Detecting KRAS mutations in pancreatic cystic neoplasms: droplet digital PCR versus targeted next-generation sequencing

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RESEARCH LETTER

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Introduction

DNA-based testing of pancreatic cyst fluid (PCF), obtained by endoscopic ultrasound fine needle aspiration (EUS-FNA) may improve diagnostic accuracy for differentiation between types of pancreatic cystic neoplasms (PCN),[1] which is currently suboptimal. Mutations in DNA (a.o. KRAS, GNAS) can differentiate non-mucinous from (pre)-malignant, mucinous PCN. Various techniques are available to detect mutations in DNA; including targeted next generation sequencing (NGS), like Ion Torrent NGS, and since more recently droplet digital PCR (ddPCR). Molecular tests involving ddPCR are not widely used in clinical setting. DdPCR requires only 1 ng of DNA for a 0.1% sensitivity,[2] and is therefore a potentially suitable technique for DNA-based testing of PCF, which tends to contain low DNA concentrations. In comparison, NGS is able to detect mutations in multiple genes simultaneously[3] on around 10 ng of DNA and has a limit of detection of 3–5%.[4] The aim of this pilot study was to evaluate whether ddPCR is suitable for measuring KRAS-mutations in PCF, and to compare it with NGS.

Materials and methods

Patients with PCN referred for evaluation with EUS-FNA and/or surgery in the Amsterdam UMC (2007–2014) were eligible for enrolment. Analyses were restricted to patients with surgical histopathology. PCN were classified as mucinous (IPMN or MCN) or non-mucinous (SCN, cNET or SPN). Level of dysplasia was recorded as the highest neoplastic grade detected during pancreatectomy.[5] PCF was thawed at 37 °C and DNA was extracted using the Qiagen DNAmicrokit. DNA was quantitated on the Qubit V.2.0 Fluorometer using the dsDNA HS Assay Kit (Thermo Fisher Scientific, USA).

ddPCR

KRAS-alterations in codons 12 and 13 were detected using KRAS Screening Multiplex Kit (Bio-Rad). QuantaSoft Software version 1.7.4 was used for data analysis, including fractional abundance (FA). To set cut-off thresholds for mutation calling, we used duplicate dilution series of cell lines Capan1 and BxPC3, homozygously KRAS-mutated and wt-KRAS, respectively.[6] To identify double positive droplets, we mixed Capan1 and BxPC3 1:1 (10 ng/µl), and diluted to 50%, 10%, 1%, 0.1%, and 0.01%. Mutations were detected in 1%, but not 0.1% diluted samples. The cut-off threshold to call a mutation was set at 5 positive mutational droplets (Channel 1 > 8000, Channel 2 6000–8000). With <100 positive droplets the run is considered unreliable.

Ion Torrent NGS

We used a custom targeted sequencing panel (Ion AmpliSeq Designer) including KRAS codons 12, 13, 61. DNA libraries were produced using Ion AmpliSeq™ Library Kit 2.0 (Life Technologies, France). Libraries were bar-coded and sequenced on a 316 chip in the Personal Genome Machine system (Ion Torrent, Life Technologies). Torrent suite software v4.4.0 and SeqNext software v4.1.2 (JSI Medical Systems, Germany) were used for processing and analysing data. The minimum threshold was >1000 reads for both KRAS-amplicons.

Statistical analysis

Continuous data were reported as mean ± standard deviation (SD) or median and interquartile range (IQR). Categorical data were reported as frequency or percentage. Technical success was defined as cases in which the method could be successfully applied. Sensitivity was defined as true positive cases divided by true positive plus false negative cases. Accuracy was defined as the successful samples in which the outcome corresponded with the histopathological outcome. Statistical analysis was performed using SPSS V26.

Results

Twenty-four patients were included in our analysis, predominantly female (63%), with a median age of 61 years [SD ± 15] (Table 1). The concentration of PCF-derived DNA ranged from 0.26 to 78.4 ng/µl, with a median of 3.3 ng/µl. Histopathology showed 3 SCN, 1 cNET (LGD), 7 MCN (LGD), and 13 IPMN (9 LGD, 1 HGD and 3 invasive cancer; Table 2).

The mean number of KRAS-mutational droplets was 218.2 [SD ± 414.0], the mean number of positive wt-droplets was 2015.3 [SD ± 2825.0], and the mean FA was 8.2 [SD ± 13.1]. When using cut-off values of ≥5 KRAS-mutational droplets, ≥100 positive droplets, and FA ≥0.25, results were satisfactory for 22/24 samples, a technical success rate of 92%. We identified

Table 1 Baseline characteristics of patients and cyst fluid

ID	Final diagnosis	Sex	Age (years)	Route of obtaining PCF	Fluid viscosity	EUS morphology	CEA	Amylase	Cytology	DNA concentration PCF (ng/μl)
1	SCN	Male	30	PA	serous	Atypical pseudocyst	0.3	56		6.9
2	SCN	Male	69	EUS-FNA	serous	SCN	0.2	64		0.1
3	SCN	Female	50	EUS-FNA	mucinous	SB-IPMN	0.3	246		0.1
4	Cystic NET	Male	70	PA		SB-IPMN				41.2
5	MCN	Female	54	PA	mucinous	MCN	343	220		12.4
6	MCN	Female	41	EUS-FNA	serous	MCN	472	17,500		0.7
7	MCN	Female	52	EUS-FNA	serous	MCN	77.9	43,738		1.0
8	MCN	Female	32	EUS-FNA		MCN	677.5	12	satisfactory	0.7
9	MCN	Female	61	EUS-FNA	mucinous	MCN			satisfactory	0.0
10	MCN	Female	52	EUS-FNA		MCN	981.6	1900		7.3
11	MCN	Female	22	EUS-FNA	serous	MCN	66.1	39,414		0.3
12	SB-IPMN	Male	53	PA	mucinous	MCN			satisfactory	20.0
13	SB-IPMN	Female	68	PA		SB-IPMN				11.0
14	SB-IPMN	Female	62	PA	serous	SCN	1667	185		78.4
15	MT-IPMN	Male	64	EUS-FNA	mucinous	MT-IPMN	71.6	3274		0.7
16	MT-IPMN	Male	76	EUS-FNA		MT-IPMN				0.4
17	MT-IPMN	Male	61	EUS-FNA	mucinous	SB-IPMN	232.2	139,852		4.0
18	MT-IPMN	Male	74	EUS-FNA		Atypical	115	16,210	satisfactory	7.4
19	MT-IPMN	Female	46	EUS-FNA	mucinous	MT-IPMN	44.9	606,300	satisfactory	5.8
20	SB-IPMN	Female	80	EUS-FNA	mucinous	SB-IPMN	136.5	115,200	satisfactory	2.6
21	SB-IPMN	Female	65	EUS-FNA	serous	SB-IPMN	13.8	184,734		1.2
22	PDAC in IPMN	Female	67	PA		MT-IPMN				2.6
23	PDAC in IPMN	Male	70	EUS-FNA		SB-IPMN			satisfactory	8.8
24	PDAC in IPMN	Female	46	EUS-FNA	mucinous	MCN		26		42.6

EUS, endoscopic ultrasound; *FNA*, fine needle aspiration; *PCF,* pancreatic cyst fluid; *CEA,* carcinoembryonic antigen*; PA,* pathology; *SCN*, serous cystic neoplasm; *NET,* neuro-endocrine tumor*; SB*, side-branch; *IPMN,* intraductal papillary mucinous neoplasm; *MT*, mixed type; *MCN*, mucinous cystic neoplasm; *PDAC*, pancreatic ductal adenocarcinoma.

12 KRAS-mutation positive cases (54%). The sensitivity and accuracy of ddPCR were 63% and 68%, respectively.

In 13/24 cases Ion Torrent library preparations and NGS runs were successful; a technical success rate of 54%. In 2 cases a KRAS-mutation was detected, resulting in sensitivity and accuracy of 22% and 42%. We also looked at GNAS-mutations, which were identified in 3 cases.

In 11/13 cases in which both techniques were successfully performed, results were concordant (85%). In 9 cases, ddPCR was successful, identifying 8 KRAS-mutations, while NGS was not, and in 2 cases neither technique was successful. DdPCR detected KRAS-mutations in 85% of IPMN, and 20% of MCN with LGD. When combining ddPCR with NGS, GNAS-mutations were detected in 75% of IPMN, and KRAS and/or GNAS-mutations were detected in all but one IPMN (92%). Overall, a mutation was identified in PCF by either NGS and/or ddPCR in 13/22 samples (59%), which was accurate in 73%.

Discussion

This pilot study found that ddPCR is superior to NGS for detecting KRAS-mutations in PCF in success rate and concordance to histopathology. This might resolve an issue related to insufficiency of materials for DNA-based testing of PCF.

Several studies have shown the additional diagnostic value of KRAS-mutational status,[7] reporting 89% sensitivity and 100% specificity for detection of KRAS and/or GNAS-mutations in mucinous PCN.[8] Another study already demonstrated the feasibility of ddPCR, detecting methylated-DNA markers in PCF.[9]

Our study demonstrates that for the detection of mutations in PCF the technical success rate is a limitation of NGS compared to ddPCR (54 vs 92%), due to NGS requiring more DNA. Furthermore, ddPCR detected KRAS-mutations with higher sensitivity and accuracy than did NGS (62 vs 22% and 68 vs 42%, respectively), due to the limit of detection of NGS in low tumor cellularity samples. This was clear even from this small set of samples.

Table 2 Concordance between ddPCR and NGS on PCF

Pathology			ddPCR	NGS		
ID	**Diagnosis**	**Dysplasia**	**Mutational droplets**	**Wt droplets**	**ddPCR**	**Ion Torrent NGS**
1	SCN	No dysplasia	0	290	no mutation	no mutation
2	SCN	No dysplasia	0	103	no mutation	no mutation
3	SCN	No dysplasia	0	26		
4	Cystic NET	Low-grade	0	416	no mutation	no mutation
5	MCN	No dysplasia	2	2259	no mutation	no mutation
6	MCN	No dysplasia	0	116	no mutation	
7	MCN	No dysplasia	1	636	no mutation	no mutation
8	MCN	No dysplasia	14	1495	***KRAS***	
9	MCN	No dysplasia	5	44		
10	MCN	No dysplasia	3	746	no mutation	no mutation
11	MCN	No dysplasia	0	298	no mutation	no mutation
12	SB-IPMN	Low-grade	1014	7641	***KRAS***	
13	SB-IPMN	Low-grade	799	6883	***KRAS***	*GNAS* (3′UTR C > T)
14	SB-IPMN	Low-grade	1147	7675	***KRAS***	
15	MT-IPMN	Low-grade	55	47	***KRAS***	*GNAS* (3′UTR G > A)
16	MT-IPMN	Borderline	0	746	no mutation	*GNAS* (3′UTR C > A)
17	MT-IPMN	Borderline	16	649	***KRAS***	
18	MT-IPMN	Borderline	33	360	***KRAS***	***KRAS*** (12 G > V)
19	MT-IPMN	Borderline	1333	2908	***KRAS***	
20	SB-IPMN	Borderline	35	362	***KRAS***	
21	SB-IPMN	High-grade	21	210	***KRAS***	
22	PDAC in IPMN	invasive carcinoma	501	1166	***KRAS***	
23	PDAC in IPMN	invasive carcinoma	260	1242	***KRAS***	***KRAS*** (12 G > D)
24	PDAC in IPMN	invasive carcinoma	0	9019	no mutation	no mutation
	Total number of samples run				24	24
	Technically successful				22	13
	Technical success rate (%)				**92**	**54**
	***KRAS* identified**				12	**2**
	Prevalence *KRAS* (%)				54	17
	Sensitivity (%)				**63**	**22**
	Number of accurate samples				15	5
	Accuracy (%)				**68**	**42**
	***KRAS*/*GNAS* identified**					5
	Prevalence mutation (%)					39
	Sensitivity (%)					**50**
	Number of accurate samples					8
	Accuracy (%)					**62**

Mutational analysis on PCF using ddPCR (***left***) and Ion Torrent targeted NGS (***right***), showing number of positive mutational and wild-type droplets and mutational calls. Cut-off value to call a mutation was ≥5 positive mutational droplets and ≥100 positive mutational and/or wild-type droplets.Left empty indicates unsatisfactory run. *PCF*: pancreatic cyst fluid; *Wt*: wild-type; *SCN*, serous cystic neoplasm; *NET,* neuro-endocrine tumor*; SB*, side-branch; *IPMN,* intraductal papillary mucinous neoplasm; *MT*, mixed type; *MCN*, mucinous cystic neoplasm; *PDAC*, pancreatic ductal adenocarcinoma.

Even with improved DNA isolation methods, (multiple/multiplex) ddPCR runs are preferred for fluids with ultralow DNA contents, while for high-yield DNA specimens multi-gene panel NGS may be preferred. For implementation of ddPCR in clinical setting, the optimum threshold for the number of positive droplets requires validation, specifically with respect to the risk of false positive results when testing for single gene mutations using ultrasensitive detection methods.[10]

In conclusion, ddPCR has the potential to detect mutations in a highly sensitive manner, even in low tumor cell-content PCN specimens. To further validate the use of ddPCR for diagnostics, it is necessary to conduct studies with a larger number of PCF samples ranging from completely benign to (pre)-malignant, determine the cut-off values to call a mutation, and interrogate multiple genes per sample when feasible.

Author's contribution

- N.C.*M. van* Huijgevoort: conception and design; analysis and interpretation of the data; drafting of the article; final approval of the article.
- J.B.G. Halfwerk: performed experiments, analysis and interpretation of the data
- S.J. Lekkerkerker: designed and performed experiments, analysis and interpretation of the data; critical revision of the article for important intellectual content; final approval of the article.
- F.R. Ramp: performed experiments and analyzed data
- R.J.A. Reinten: interpretation of the data
- P. Fockens: critical revision of the article for important intellectual content; final approval of the article.
- M.G. Besselink: critical revision of the article for important intellectual content; final approval of the article.
- O.R. Busch: critical revision of the article for important intellectual content; final approval of the article.
- M.J. van de Vijver: critical revision of the article for important intellectual content; final approval of the article.
- J. Verheij: critical revision of the article for important intellectual content; final approval of the article.
- J.E. van Hooft: conception and design; analysis and interpretation of the data; drafting of the article; critical revision of the article for important intellectual content; final approval of the article.
- F. Dijk: conception and design; designed and performed experiments, analysis and interpretation of the data; drafting of the article; final approval of the article.

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- F. Dijk: None
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Ethics

This study was reviewed and approved by the Medical Ethics Review Committee of the Amsterdam UMC, location Academic Medical Center. All patients provided written informed consent prior to sampling and analysis.

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Conflicts of interest

None to declare.

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