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Original Article

DNA integrity as biomarker in pancreatic cyst fluid

Wesley K Utomo[1], Vincent T Janmaat[1], Auke P Verhaar[1], Jérôme Cros[2], Philippe Lévy[3], Philippe Ruszniewski[3], Mirella S Vredenbregt-van den Berg[4], Guido Jenster[4], Marco J Bruno[1], Henri Braat[1], Gwenny M Fuhler[1], Maikel P Peppelenbosch[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Department de Pathology, Hôpital Beaujon, INSERM U1149, Université Paris Diderot, Clichy, France; [3]Service de Pancréatologie-Gastroentérologie, Pôle des Maladies de l'Appareil Digestif Université Denis Diderot-Paris VII Hôpital Beaujon, Clichy Cedex, France; [4]Department of Urology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

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Abstract: Identification of pancreatic cysts with malignant potential is important to prevent pancreatic cancer development. Integrity of cell free DNA (cfDNA) has been described as tumor biomarker, but its potential for pancreatic cancer is unclear. While normal apoptotic cells release uniformly truncated DNA, malignant tissues release long fragments of cell free DNA (cfDNA). We measured 247 base pair (bp) and 115 bp DNA fragments of ALU repeats by qPCR in serum from healthy controls and pancreatic cancer patients, and in cyst fluid from pancreatic cyst patients. No differences in total cfDNA (ALU115) and cfDNA integrity (ALU247/115) were observed between sera from healthy controls (n=19) and pancreatic cancer patients (n=19). Although elevated as compared to serum, but no differences in cfDNA were found in cyst fluid from high risk (n=10) and low risk (n=20) cyst patients. We conclude that cfDNA integrity is not a useful marker to identify (pre)malignant pancreatic lesions.

Keywords: Cell free DNA, circulating, serum, pancreatic cyst fluid, PCR, ALU repeats

Introduction

Pancreatic cystic neoplasms (PCN) can give rise to pancreatic cancer, with intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) showing a high malignant potential, whereas serous cystic adenomas and solid pseudopapillary neoplasms have a more favorable prognosis [1]. Development of pancreatic cancer may be prevented by resection of the cysts with high risk malignant potential. Unfortunately, current imaging and diagnostic techniques have difficulty distinguishing low risk cysts from high risk and transformed cysts, which in some cases leads to unnecessary surgery [2]. Thus, better diagnostic tools are urgently needed. Patients with neoplastic diseases often have an increased amount of free circulating, cell-free DNA (cfDNA) in their peripheral blood, which originates from the tumor [3-5]. This cfDNA is not all of an equal length. While apoptotic cells release small, ~180 base pair (bp) DNA fragments, necrotic cells release larger fragments of irregular size [6]. Whereas apoptosis is a normal physiological process occurring in all cells that need to be cleared from the body, necrosis is a potentially harmful form of cell death, which occurs under pathological conditions, including cancer. Thus, the presence of longer DNA fragments in serum is taken as a sign of enhanced necrosis taking place in the body and is thought to be indicative of disease [7]. In order to reliably measure such DNA fragments, researchers have employed the abundant presence in the human genome of DNA ALU repeats - repetitive ~300 bp sequences of retrotransposon origin found in genomic introns [8]. Using different primers, fragments of these ALU repeats can be detected of either >200 bp (indicative of necrotic DNA), or of <200 bp (detecting both necrotic and apoptotic DNA). Detection of these longer cfDNA fragments and their relative abundance compared to short cfDNA fragments in sera appears to be a promising tool for diagnosis and prognostic prediction of malignancies [9, 10]. However, the percentage of cfDNA originating from tumor cells has been estimated to

range from 10% to 90% of total cfDNA, and applicability of measuring cfDNA length (i.e. DNA integrity) in serum may therefore depend on the type of disease [6]. Thus, while ALU-repeat measurements have been shown to adequately predict colorectal and breast cancer, the presence of pancreatic cancer could not be diagnosed by high length cfDNA fragments in serum [11]. We speculated that pancreatic cyst fluid, coming from a small and enclosed environment, would provide a more suitable biological fluid in which to search for tumor markers. Therefore we compared DNA integrity in fluid from high risk and low risk cysts.

Material and methods

Pancreatic cyst fluid acquirement

Pancreatic cyst fluid of patients undergoing surgery was collected from two separate biobanks (Erasmus MC Rotterdam, the Netherlands, MEC-2008-233 and MEC-2012-107, and Hôpital Beaujon, Clichy France; DEC-2009-938). Fluid was obtained by endoscopic ultrasound-fine needle aspiration (EUS-FNA) or post-resection and stored sterile at -80°C until analysis. Samples were selected so as to represent the different groups of pancreatic cyst based on malignant potential. Cysts with histologically confirmed low grade and intermediate dysplasia were grouped under 'low grade dysplasia', and cysts with high grade dysplasia or invasive carcinoma were considered 'high grade dysplasia'.

Serum acquirement

Patients with pancreatic adenocarcinoma who were eligible for surgery were included at the EMC. Healthy controls (mean age 60±4 years) were collected from the biorepository of the Rotterdam arm of the ERSPC [12, 13] (MEC 138.741/1994/152). Serum was obtained by whole blood centrifugation in serum separator tubes (BD-Vacutainer), aliquoted and stored at -80°C until analysis.

Sample preparation and qPCR

To digest proteins that might confound results, both cyst fluid and serum were mixed with a buffer containing 25 ml/l Tween 20, 50 mmol/l Tris, 1 mmol/l EDTA, and 0.8 mg/ml proteinase K in a 1:1 ratio. Subsequently, the mix was incubated at 50°C for 20 minutes, followed by heat inactivation at 95°C for 5 minutes. Next, the samples were centrifuged at 10,000 g for 5 minutes and 0.2 µl of the supernatant was used in the qPCR reaction. This protocol was also described earlier [14]. After preparation of samples, DNA integrity was determined by measuring the presence of ALU repeat fragments of 115 bp size and of 247 bp size, using previously described primers [15]. The ALU115 primers are designed to amplify both the shorter and the longer fragments, and are therefore indicative of total circulating cfDNA (including DNA released from both apoptotic and necrotic cells) whereas the ALU247 primers only amplify the longer DNA fragments, and thus detect of tumor DNA. To measure the absolute concentration of DNA in the samples, we constructed a calibration curve using genomic DNA derived from Huh7 cell lines at a concentration ranging from 2.97 pg/µl up to 297 ng/µl. The absolute concentration was measured (in the most concentrated sample) using the Nanodrop (Thermo Scientific). This was subsequently used in a serial dilution and used as a template in triplicate on each qPCR-plate measured. The same serial dilutions were used to produce standard curves for all qPCR runs.

For the qPCR reaction of the ALU repeats, previously published primers were used: ALU115 forward, CCTGAGGTCAGGAGTTCGAG; ALU115 reverse, CCCGAGTAGCTGGGATTACA; ALU247 forward, GTGGCTCACGCCTGTAATC; ALU247 reverse, CAGGCTGGAGTGCAGTGG. The total volume of the qPCR reaction mix was 25 µl, consisting of 12.5 µl SYBR Green (Life technologies), 2.5 µl 10 µM forward and reverse primer, 9.8 µl Microbial DNA-Free Water (Qiagen), and 0.2 µl template.

The qPCR was run at 95°C for 10 minutes, and subsequently at 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds for 40 cycles using the StepOnePlus™ Real-Time PCR System (Life technologies). ALU repeat expression levels were measured in duplicate. The genomic DNA used for the calibration curve and negative controls were measured in triplicate.

Analysis

Using the data obtained from the serial diluted genomic DNA, we constructed a calibration curve on each plate measured using the

Table 1. Patient characteristics of the serum samples

Number of patients	19
Age, years	
Range	24-82
Mean (SD)	65 (13)
Gender, n (%)	
Male	11 (57.9)
Female	8 (42.1)
Disease location (%)	
Pancreas corpus	3 (15.8)
Pancrease head/uncinate	5 (26.3)
Pancreas head	7 (36.9)
Bile duct	3 (15.8)
Papilla	1 (5.2)

Graphpad Prism 5. From this, we derived the intercept and slope of the curve using a nonlinear regression model and recalculated the absolute concentration of the samples from measured Ct values using the following formula:

Absolute concentration = 10^((Ct-intercept)/slope)

Finally, to obtain ALU247/115 ratios, the absolute concentration of ALU247 was divided by the absolute concentration ALU115 measured in the samples. Mean differences were analysed using Mann-Whitney U test. A *p*-value of <0.05 was considered statistically significant.

Results

While previous reports were unable to find a relationship between pancreatic cancer and cfDNA length in serum, we wanted to verify this in our own cohort (see **Table 1**). The mean total circulating cfDNA, as represented by ALU115-qPCR values, was 20±3 pg/µl in control sera (n=19) vs 36±14 pg/µl in sera from pancreatic cancer patients (n=19) (P=0.559) (**Figure 1A**), whereas the mean amount of circulating tumor DNA, as determined by ALU247-qPCR values, was 8±1 and 14±6 pg/µl (P=0.793). The ratio of ALU247/ALU115, allowing quantification of the integrity of the cfDNA, was 0.41±0.02 and 0.40±0.05 for healthy controls and pancreatic cancer patients, respectively (P=0.267). Thus, no increased total cfDNA or tumor-associated DNA was detected in sera from pancreatic cancer patients.

Next, we analysed cyst fluid obtained from 40 pancreatic cyst patients, 23 of which had low risk cysts, and 17 had high risk cysts. In 10 of these samples, we were unable to perform a reliable analysis due to the mucinous nature of the fluid. In the remaining samples (**Table 2**), we observed drastically higher levels of cfDNA as compared to sera, presumably due to the enclosed nature of these cysts. However, the mean amount of total and tumor circulating cfDNA did not differ between the high risk (n=10) and low risk (n=20) samples: 44,463± 39,228 vs 33,021±20,004 pg/µl for ALU115 (P=0.10) and 27,254±24, 2682 vs 22,118± 13,774 pg/µl for ALU247 (P=0.18), respectively (**Figure 1B**). Furthermore, no significant differences in ALU247/ALU115 ratio between high risk and low risk cysts (0.63±0.07 vs 0.66±0.06, P=0.34) was seen. Overall, ratios were higher than in serum, with some samples reaching almost 1. Thus, the nature of cyst fluid makes it a less suitable compartment to determine necrotic/apoptotic cfDNA ratios.

Discussion

Measurement of necrotic cell-derived long cfDNA fragments in serum has been suggested for the early detection of tumors. However, usefulness of this tool in pancreatic diseases has so far not been shown, and we were unable to find increased levels of necrotic cell-derived cfDNA in sera from pancreatic cancer patients. As pancreatic cancer can derive from PCN, we speculated that cyst fluid would present the ideal biological fluid to detect premalignant lesions. Indeed, total levels of cfDNA observed in cyst fluid were almost 1000 fold higher as compared to sera. Nevertheless, we did not observe differences in cfDNA length between high risk and low risk cysts. While the apoptotic process reduces DNA to 180-200 bp fragments, incomplete cleaving of the DNA may result of the presence of multimers of these fragments, which can subsequently also be detected by ALU247 primers. This background 'noise' of 180 bp multimers accounts for the fact that a signal is detected in the ALU247 PCR in samples were no necrotic cfDNA is expected (i.e. healthy serum), and detection of tumor-derived, necrotic DNA depends on a relative increase in the abundance of long cfDNA, and hence a shift in DNA integrity (ALU247/ALU115 ratio). It is conceivable that pancreatic tumor cells produce too little necrotic cfDNA to be

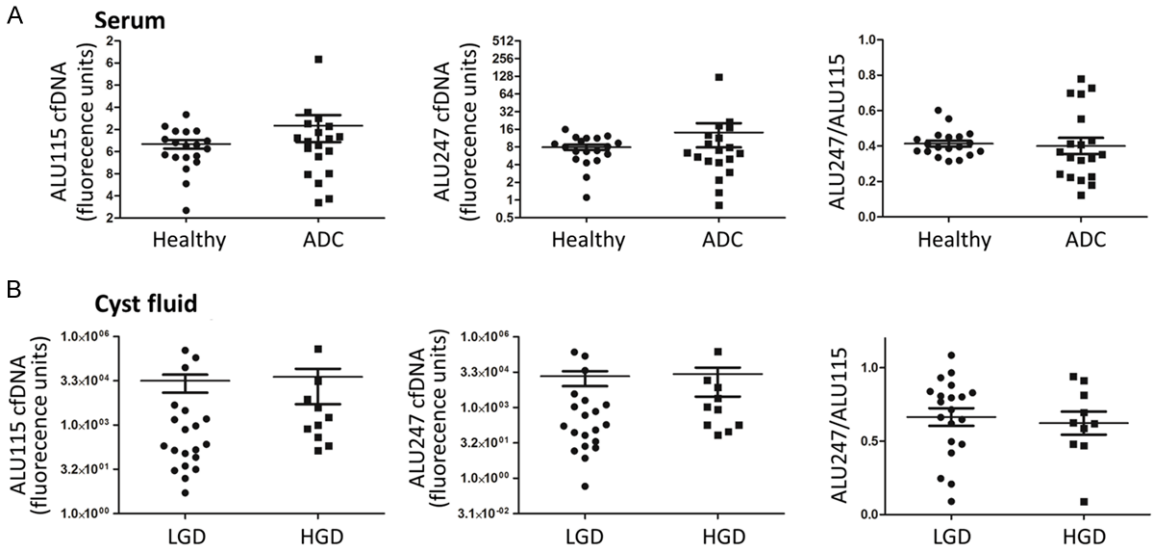

Figure 1. Levels of cfDNA in serum or pancreatic cyst fluid do not identify high risk/adenocarcinoma patients. PCRs detecting short, apoptotic cell-derived cfDNA (ALU115) and longer cfDNA fragments (ALU247) were performed on (A) serum from healthy donors (n=19) and patients with pancreatic adenocarcinoma (ADC, n=19) and (B) pancreatic cyst fluid from patients with low grade dysplasia (LGD, n=20) and high grade dysplasia (HGD and ADC, n=10). cfDNA was detected by ALU115 and ALU247, and DNA integrity was calculated (ALU247/ALU115). No differences in cfDNA levels and cfDNA integrity were observed.

Table 2. Patient characteristics of the cyst fluid samples

	Total	LGD	HGD
Number of patients	30	20	10
Age, years			
Range	19-85	19-79	52-85
Mean (SD)	59.5 (15.3)	54.7 (15.4)	69.2 (10)
Gender			
Male	10 (33.3%)	3 (15%)	7 (70%)
Female	20 (66.7%)	17 (85%)	3 (30%)
Diagnosis			
IPMN	17 (56.7%)	8 (40%)	9 (90%)
MCN	13 (43.3%)	12 (60%)	1 (10%)
Disease location (%)			
Pancreas head	6 (20%)	2 (10%)	4 (40%)
Pancrease corpus	5 (16.7%)	3 (15%)	2 (20%)
Pancreas tail	18 (60%)	15 (75%)	3 (30%)
Unknown	1 (3.3)	0 (0%)	1 (10%)

detected above background levels. Additionally, in cyst fluid, high levels of total cfDNA levels present may preclude detection of additional long cfDNA fragments.

We acknowledge several limitations to our study. Of the cystic fluid samples selected for this pilot study, 15 of 30 were obtained after resection, with ischemic damage potentially causing necrosis. However, subanalysis of the re-resection and post-resection obtained samples did not show significant differences in total cfDNA levels or ALU247/ALU115 ratios (not shown).

A second limitation is the low number of high risk cyst fluid samples in our analysis. The mucinous nature of the fluid prevented accurate analysis in ~25% of cases. As mucinous cysts show a higher malignant potential, it is not surprising that many of the excluded samples were high risk. This means that the intrinsic nature of high risk cyst fluid makes them less suitable for this type of analysis.

In conclusion, our data suggest that the use of cfDNA integrity in pancreatic cyst fluid is not suitable to distinguish low risk from high risk cysts.

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Disclosure of conflict of interest

None.

Abbreviations

qPCR, quantitative polymerase chain reaction; EUS-FNA, endoscopic ultrasound-fine needle aspiration; PCN, pancreatic cystic neoplasms; IPMN, intraductal papillary mucinous neoplasms; MCN, mucinous cystic neoplasms; cfDNA, cell free deoxyribonucleic acid.

Address correspondence to: Maikel P Peppelenbosch, Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Gravendijkwal 230, 3015CE, Rotterdam, The Netherlands. Tel: +31(10)7063792; E-mail: m.peppelenbosch@erasmusmc.nl

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