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RESEARCH PAPER

Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer

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ABSTRACT

Pancreatic cancer presents with a dismal mortality rate and is in urgent need of methods for early detection with potential for timely intervention. All living cells, including cancer cells, generate exosomes. We previously discovered double stranded genomic DNA in exosomes derived from the circulation of pancreatic cancer patients, which enabled the detection of prevalent mutations associated with the disease. Here, we report a proof-of-concept study that demonstrates the potential clinical utility of circulating exosomal DNA for identification of *KRAS*^{G12D} and *TP53*^{R273H} mutations in patients with pancreas-associated pathologies, including pancreatic ductal adenocarcinoma (PDAC), chronic pancreatitis (CP) and intraductal papillary mucinous neoplasm (IPMN), and in healthy human subjects. In 48 clinically annotated serum samples from PDAC patients, digital PCR analyses of exosomal DNA identified *KRAS*^{G12D} mutation in 39.6% of cases, and *TP53*^{R273H} mutation in 4.2% of cases. *KRAS*^{G12D} and *TP53*^{R273H} mutations were also detected in exosomal DNA from IPMN patients (2 out of 7 with *KRAS*^{G12D}, one of which also co-presented with *TP53*^{R273H} mutation). Circulating exosomal DNA in 5 out of 9 CP patients enabled the detection of *KRAS*^{G12D} mutation. In 114 healthy subject-derived circulating exosomal DNA, 2.6% presented with *KRAS*^{G12D} mutation and none with *TP53*^{R273H} mutation. This study highlights the value of circulating exosomal DNA for a rapid, low-cost identification of cancer driving mutations. The identification of mutations in IPMN patients and healthy subjects suggests that liquid biopsies may allow potential assessment of cancer risk but with a cautionary note that detection of clinical cancer cannot be assumed.

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Introduction

Despite current treatment modalities, pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with a median overall survival between 6–12 months for patients with metastatic disease, and a 7.7% 5-year survival.[1,2] The genetics of PDAC indicate that about 50% of the patients exhibit *KRAS*^{G12D} mutation and about 12% possess *TP53* mutation at codon 273, with R273H being the most common point mutation.[3-7] Similarly, *KRAS* mutations, at lower prevalence, have been observed in patients with chronic pancreatitis (CP)[8-10] and intraductal papilliary mucinous neoplasm (IPMN).[11,12] In recent years, many different studies used cell-free DNA (cfDNA) isolated from the blood of pancreatic and colorectal cancer patients to identify *KRAS* mutations.[5,13-17] Circulating cfDNA originates from dying cells in injured tissue or accumulates as a consequence of physiologic cell turnover, thus possibly contributing to a decreased sensitivity and making it more challenging to identifying cancer specific mutations.[18,19]

Exosomes are 40–150 nm extracellular vesicles that contain DNA, RNA and proteins.[20,21] All living cells, including cancer cells, generate exosomes and cancer cells generate higher levels of exosomes than normal cells.[22,23] Recently, genomic DNA was discovered in serum-derived exosomes from healthy donors and cancer patients, with utility to identify cancer specific mutations.[24] This original finding was subsequently validated by other research groups.[25-27] Circulating exosomal DNA may offer a superior advantage to circulating cfDNA as exosomes contain larger fragments of DNA, making this source advantageous for PCR based detection of mutations. Moreover, exosomes originate from live cells. The ease of exosomes isolation from the blood has led to a strong push to explore the cancer diagnostic utility of circulating exosomes. This proof-of-concept study here highlights the potential use of circulating exosomal DNA in detection of cancer specific mutations.

Results

Defining digital PCR parameters using exosomal DNA derived from cancer cells

We previously reported the detection of a heterozygous *KRAS* mutation at codon 12 (KRASG12D c35G>A) and a

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homozygous *TP53* mutation at codon 273 (TP53R273H c.818G>A) using the genomic DNA from PDAC cell line, Panc-1, derived exosomes.[24] To determine the detection limit of $KRAS^{G12D}$ and $TP53^{R273H}$ mutations in exosomes by digital PCR, we used exosomal DNA derived from Panc-1 cells as well as HMLE cells (wild-type for KRAS at codon 12 and wild-type for TP53 at codon 273). Nanoparticle tracking analysis showed that the exosomes enriched from cell culture supernatant displayed the characteristic particle size distribution that peaks between 100 and 200nm (Fig. 1A). Exosomal DNA from HMLE cells (n = 3 repeats) indicated a 0 ± 0% rate of detection for $KRAS^{G12D}$ mutation and 0 ± 0.05% rate of detection for $TP53^{R273H}$ mutation, validating their wild-type status at both loci. In contrast, exosomal DNA from Panc-1 cells (n = 3 repeats) indicated a 59.62 ± 0.89% rate of detection for $KRAS^{G12D}$ mutation and 99.67 ± 0.13% rate of detection for $TP53^{R273H}$ mutation, validating their reported genotype for each mutation. Known amounts of HMLE exosomal DNA were used to determine the false positive detection rates upon digital PCR analyses for $KRAS^{G12D}$ and $TP53^{R273H}$ mutations. A stringent threshold of 8800 A. U. (arbitrary units, referring to the intensity of mutant allele (Fig. 2), for each mutation was used, reflecting an average false positive rate of 0.040% and 0.034% for $KRAS^{G12D}$ and $TP53^{R273H}$ mutation, respectively (Fig. 1B). Importantly, the wild-type HMLE exosomal DNA concentration range in these experiments reflects the range of exosomal DNA concentration obtained from serum samples of patients (*vide infra*). Whether relatively low or high concentrations of exosomal DNA were used, the average false positive rate remained consistent (Fig. 1B), supporting the reliability of the chosen limit set for defining false positives (< 8800 A. U.). To define the sensitivity of detection of $KRAS^{G12D}$ and $TP53^{R273H}$ mutations, we next performed a titration experiment, in which different amounts of Panc-1 mutant exosomal DNA were mixed with different amounts of wild-type HMLE exosomal DNA. These mixtures reflected the 'spiked-in' mutation at an expected frequency of 0.05, 0.1, 0.25, 0.5, 1.0, 5.0 and 10% (Fig. 1C). The titration experiment revealed that the sensitivity of detection for both mutations by digital PCR was 0.25% (Fig. 1C), below which mutant alleles could not be detected reliably, especially at low concentrations (0.5ng in Fig. 1C). We reasoned that detection was limited by the low concentration of exosomal DNA, and decided that a functional abundance above 0.25% was a conservative and reliable threshold to accurately define samples as 'positive' or 'negative' for the 2 mutations. This threshold was previously reported when using droplet digital PCR to detect low-prevalence somatic mutation.[28] Taking into consideration the respective false positive rates for each mutation, we

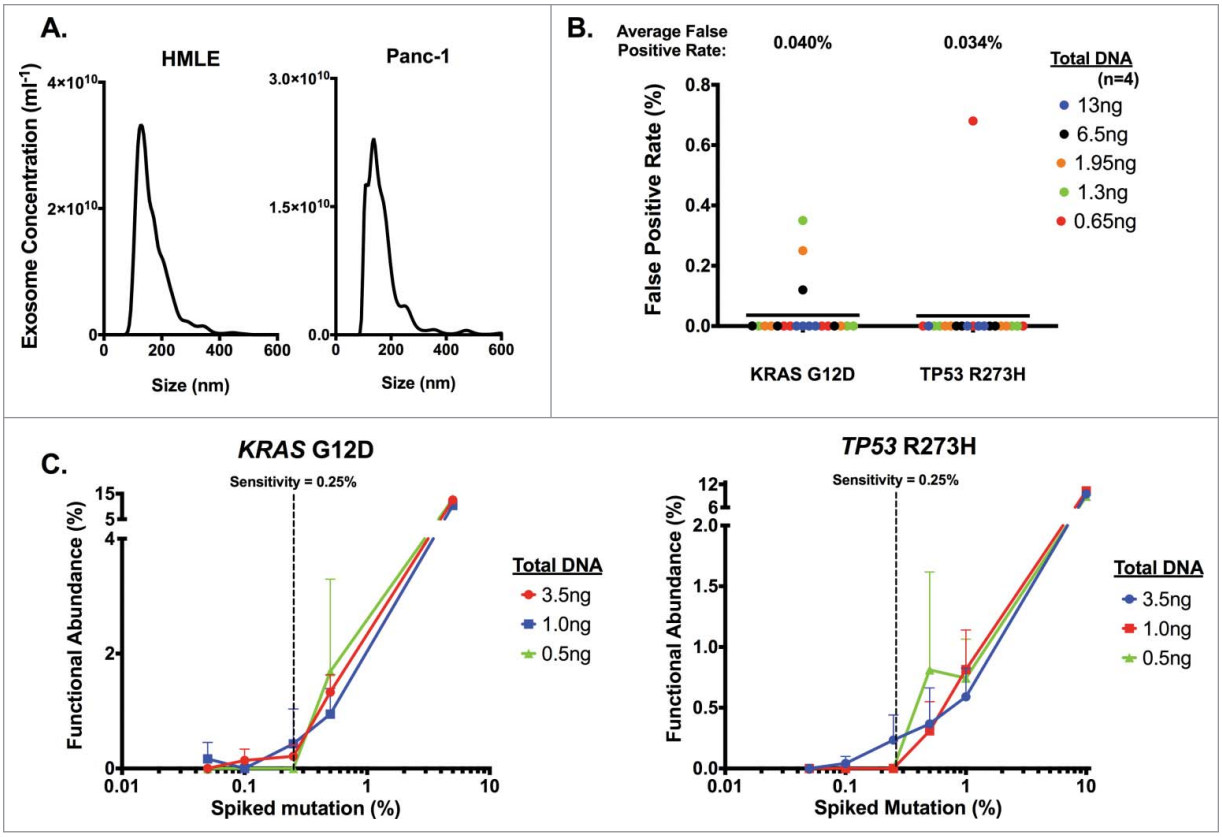

Figure 1. Definition of digital PCR parameters using cell line-derived exosomal DNA. Exosomes were pelleted from HMLE and Panc-1 cell culture supernatant with sequential filtration and ultracentrifugation, and DNA was extracted from purified exosomes. (A) Concentration and size distribution of HMLE (left) and Panc-1 (right) cell-derived exosome were analyzed using nanoparticle tracking analysis. (B) Determination of the average false positive rate using HMLE exosomal DNA. ng: nanograms. (C) Determination of the sensitivity threshold by measuring the relative functional abundance of titrated mixture of Panc-1 exosomal DNA spiked in HMLE exosomal DNA samples. Defined percentages of spiked Panc-1 exosomal DNA (with $KRAS^{G12D}$ mutation and $TP53^{R273H}$ mutation) with HMLE exosomal DNA were expressed as an expected percentage of spiked mutation, and dPCR analyses were run using the listed amount of DNA input (n≥ 2 for all other points except n = 1 for 5% in $KRAS^{G12D}$ and 10% in $TP53^{R273H}$). The sensitivity threshold was set to 0.25% to reliably detect either $KRAS^{G12D}$ (left) or $TP53^{R273H}$ (right) mutations.

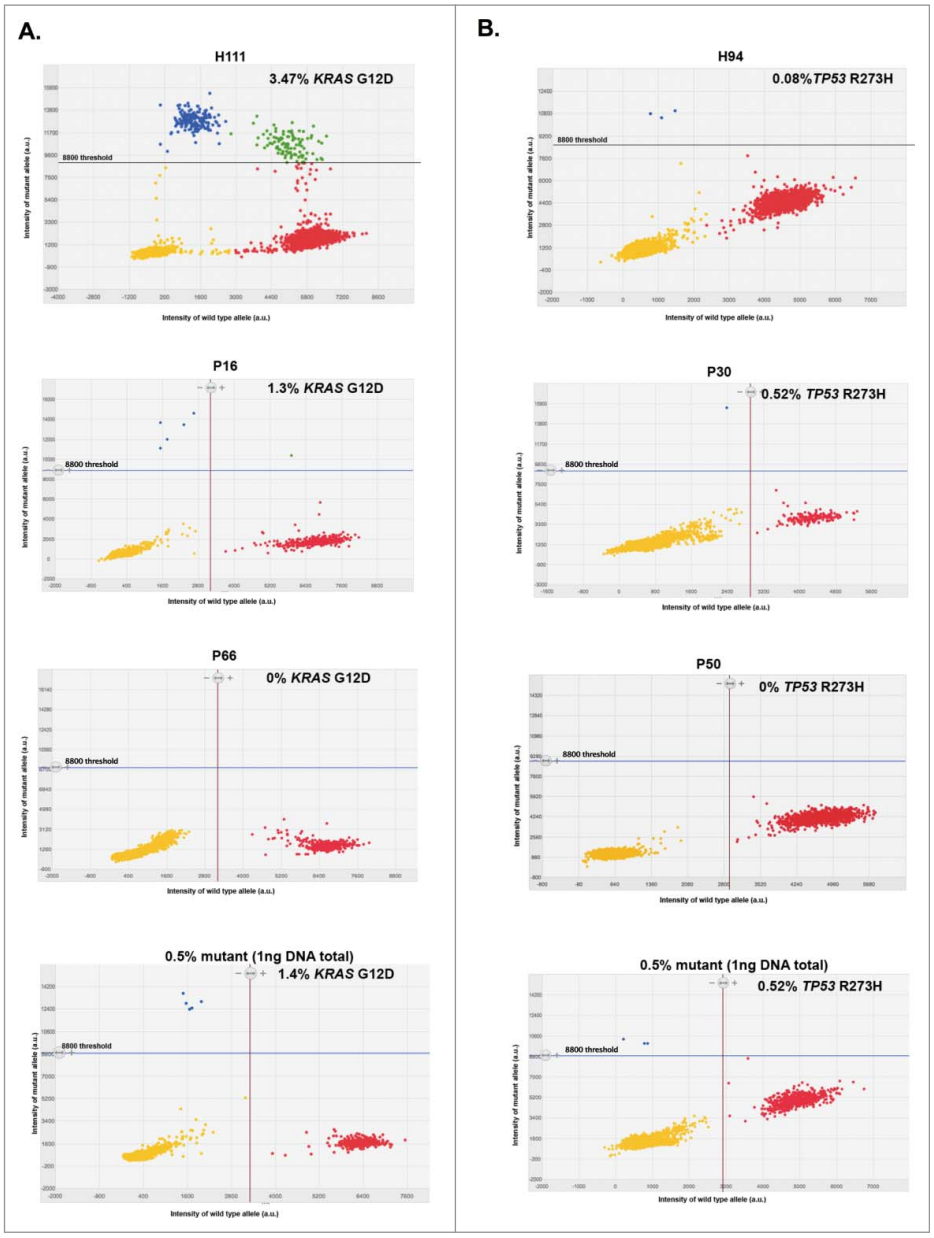

Figure 2. Representative 2D intensity scatter plot of wild-type and mutant amplicon for *KRAS*^{G12D} (A) *KRAS*G12D and (B) *TP53*R273H, PDAC patients (P16, P50), an IPMN patient (P30) and a CP patient (P66), and control spiked-in reactions (0.5% mutant). Threshold for mutant was set to 8800. Yellow, no DNA; blue, mutant; red, wild-type; green, mutant and wild-type, ng: nanograms.

defined a sample as 'positive' for a given mutation if 1) the intensity of mutant allele was higher than 8800 A.U. (Fig. 2) and 2) the functional abundance, with the average false positive rate deducted (as described in experimental procedures), was greater than 0.25%.

Detection of KRAS^{G12D} and TP53^{R273H} mutations from circulating exosomal DNA of patients and healthy individuals

Exosomes from the serum of healthy subjects and patients (PDAC, IPMN, CP or others) were enriched following sequential size exclusion filtration and ultracentrifugation as described in the accompanying experimental procedures. Demographic and, when pertinent, cancer related characteristics of subjects are listed in Table S1. Our analyses included other patients with non-pancreas related disease and non-cancer pancreas pathologies distinct from chronic pancreatitis and IPMN (classified as 'others' and including autoimmune pancreatitis, common bile duct cancer, pancreatic cystadenoma, pancreatic neuroendocrine tumor (NET), duodenal adenoma, and uterine sarcoma, Table S2). Nanoparticle tracking analyses indicated that the exosome preparations contained particles with a size distribution characteristic of exosomes for both healthy donors and patients (Fig. 3A). Despite the low volume of some of the serum samples (as low as 150 μl, Table S2 and S3), sufficient amount of exosomal DNA, as defined by the Qubit detection sensitivity, was obtained and with a circulating exosomal DNA concentration range of 0.102 – 1.35 ng/μl for patients (0.11 – 1.22 ng/μl for PDAC patients, 0.106–0.584 for IPMN, 0.13–1.35 for CP, 0.102–0.476 for others), and of 0.212 – 19.7 ng/μl for healthy subjects (Fig. 3B). The minimal amount of exosomal

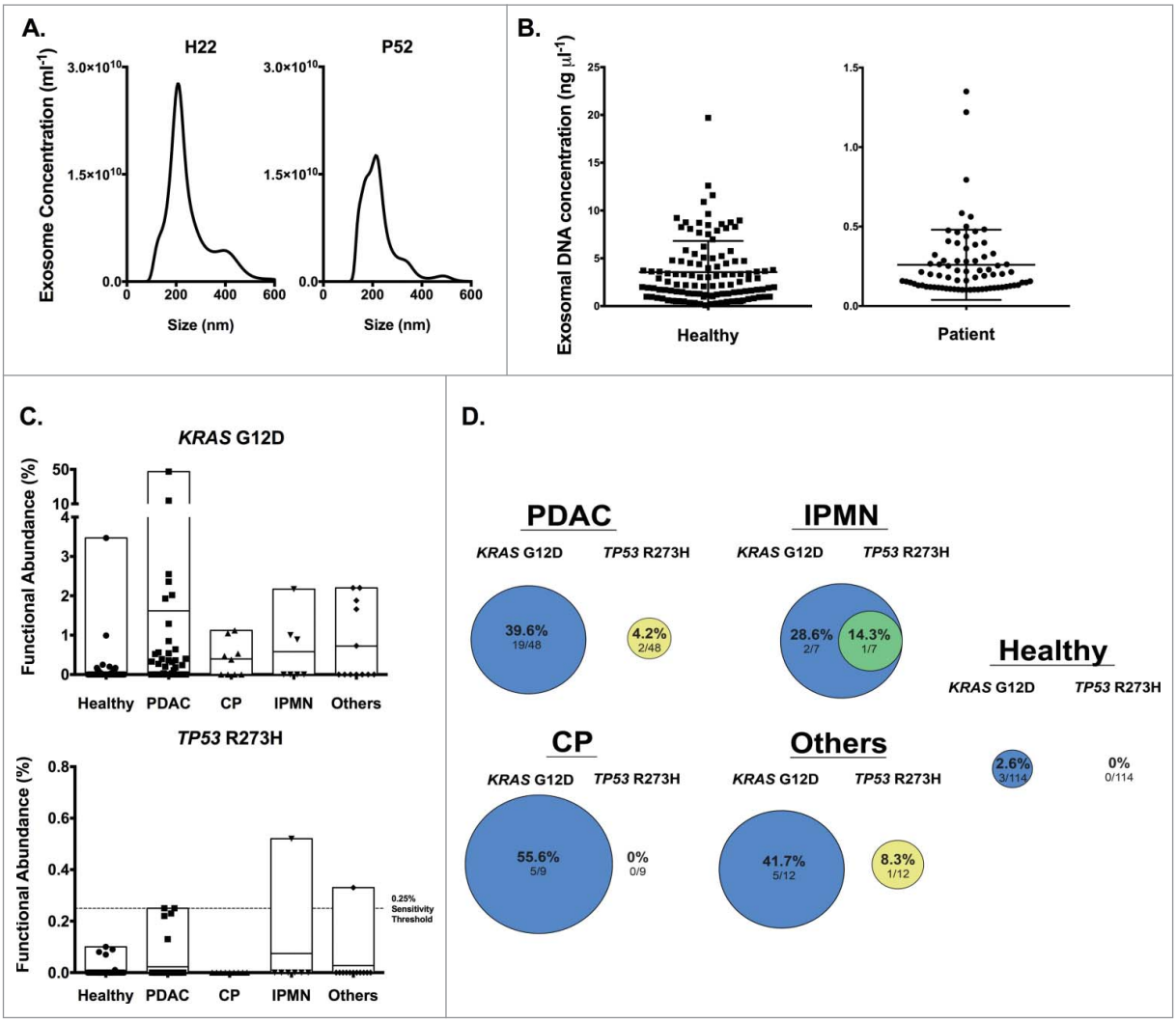

Figure 3. Identification of $KRAS^{G12D}$ and $TP53^{R273H}$ mutation in exosomal DNA from serum samples of cancer patients and healthy donors. (A) Representative concentration and size distribution of exosome using nanoparticle tracking analysis, from a healthy donor (left) and a PDAC patient (right). (B) Serum derived exosomal DNA concentration in healthy donors and patients. (C) Functional abundance of $KRAS^{G12D}$ (top) and $TP53^{R273H}$ (bottom) mutations for the indicated groups. (D) Percent distribution of $KRAS^{G12D}$ and $TP53^{R273H}$ mutations in the indicated groups with overlapping circles indicating individuals with both mutations. For PDAC: n = 48 patients, IPMN: n = 7, CP: n = 9, Others: n = 12, Healthy: n = 114.

DNA used for digital PCR analyses was thus 0.663 ng. Samples with DNA concentration greater than 8 ng/μl were diluted 1:3, equating to a 42.68 ng maxima.

Informed by the thresholds established by our control experiments (Fig. 1 and experimental procedures), defined clusters of mutant and wild-type populations of positive wells were identified by digital PCR (Fig. 2) and the data informed on the positivity for detection of $KRAS^{G12D}$ and $TP53^{R273H}$ mutations. For PDAC patients, $KRAS^{G12D}$ and $TP53^{R273H}$ mutations were detected in 39.6% (19/48) and 4.2% (2/48) of the samples, respectively (Fig. 3C–D and Table S2). The highest functional abundance observed was 47.45% (patient P8) and 0.25% (patients P27 and P65) for $KRAS^{G12D}$ and $TP53^{R273H}$ mutations, respectively (Fig. 3C, Table S2). We found that 3 out of 7 IPMN patients harbored the $KRAS^{G12D}$ mutation with the highest observed functional abundance at 2.17%, and one of these patients also co-harbored the $TP53^{R273H}$ mutation (0.52% functional abundance) (Fig. 3C–D). In CP patients, $KRAS^{G12D}$ mutation was found in 5 out of 9 of the serum samples (highest functional abundance of 1.12%), but none with the $TP53^{R273H}$ mutation (Fig. 3C–D). Five out of 12 (41.7%) patients diagnosed with other diseases (autoimmune pancreatitis, common bile duct cancer, pancreatic cystadenoma, pancreatic neuroendocrine tumor, duodenal adenoma, and uterine sarcoma, Table S2) harbored the $KRAS^{G12D}$ mutation (highest functional abundance of 2.20%), and only 1 had the $TP53^{R273H}$ mutation (functional abundance of 0.33%). In healthy subjects, the $KRAS^{G12D}$ mutation was observed in 2.6% (3/114) of individuals, with highest functional abundance of 3.47% (Fig. 3C and Table S3). $TP53^{R273H}$ mutation was not detected in the exosomal DNA from healthy subjects.

Discussion

The use of circulating cfDNA as a diagnostic and monitoring tool for cancer management, and also perhapsas as a screening tool for early detection of cancer is suggested by emerging studies. Such potential could likely be achieved for cancer types

with high mutation frequency in specific loci.[19] This study indicates that even small amount of serum, as low as 150 microliters, can be used to isolate exosomes and purify DNA to identify cancer specific mutations. We identified, in 48 serum samples from PDAC patients, $KRAS^{G12D}$ mutation in 39.6% of the samples and $TP53^{R273H}$ mutation in 4.2% of them, leaving 27 samples without these 2 specific mutations. These results are relevant as the prevalence of $KRAS^{G12D}$ mutation identified using PDAC tumor tissue is approximately 40–50%.[7] Therefore, this exosomal DNA study likely captures most of the anticipated $KRAS^{G12D}$ mutations in PDAC patients. This also appears to be the case for $TP53^{R273H}$ mutation, with an estimated 7% of patients possibly presenting with a c.818G>A substitution reflecting the R273H mutation. Previous studies have used serum or plasma-derived cfDNA from PDAC patients to define a detection rate of specific mutation in tumor tissue and corresponding (matched) serum/plasma samples, and in some cases, also evaluated a limited number of healthy donors.[13,29] Using droplet digital PCR analyses of cfDNA derived from 1 mL of serum, a 36.3% detection rate of $KRAS^{G12D}$ mutation was reported in a cohort of 66 patients with pancreatic cancer, and in 5% (1 out of 20) of healthy donors.[16] Our results using circulating exosomal DNA appear to provide similar sensitivity for mutation detection. Future studies using larger cohorts will address whether cfDNA or exosomal DNA offer better sensitivity and/or specificity, or be of equal value. Nonetheless, our study shows that circulating exosomal DNA can be used to identify mutations using high-throughput techniques such as digital PCR. Importantly, while $KRAS^{G12D}$ mutations were noted in 2.6% of our large cohort of healthy donors (n = 114), *TP53* mutation (here, R273H associated with c.818G>A mutation) was not detected in healthy subjects. Detection of such mutations in healthy subjects.[16,30]

Our study, using a large number of healthy subjects to detect cancer associated mutations in liquid biopsy, leads to a cautionary note that identification of driver mutations may not signify presence of disease. On the other hand, patients with CP (at higher risk of developing PDAC lesions) appear to present with a greater rate of *KRAS* mutation in our study; and this is also supported by other studies using cfDNA (5–20%)[16,29] as well as in analyses of pancreatic tissue.[8,9] In previous studies, *KRAS* mutations in IPMN patients were identified using tissue samples[11,12,31-34] and pancreatic juice[31,35]; however, *KRAS* mutations (G12D and G12V) were not detected in cfDNA of IPMN patients.[36] With regards to *TP53* mutation, immunostaining and PCR analyses of pancreas from CP patients failed to detect them,[10,37] supporting our results. *TP53* mutations, typically associated with disease progression, were detected in tissue samples[32,34] and pancreatic juice[35] of IPMN patients. However, both mutations ($KRAS^{G12D}$ and $TP53^{R273H}$) in the same IPMN patient had not been reported but was identified in our study for one IPMN patient. We noted in our analyses that 4 of the 5 highest functional abundances for the $KRAS^{G12D}$ mutation were in PDAC exosomal DNA samples, supporting previous studies in which higher concentration of *KRAS* mutation in pancreatic juice was able to distinguish PDAC and IPMN patients.[38]

Using multiple stringent criteria for the false positive rate, intensity and sensitivity, we noted that there were patients with sensitivity values just above or below 0.25% (Table S2). As reported by Uchiyama et al.,[28] these samples may be in the "gray zone" of detection due to low exosomal DNA concentration, which may be a potential limitation for liquid biopsy; however, we expect that improvement in exosomal DNA isolation techniques will increase sensitivity of digital PCR, and thus, improve detection of DNA with low mutation rates. Our study, in combination with other studies, suggests that the use of circulating exosomal DNA analyses, together with other nucleic acid analyses might offer a more reliable screening tool for early detection of pancreatic neoplasia. Nucleic acids, including DNA and RNA species, are presumably protected from degradation in the blood stream by exosomes encapsulation; hence, exosomal nucleic acid analyses may refine the sensitivity of the assay for early detection of cancer. Indeed, while identification of highly prevalent mutations in circulating exosomal nucleic acids by itself may not offer a specific diagnosis, this approach, when combined with imaging modalities and other diagnostic procedure, may enhance personalized care of high-risk individuals. Circulating exosomal nucleic acid analyses may allow us to further develop precision medicine techniques for tailoring personalized care, with early detection and cancer prevention in mind.

Materials and methods

Healthy individual and PDAC patient serum samples

The study was performed on 171 patients who underwent major surgical pancreatic resection due to several underlying diagnosis at the Department of General, Visceral and Thoracic surgery, University Medical Center of Hamburg, Germany between 2003 and 2013. The diagnosis was confirmed upon histopathological analyses of the resected material. The study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany (PVN1045). Written consent for using the samples for research purposes was obtained from all patients before surgery or blood drawing. Blood samples of patients with suspected PDAC were obtained from central venous catheter from each patient directly before surgery. After collection of the blood samples, serum was separated from the blood by centrifugation at 3400×*g* for 10 minutes and these serum samples were kept frozen at −80°C. Samples were chosen randomly from the pre-existing tumor bank unaware of the underlying disease at the time of exosome analysis. Histopathologically proven diagnoses (PDAC, IPMN, chronic pancreatitis and in a few cases autoimmune pancreatitis, common bile duct cancer, pancreatic cystadenoma, pancreatic neuroendocrine tumor, duodenal adenoma, and uterine sarcoma) were revealed after digital PCR analyses of serum-derived exosomal DNA. None of the patients with malignant disease received preoperative cancer related therapies.

Blood samples of healthy individuals were collected by MD Anderson Blood Bank. Serum samples were collected in Vacutainer Plus plastic serum tube (Becton Dickinson) and stored at room temperature until the end of the day. Samples were then spun at 3000*xg* for 20 minutes and then stored at 4°C overnight. The serum was then extracted the next day and the samples were stored at −80°C.

Cell lines

A pancreatic cancer cell line, Panc-1 (CRL-1469) was purchased from ATCC and cultured in RPMI 1640 (Corning) completed with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Gibco). HMLE cell line was a gift from Dr. Sendurai Mani at MD Anderson and cultured in DMEM/Ham's F12 50/50 media (Corning) completed with 5% horse serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (Gibco) and 100 ng/ml cholera toxin (Sigma Aldrich). All cells were cultured in 37°C and 5% CO_2. The cells routinely tested negative for mycoplasma. The *KRAS* and *TP53* genotypes of the cells were confirmed by Sanger sequencing.

Exosome isolation

For cell culture supernatant derived exosomes, the cell lines were cultured until 80% confluence, washed with phosphate buffered saline (PBS), and 20ml of serum free media was added to the cells for 48 hours. This conditioned medium was then collected for exosome isolation (*vide infra*). Serum samples from healthy donors or PDAC patients were thawed at 37°C before exosomes extraction. The starting volumes of plasma are listed in Table S2. Serum samples and cell line-conditioned medium were centrifuged at 800*xg* for 5 minutes, then 2000*xg* for 10 minutes. The supernatant was filtered using a 0.2μm syringe filter (Corning or Whatman). For serum samples, the filter was washed with PBS to maximize sample recovery, and samples were brought up to 11ml with PBS. Exosomes were pelleted by ultracentrifugation (Beckman Coulter Optima L-90K) at 198,000*xg* for 4 to 8 hours for serum samples (SW-41 Ti rotor), and at 134,000*xg* for 2 to 3 hours for cell lines (SW-32 Ti rotor). Exosome pellets were resuspended in 200μl PBS and stored at −80°C. The enrichment of exosomes in our preparations was verified using nanoparticle tracking analysis (Malvern NanoSight LM10-HS) and manufacturer's software (v3.1 with camera level set to 9 for serum and 13 for cell line, and detection threshold to 5).

DNA extraction

DNA was extracted from exosomes using QIAamp DNA Micro kit (Qiagen) according to the manufacturer's instructions with the following modifications: exosomes lysis was performed at 56°C for 1 hour, and DNA was eluted with 25μl of elution buffer. DNA concentration was quantified using Qubit dsDNA high sensitivity assay according to manufacturer's instructions (Thermo Fisher). The samples with detectable DNA concentration were subsequently used for *KRAS* and *TP53* mutation analysis by digital PCR. Sufficient amount of DNA was obtained from 49% (76/156) of patient and 66% (114/171) of healthy serum samples.

Digital PCR

We aimed to detail the experimental procedures following the guidelines proposed for reporting digital PCR data[39] and proceeded with analyses of our results keeping in mind reported considerations on sensitivity and specificity.[40] To identify $KRAS^{G12D}$ and $TP53^{R273H}$ mutations in human sample exosomal DNA, digital PCR was performed using Taqman SNP Genotyping assays on the Quantstudio 3D system (Thermo Fisher) according to manufacturer's instructions. The probe assay ID used are AH6R5PI (KRAS G12D c35G>A (WT:C —> MUT:T) and AHWSLEX (TP53 R273H c.818G>A (WT: C —> MUT:T). For human samples, 6.5μl of DNA was added regardless of DNA concentration. Samples with DNA concentration greater than 8 ng/μl was diluted 1:3 in elution buffer to ensure proper cluster separation during analysis. The functional abundance was calculated and reported as a percentage using the formula below:

$$\text{Functional Abundance}(\%) = \text{Total number of mutant alleles}/\text{Total number of alleles (mutant and wildtype)} - \text{average false positive rate.}$$

The analysis of the digital PCR data was performed with the manufacturer's software (QuantStudio 3D Analysis Suite). The detection limit of the assay was determined using exosomal DNA extracted from cell lines. Exosomal DNA extracted from HMLE cell lines were used as wild-type DNA, while that extracted from Panc-1 was used as mutant DNA for *KRAS* (heterozygous G12D) and *TP53* (homozygous R273H). False positive rate and threshold (intensity) limit for mutant alleles were determined using wild-type DNA at concentrations representative of that of samples - 4 replicates at 0.1, 0.2 and 0.3 ng/μl for patients (totaling 0.65, 1.3, 1.95ng of DNA), and 1 and 2 ng/μl for healthy donors (totaling 6.5 and 13ng of DNA). The average false positive rate was calculated from all replicates (Fig. 1B), and used to calculate the reported functional abundance. A titration of mutant exosomal DNA from 10% to 0.01% with a total of 0.5, 1 and 3ng DNA was performed (Fig. 1C), determining the functional abundance threshold at 0.25%. We also conducted a 'no template' control (n = 1) and did not detect any mutant or wild-type alleles.

Disclosure of potential conflicts of interest

MD Anderson Cancer Center and R. Kalluri hold patents in the area of exosomes biology, which are licensed to Codiak Biosciences Inc. MD Anderson Cancer Center and R. Kalluri are stock equity holders in Codiak Biosciences Inc. R. Kalluri receives research support from Codiak Biosciences and serves as a member of the board of directors.

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