10% [1]. The lethality of pancreatic cancer is attributed to delayed presentation, early recurrence, and resistance to chemotherapy [2–6]. Surgical resection of local disease provides the greatest chance of cure, yet, approximately 80% of resected patients die of subsequent metachronous disease with the overwhelming pattern of recurrence being systemic. Thus, understanding the mechanisms of the underlying nature of systemic disease after margin-free resection can guide the development of more effective therapeutic strategies.

One potential mechanism of cancer dissemination is through circulating tumor cells (CTCs). CTCs are cancer cells shed from a solid tumor into the circulation. The presence of CTCs is a strong prognostic factor for the overall survival in patients with various cancers, such as breast

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cancer, prostate cancer, and pancreatic cancer [7–9]. A growing body of evidence supports a direct role of CTCs in the development of metastasis in a variety of cancers [8,10]. The significance of CTCs in PDAC is unclear [11]. Previous work from our group demonstrated the presence of at least two distinct CTC phenotypes and that the mesenchymal-like phenotype expressing both epithelial and mesenchymal markers is correlated with cancer recurrence and decreased overall survival of patients with pancreatic cancer [8,12].

Pancreatic cancer is perhaps one of the best-characterized cancers at the genetic level. However, due to technical challenges, we have limited information about the genetics of pancreatic cancer CTCs. DNA analysis by next-generation sequencing (NGS) has revolutionized the analytical spectrum to decrypt cancer genomes by detection of copy number variations (CNV), single nucleotide variants (SNV), and structural rearrangements. Hence, singe-cell DNA sequencing of CTCs specifically may provide a unique insight into the biological process of metastasis. Most existing single-cell sequencing approaches require whole-genome amplification (WGA) prior to library construction [13–20]. This step introduces PCR errors and decreases the uniformity of chromosomal coverage [21–23], leading to false calls on the single-nucleotide variant (SNV) and copy-number variation (CNV) analysis [24]. Single-cell library preparation without pre-amplification has been recently reported for whole-genome sequencing (WGS) and CNV analysis [25]. However, the reported technique is not suitable for targeted SNV analysis.

We developed a novel method for SNV analysis without pre-amplification using targeted single-cell next-generation sequencing (scNGS). This method is based on digital NGS (dNGS), a technique that we previously established. It uses 45 *g. e.* (150 *pg*) of cell-free DNA (cfDNA), and can detect low abundance mutations of cfDNA (from 0.1% to 1%) in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer [26]. In brief, the method includes the isolation of single cells, DNA extraction from single cells, library preparation without whole genome amplification, and application of next-generation sequencing, which also is less than one-tenth of the total cost of other single-cell NGS methods. The use of this technique can be extended to identify the genetic features of CTCs from the peripheral blood of patients with PDAC, which helps achieving a more accurate identification of CTCs comparing to the current and broadly used immunofluorescent method as a liquid biopsy biomarker for the detection of cancer and its reoccurrence and as a monitor marker for the surgical or chemotherapeutic treatments.

2. Materials and methods

2.1. Cell line and culture conditions

The human pancreatic cancer cell line AsPC-1 (also called CRL-1682™, ATCC: American Type Culture Collection [ATCC]) was used in this study using appropriate culture conditions described previously [27,28]. For enumeration, cells were counted by Countess® II Automated Cell Counter (ThermoFisher SCIENTIFIC).

2.2. Cases

This study was approved by the Institutional Review Board (IRB) of the Johns Hopkins Hospital. Cases #1-#12 were collected from August 2016 to August 2017. Three patients with late-stage of PDAC were identified by Pancreas Multidisciplinary Clinic (PMDC) of the Johns Hopkins Hospital. Informed consent signed by the patients.

2.3. DNA extraction

Genomic DNA was extracted from either cell lines or from peripheral circulating tumor cells using the ARCTURUS® PicoPure® DNA Extraction Kit (ThermoFisher SCIENTIFIC) according to the manufacturer's instructions. DNA samples were quantified using Quantifiler® Human DNA Quantification Kit (ThermoFisher SCIENTIFIC).

2.4. Targeted next-generation sequencing

The three most frequent gene mutations in PDAC were sequenced using an Ampliseq Custom panel (*KRAS*, *TP53*, and *SMAD4*). The full list for the covered exons is listed at Table S1. Next-generation sequencing was performed using an Ion Torrent system (LifeTechnologies) according to the manufacturer's protocols, and publications described previously [26,29]. The minimum depth of sequencing libraries was 500x.

2.5. Isolation by Size of Epithelial Tumor Cells

The Isolation by Size of Epithelial Tumor Cells (ISET), a size-based isolation assay (Rarecells, France), was utilized for CTC enrichment and isolation. All blood samples are processed following manufacturing protocol [1]. The membranes are stored in −20 °C covered in foil and can be used for downstream applications, such as Hematoxylin-eosin (H&E) and immunofluorescence (IF). Dual staining of H&E and IF was carried out in different membrane cores per sample for CTC assessment. Conjugated immunofluorescent antibodies for DAPI (600 m s), pan-cytokeratin (Abcam AF488, 1/100, 1sec), CD45 (Bios AF647, 1/100, 3sec), and Vimentin (Abcam AF594, 1/100, 1sec) were used. CTCs were stratified as epithelial (PanCK+/CD45-) and epithelial/mesenchymal-like (PanCK+/Vimentin+/CD45-) cells. CTC morphology criteria included high nuclear-cytoplasmic ratio and nuclear irregularities. Restrictions in cell size were not applied.

2.6. Flow cytometry (FACS) and spiked-in tumor cells

The blood sample was processed by adding the RosetteSep™ Human CD45 Depletion Cocktail (Stemcell Technologies, Cambridge MA, USA) with antibodies targeted towards white blood cells expressing CD45 and CD66b proteins and red blood cells forming tetrameric antibody complexes which were depleted via gradient centrifugation (1200×*g* for 20 s) over a buoyant density medium. The targeted cells were washed with a 4x volume of HBSS buffer (Hank's Balanced Salt Solution with 2% Fetal Bovine Serum) and centrifuged at 300×*g* for 10 s. The pellet with the target cells was stained with anti-EpCAM (Abcam ab112068 VU-1D9, PE-conjugated) and anti-CD45 (Biolegend 368511, APC-conjugated) antibodies at a concentration of 1:100 each, and incubated in ice and in the dark for 30 s. The stained pellet is diluted in 25 mL of HBSS buffer and centrifuged at 300×*g* for 10 s to be washed twice. The pellet was resuspended in 500 µl of HBSS buffer, and the cells are kept in ice for up to 4–6 h before sorting.

Single-cell sorting was performed with the MoFlo XDP Cell Sorter (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Following the manufacturing protocol, the utilization of the antibodies was performed for negative and positive control samples from peripheral blood of a healthy volunteer ($CD45^+$, EpCAM-) and pancreatic cancer cell line AsPC-1 ($CD45^-$, EpCAM+), respectively. Prior to sorting, cells are stained with Propidium Iodide (PI).

2.7. Sequence variant calling

Variants identified by the NextGENe software Version 2.4 (SOFTGENETICS, State College, PA) were filtered to select only variants with a nucleotide score of ≥30 (reference nucleotide score + mutant nucleotide score/indel score) and without a strand bias. Synonymous somatic mutations were not tabulated. All SNVs were double checked by Integrative Genomics Viewer (IGV, Broad Institute).

In this single-cell study, we used the same cut-off values from the digital NGS scoring system we previously published. A score of 1 (scNGS socre 1) determines *KRAS* G12/G13/Q61, 2 for hotspots of *TP53* & *SMAD4*, and 3 for non-hotspots [26].

3. Results

3.1. Overview of method

The diagram of the design for targeted scNGS is shown in Fig. 1. This includes the feasibility of assessing for: i) single cell isolation and DNA extraction, ii) targeted library preparation without whole genome amplification, and iii) next-generation sequencing and bioinformatics pipeline for SNV callings. We did quality checks (QCs) for all steps from the entire processing to make sure they pass the QCs.

3.2. Cell line separated into single cells and the barcoded library construction

1000 AsPC-1 cells were suspended in 1000 μl phosphate-buffered saline (PBS) (1 cell/*ul*). Serial volumes of 5, 10, 20, 40, and 80 μl of cell/PBS suspensions were aliquoted into 1.5-ml DNase/RNase-free tubes, which led to 5, 10, 20, 40, and 80 cells per tube. The cell pellets were formed after centrifugation at 1000 g for 3 min. The supernatant was removed without disturbing the pellets.

DNA was extracted from the cell pellets. We used the Quantifiler® Human DNA Quantification Kit to quantify the DNA yield, which showed the equivalent molecules matched to the cell numbers (data not shown).

Samples of 5-cells, 10-cells, and 20-cells were sequenced in duplicate with identical barcodes #1 - #12 (Fig. 2A). Samples of 40-cells and 80-cells were sequenced with barcodes #13 - #16. Barcodes #1, #2, #5, #6, #9, #10, #13, and #15 covered AmpliSeq custom panel pool 1. Barcodes #3, #4, #7, #8, #11, #12, #14, and #16 covered AmpliSeq custom panel pool 2. Pools 1 and 2 were designed with overlapping hotspots for *KRAS* codons 12 and 13. The barcoded libraries were constructed following protocols previously described in detail [26,30].

3.3. Targeted single-cell next-generation sequencing in separated individual tumor cells

The amplicons for all 16 barcoded libraries were loaded into a 316 v2 Chip (Life Technologies) in Ion Torrent PGM (Life Technologies), which were amplified and sequenced successfully (Fig. 2B). *KRAS* p. G12D (homozygous) mutation was detected in all 16 barcoded libraries which is consistant with the designed panels. *TP53* p. C135Afs and *SMAD4* p. R100T mutations were detected in all eight barcoded libraries (barcodes #3, #4, #7, #8, #11, #12, #14, and #16), which is consistent with the designed panels (Fig. 2A and B and Table S1). The detections of the mutations we had (*KRAS* p. G12D, *TP53* p. C135Afs, *SMPAD4* p. R100T) is consistant with the genotype of AsPC-1 as described in the ATCC Pancreatic Panel database and previous publications [26].

3.4. Isolation and characterization by size of epithelial tumor cells for spiked-in tumor cells in donor blood

To evaluate this technique with blood as the dilutant, 1000 cells of AsPC-1 were spiked into 10 ml fresh blood of an anonymous healthy donor, as shown in Fig. 3A (top). A portion of this sample was filtered using the Isolation by Size of Epithelial Tumor Cells (ISET) instrument in a technique previously described (Fig. 3B) [8]. Three membrane cores were then placed into a 1.5-ml DNase/RNase-free tube for DNA extraction using ARCTURUS PicoPure DNA Extraction Kit (ThermoFisher SCIENTIFIC). Extracted DNA was measured by Human DNA Quantification Kit yielding 61.6 ng DNA (18,666 genome equivalents [*g. e.*], 3.3 *pg* ≈ 1 *g. e.*, normal diploid cells have 2 *g. e.* of DNA). For library construction, 66 *pg* of DNA (equivalent to 10 cells) was used per barcode. DNA extracted from the membranes of ISET was used for library construction with a targeted NGS, including 410 barcodes for two Ampliseq custom panel pools (20 *g. e.* per barcode per custom pool).

All 410 barcoded libraries derived from ISET were loaded into a 318v2 Chip for sequencing. After the succeeded sequencing run, we did the analysis with the NEXTGENe software and IGV of detecting the mutations in each barcoded library showing *KRAS* p. G12D, *TP53* p. C135Afs, and *SMAD4* p. R100T with scNGS scores of 86, 7, and 14, respectively. AsPC-1 is known to harbor these mutations.

3.5. Flow cytometry (FACS) for spiked-in tumor cells in healthy donor blood

Using a different single-cell isolation technique, fluorescence-

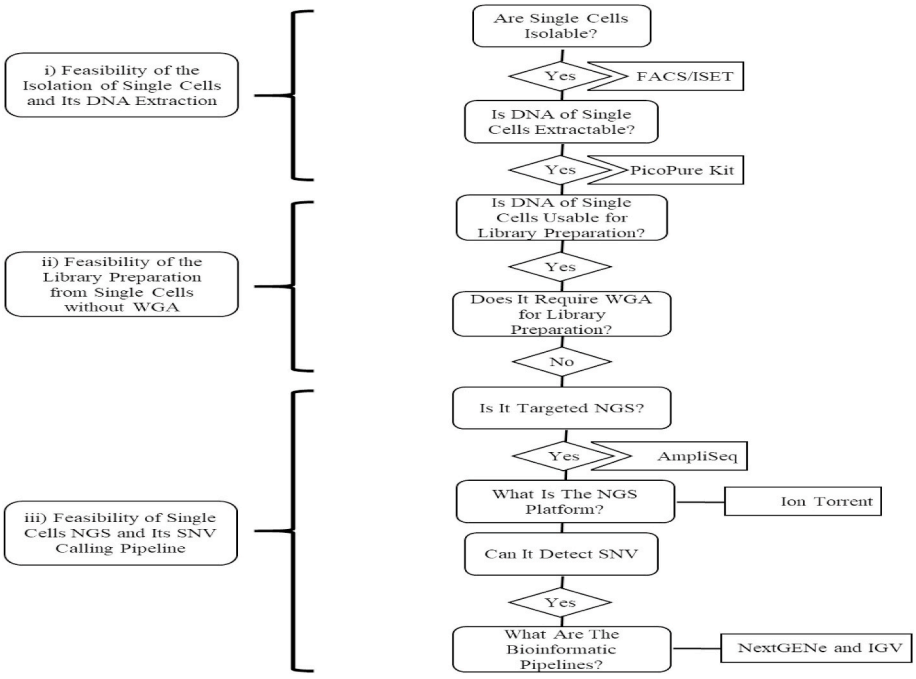

Fig. 1. Flow diagram of targeted scNGS with appropriate considerations.

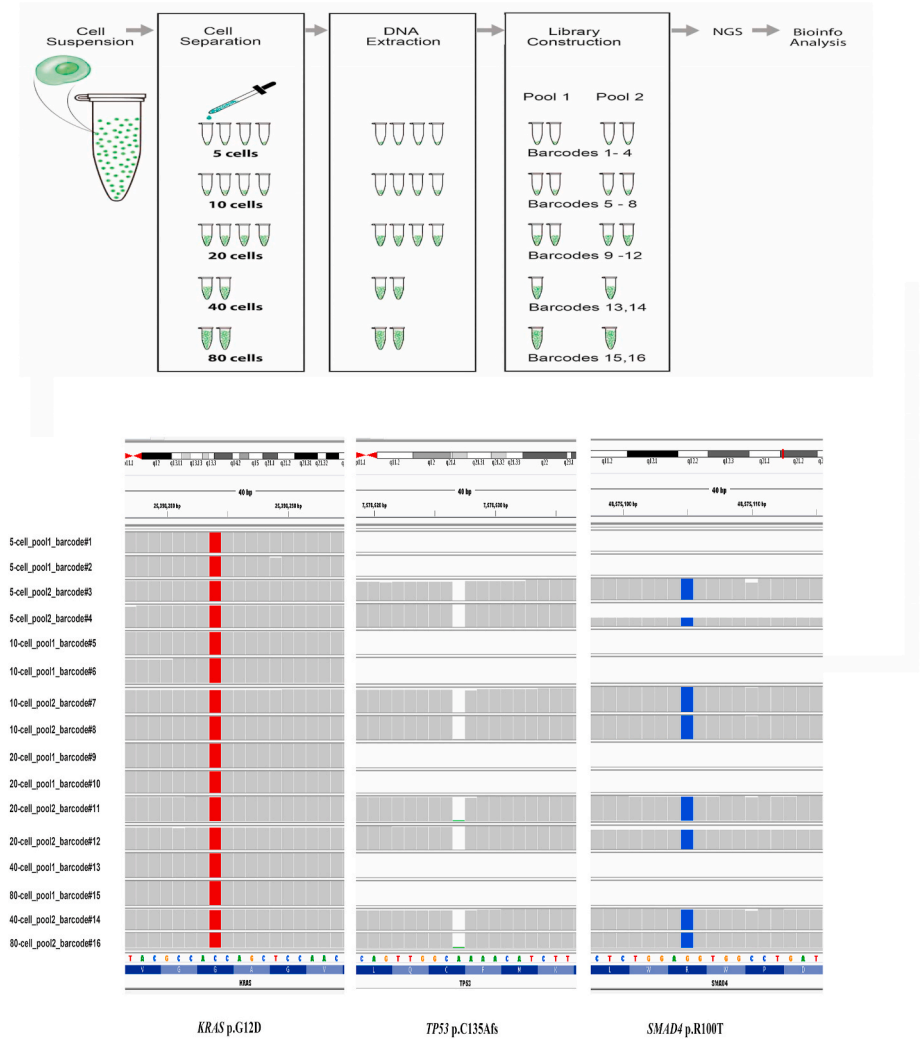

Fig. 2. A. Cells are divided into different aliquots for DNA extraction, library preparation with identical barcodes, and next-generation sequencing. B. All barcoded libraries showed mutations for *KRAS*, *TP53*, and *SMAD4*.

activated cell sorting (FACS) was utilized to isolate 7500 AsPC-1 cells that were spiked into 15 ml of fresh venous blood drawn from a healthy volunteer (Fig. 3A bottom). FACS labeling included the epithelial cell adhesion molecule (EpCAM, an epithelial marker), CD45 (a pan-leucocyte marker), and propidium iodide (PI, a nuclear marker) (Fig. 3C). During FACS, 5, 10, 20, 50, 100, 200, and 500 events positive for EpCAM and negative for CD45 and PI were sorted into sequential 1.5 mL Eppendorf tubes. The sorted cells are transferred to dry ice immediately and stored at −80 °C. For processed peripheral blood samples from the patients with PDAC, all sorted cells are collected in a single 1.5 mL Eppendorf tube. DNA extracted from the sorted cells with 200 and 500 events was quantified by PCR, which had 1.27 ng and 2.9 ng DNA (390 *g. e.*, and 879 *g. e.*, respectively). Barcodes #1–8 were used in library construction for the two custom panel pools: #1 for 5-cell sample, #2 for 10-cell sample, #3 for 20-cell sample, #4 for 50-cell sample, #5 for 100-cell sample, #6 and #8 for 200-cell sample (random duplication), and #7 for 500-cell sample.

All eight barcoded libraries from FACS isolation were loaded into a 316 v2 Chip for sequencing. All libraries were amplified and sequenced successfully, and we found all barcoded libraries that the *KRAS* p. G12D, *TP53* p. C135Afs, and *SMAD4* p. R100T mutations of the AsPC-1 cell line.

3.6. Targeted single-cell next-generation sequencing in CTCs from patients with PDAC

To evaluate clinical applicability, we evaluated the method in patients with PDAC (Fig. 4). 15 ml of fresh blood from a 52 years-old male patient with PDAC (Case #1) was drawn for CTC isolation by FACS. FACS events staining EpCAM+/CD45- (total 520) were collected for DNA extraction. A total of 2.376 ng of DNA was obtained. Library construction utilized 720 *g. e.* of DNA with 18 barcodes for two custom panel pools for scNGS. All barcoded libraries were loaded into a 316 v2 Chip for sequencing. A *KRAS* p. G12D mutation (scNGS score 3) was identified successfully for this patient. To assess for CTC burden in this patient, a separate blood sample was processed and stained using ISET. Immunofluorescent (IF) staining with Pan-CK, Vimentin, and CD45 [8,12], showed a total of 18 cells per mL of blood, 15 for Pan-CK+/Vimentin-/CD45- (epithelial CTC) and 3 for Pan-CK+/Vimentin+/CD45- (mesenchymal-like CTC).

In Case #2, two blood samples (2a and 2b) were available from a previously chemo naïve, newly diagnosed, resectable 68 years-old female PDAC patient by pre-surgical imaging but with oligometastases during the exploration. One sample was collected before the surgical incision (Case #2a], and the other was at five days post-surgical exploration (Case #2b]. Both blood samples were filtered on ISET. The total numbers of CTC per mL blood from the pre-explorative and

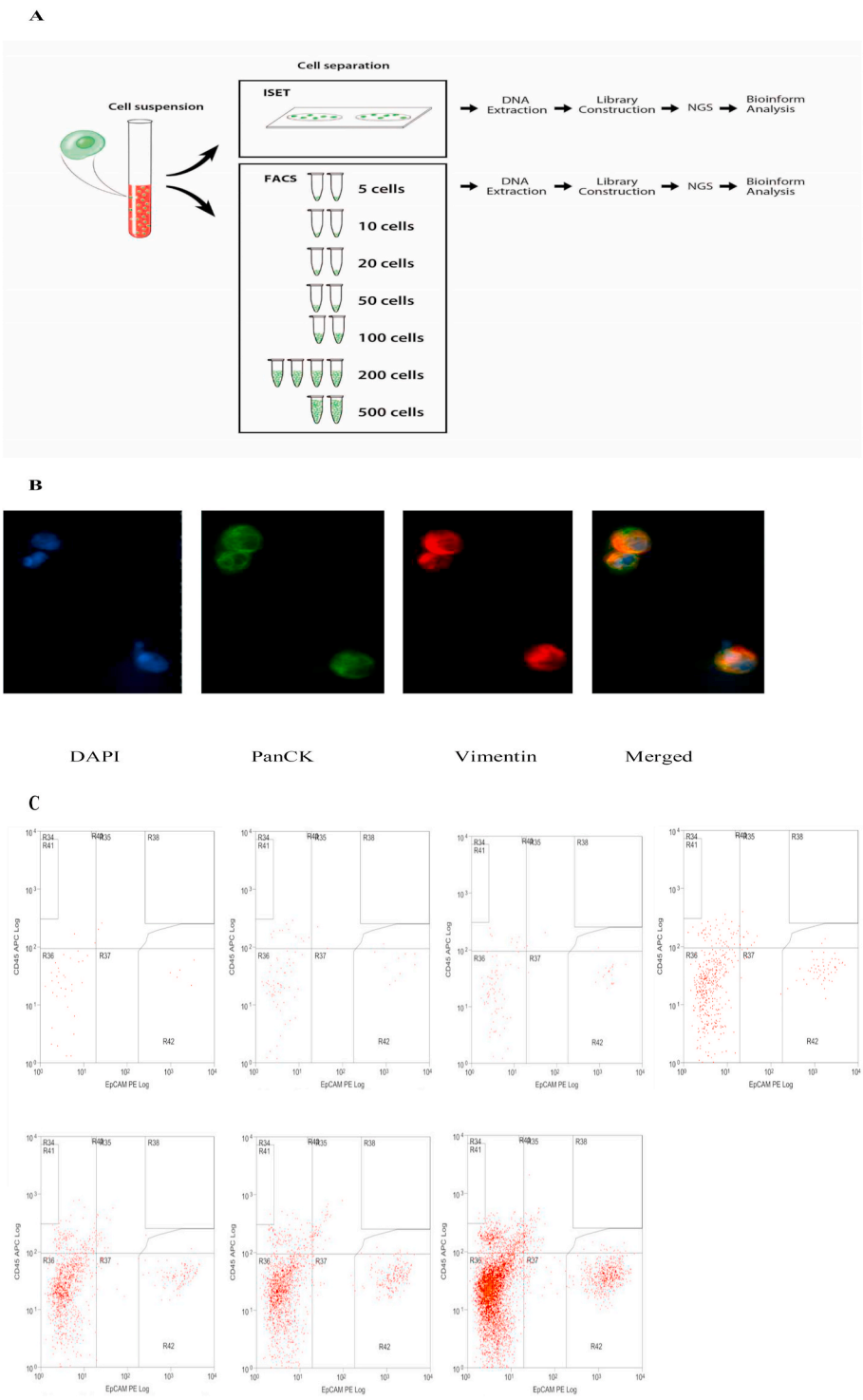

Fig. 3. A. Cells spiked into 10 ml of healthy donor blood for CTC collection using ISET (top), or cells are spiked into 15 ml of healthy donor blood for CTC collection using FACS (bottom). B. AsPC-1 on ISET membrane with immunofluorescent staining (DAPI [blue] for nucleus, PanCK+ [green], and Vimentin+ [red]) with 400x margination. C. AsPC-1 showing EpCAM+/CD45-in gate R42 is used for sequencing with the cells of 5, 10, 20, 50 (top left to right) and 100, 200, 500 (bottom left to right). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

post-explorative samples were both 21 (Pan-CK + Vimentin-/CD45-and Pan-CK+/Vimentin+/CD45-; 18 and 3; 19 and 2, respectively). We used two membrane cores of each sample for DNA extraction. The DNA yield for pre-surgical and post-surgical samples was 28.2 ng and 15.48 ng, which were equivalent to 8545 *g. e.* and 4691 *g. e.*, respectively. 214 and 117 barcodes for scNGS were used for the library construction of pre-surgical and post-surgical samples, respectively. All libraries were loaded into an Ion 530 chip on Ion Torrent S5XL sequencer (Life Technologies). Both samples showed *KRAS* p. G12D, *TP53* p.M246V, *SMAD4* p. R496H with high scNGS scores of 3/6/3, and 1/8/4(*KRAS/TP53/SMAD4)*, respectively.

A third case (Case #3), derived from an 89 years-old male PDAC patient with late-stage disease, showed ten cells (Pan-CK+/Vimentin-/CD45-and Pan-CK+/Vimentin+/CD45-, 8 and 2, respectively) by ISET. We used three membrane cores for DNA extraction. The DNA yield was equivalent to 2225 *g. e.* Fifty-two barcodes for scNGS were used for the library construction. All libraries were loaded into an Ion 318v2 chip on Ion PGM sequencer. This sample showed *KRAS* p. G12D, *TP53* p. G245S, *SMAD4* p. Q248X with a high scNGS score of 4/3/4 (*KRAS/TP53/SMAD4)*.

Cases #4–6 were derived from three patients with stage IV PDAC and showed 13, 27, and 6 CTCs per mL blood by ISET, respectively. One

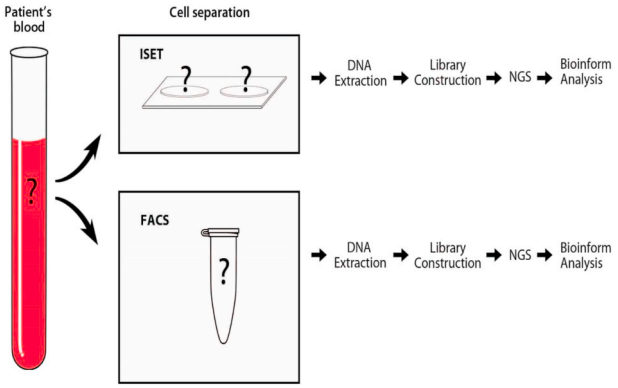

Fig. 4. 10 ml of fresh blood from a pancreatic cancer patient was used for CTC collection using ISET, DNA extraction, and targeted next-generation sequencing (top); 15 ml of patient blood with pancreatic cancer was used for cell collection with FACS, DNA extraction, and targeted next-generation sequencing (bottom).

membrane core of each patient was used for DNA extraction and yielded 5140 *g. e.*, 19,345 *g. e.*, and 3734 *g. e.* respectively. Ninety-six barcodes for targeted scNGS, each covering 3840 *g. e.*, were used for the library construction. All libraries were loaded into a 540 chip on Ion Torrent S5 sequencer. As shown in Table 1, we detected *KRAS* p. G12D and *TP53* p. R248Q (scNGS score of 17/7) for Case#4, *KRAS* p. G12C (score 24) for Case#5, and two types of the mutations for both *KRAS* and *TP53* [*KRAS* p. G12V/G12D and *TP53* p. H179R/R306* (scores 7/11 and 11/4)] for Case#6, respectively. The immunofluorescent results for detected CTCs of Cases#4–6 were 13, 27, 6, respectively.

Cases #7 and #8 were two Stage IA PDAC patients, and Cases #9 and #10 were two Stage IIB PDAC patients, with DNA yields of 6200 *g. e.*, 2600 *g. e.*, 2350 *g. e.* and 3700 *g. e.*, respectively. Case #11 was a benign pancreas disease, an intraductal papillary mucinous neoplasm, and Case #12 was a control blood sample donated by a healthy donor (control), with 20,000 *g. e.* and 5800 *g. e.*, respectively. DNA from these six patients was extracted from three membrane cores which was used for subsequent library construction and NGS. We did not detect any mutations for CTC from these six patients, which is consistant with their negative CTC detected by the immunofluorescent CTC enumeration method.

4. Discussion

In this study, we describe a method to overcome several limitations inherent to commonly used methods for scNGS. Specifically, this method addresses the following: i) targeted single-cell next-generation sequencing (scNGS) no longer requires routine whole genome amplification prior to sequencing, and ii) targeted scNGS can now be used for DNA SNV analysis. Herein we demonstrate this method and successfully detected genetic mutations in CTCs from patients with PDAC. Unlike other scNGS technologies using WGA to replicate the templates, this technique can provide reliable sequencing results by eliminating the false mutation callings induced by the PCR process and polymerase. To overcome the decreased uniformity of coverage distribution on chromosomes, previous studies have utilized tens to hundreds of single-cell genomes to determine SNVs by scNGS [17,19,20,26,31].

We found that targeted scNGS results in a stable amplification of sequencing when utilized with approximately five cells (10 *g. e.*) and provide more reliable sequencing data than targeted scNGS on an individual cell. Furthermore, applying targeted scNGS on multiple cells with various subpopulations can bioinformatically overcome germline variant influences along with PCR-induced error occurrences on the first cycles of direct library preparation. For example, it is difficult to distinguish a true somatic tumor mutation, heterozygous germline single nucleotide polymorphism, or PCR-induced error from the 50% of the variant frequency in individual cell-targeted scNGS. Targeted scNGS on multiple cells helps to eliminate heterozygous germline variant callings and PCR-induced errors, especially when the multiple cells are not purely tumor cells.

All six cases of patients with late-stage PDAC had CTCs detected with mutations of either *KRAS* alone (Cases #1 and #5), or *KRAS/TP53/SMAD4* (Cases #2 and #3), or *KRAS/TP53* (Cases #4 and #6) by targeted scNGS. These findings support the notion that the cells isolated on membrane cores are, in fact, tumor-derived CTCs (Pan-CK+/Vimentin+/CD45-). Specifically, when analyzing in Case #2 where occult metastatic disease with peritoneal metastasis was found during operative exploration, both samples (Case #2a and Case #2b) detected the identical tumor-associated mutations with comparable scNGS scores. We did not detect CTC with mutations from four early-stage PDAC (Cases #7–10), nor from the benign pancreatic disease (Case #11) or from a heathy control (Case #12), which were consistant with CTC enumeration by immunofluorescent staining. In support of our findings, Effenberger et al. reported an increased likelihood of detecting CTCs in later staged PDAC [32]. More specifically, they did not find any CTCs in Stage I patients and found CTCs in only 23% of Stage II patients. Herein, we found a similar distribution in CTCs detection where no CTCs were found in our early stage patients while CTCs were detected in all patients with metastatic disease. Also of important note, we found no CTCs in the patient with IPMN and in the healthy control which helps mitigate concerns for false positive results. Similar findings are reported in the literature with no CTCs detected from 18 healthy donors [32].

In previous work, we have shown different sub-phenotypes of CTCs (epithelial CTCs and epithelial-mesenchymal transitional CTCs) by immunofluorescent stainings [8,12]. However, no evidence does exist so far, showing heterogeneous CTCs with different mutations, and one of the reasons might be the challenge of detecting the mutations of CTCs. Using the targeted scNGS technique which we developed, we have shown heterogeneous CTC populations harboring different DNA mutations such as in Case #6 with *KRAS* (p.G12D and p. G12V) and *TP53* (p. H179R and p. R306*). Case#5 showed *KRAS* p. G12C mutation, a targetable mutation by AMG 510 [33], which brings a hope for personalized medicine in pancreatic cancer with systematic treatment targeting on CTCs.

There are several limitations to note in this study. While focused on the technique, the clinical applicability should be considered pilot work, as this is a small study that shows the feasibility and methods behind targeted scNGS without WGA. We used six Stage IV PDAC cases to validate the ability of targeted scNGS for the detection of mutations. Therefore, correlations between mutations in the primary tumor and CTCs could not be assessed since the primary tumor could not be surgically resected. Unlike in spiked-in cell line experiments, which result in pure cell line cells, there is difficulty in sorting CTCs by FACS. With fewer sorted events, fewer barcodes for targeted scNGS were utilized. As the number of events declines with the use of FACS, false-negative results are possible due to the loss of CTCs during cell sorting. When using ISET, WBC contamination on each membrane may decrease the signal-to-noise ratio for CTC identification and require more barcodes to detect CTCs. Increasing barcodes needed for detection means more resources (and cost) are required for library preparation. A technique with low cost and high efficiency to enrich CTCs is still required.

Longitudinal repeated collection and analysis of CTCs can be used as a biomarker to monitor the progression of PDAC or response to therapy in real-time [34]. Genetic analysis on CTCs by targeted scNGS might lead to a more sensitive biomarker, which can be more powerful for a personalized approach in the treatment of PDAC than CTC enumeration or ctDNA alone.

In summary, we introduce targeted scNGS to identify genetic mutations in CTCs from patients with PDAC. This new sequencing technology has promising potential for broad applications using any custom-designed panels with specific interests of genes in any malignancies. It may increase the diagnostic accuracy of cytopathology specimens in

Table 1
CTC with mutations identified in the blood of pancreatic cancer patients.

	Age (yr)	Sex	Race	Diagnosis*	Blood Volume	DNA Resource	*KRAS*		*TP53*		*SMAD4*		CTC Enumeration by ISET (Numbers/mL Blood)		
							AA Change	scNGS Score	AA Change	scNGS Score	AA Change	scNGS Score	Pan-CK+/ CD45-	Vimentin+/ CD45-	Total
Case#1	52	Male	African American	Stage IV PDAC	15 mL	FACS	p.G12D	3	–	–	–	–	15	3	18
Case#2a	68	Female	African American	Stage IV PDAC	2 mL	ISET	p.G12D	3	p.M246V	6	p.R496H	3	18	3	21
Case#2b					2 mL	ISET	p.G12D	1	p.M246V	8	p.R496H	4	19	2	21
Case#3	89	Male	Caucasian	Stage IV PDAC	3 mL	ISET	p.G12D	4	p.G245S	3	p.Q248X	4	8	2	10
Case#4	73	Male	Caucasian	Stage IV PDAC	1 mL	ISET	p.G12D	17	p.R248Q	7	–	–	13	0	13
Case#5	53	Male	African American	Stage IV PDAC	1 mL	ISET	p.G12C	24	–	–	–	–	24	3	27
Case#6	62	Female	Caucasian	Stage IV PDAC	1 mL	ISET	p.G12V; p.G12D	7; 11	p.H179R; p.R306*	11; 4	–	–	5	1	6
Case#7	65	Male	Caucasian	Stage IA PDAC	3 mL	ISET	–	–	–	–	–	–	0	0	0
Case#8	63	Female	Caucasian	Stage IA PDAC	3 mL	ISET	–	–	–	–	–	–	0	0	0
Case#9	72	Female	Caucasian	Stage IIB PDAC	3 mL	ISET	–	–	–	–	–	–	0	0	0
Case#10	55	Male	Asian	Stage IIB PDAC	3 mL	ISET	–	–	–	–	–	–	0	0	0
Case#11	69	Male	Caucasian	IPMN	3 mL	ISET	–	–	–	–	–	–	0	0	0
Case#12	39	Male	Asian	Healthy Control	3 mL	ISET	–	–	–	–	–	–	0	0	0

* , AJCC 8 th edition of TMN staging.

biopsy samples that have low cellularity. It can also be applied to the primary tumor in order to study intratumoral heterogeneity. Finally, using customized genetic panels with the integration of the known germline single nucleotide polymorphisms, targeted scNGS can also be used for the detection of CNVs, including loss of heterozygosity analysis.

Author contributions

Conceived and designed the experiments: JY; acquisition of data: JY, GG, BK, JRH, VPG, JT, LY, NP, AH, and FVO; analysis and interpretation of data: JY, GG, and JH; drafted the manuscript: JY; revised the manuscript and agreed with the manuscript's results and conclusions: all the authors; obtained funding: JY, JH and CLW; study supervision: JY, JH, and CLW.

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Declaration of competing interest

There are no conflicts of interest for any of the authors. The corresponding authors had full access to all of the data and take full responsibility for the veracity of the data and statistical analysis.

Abbreviations

CNV	copy number variation
CTC	circulating tumor cells
FACS	fluorescence-activated cell sorting
NGS	next-generation sequencing
scNGS	single-cell next-generation sequencing
PDAC	pancreatic ductal adenocarcinoma
PCR	polymerase chain reaction
SNV	single nucleotide variant

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2020.08.043.

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