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Serial EUS-Guided FNA for the Surveillance of Pancreatic Cysts: A Study of Long-Term Performance of Tumor Markers

Mahmoud A. Rahal[1], **John M. DeWitt**[2], **Harsh Patel**[3], **C. Max Schmidt**[4], **Eugene P. Ceppa**[4], **Rachel E. Simpson**[4], **Stuart Sherman**[2], **Mohammad Al-Haddad**[2]

[1]Department of Internal Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

[2]Division of Gastroenterology and Hepatology, Indiana University School of Medicine, 550 University Blvd, Suite 4100, Indianapolis, IN 46202, USA

[3]Department of Internal Medicine, Ochsner Clinic Foundation, New Orleans, LA, USA

[4]Department of Surgery, Indiana University School of Medicine, Indianapolis, IN, USA

Abstract

Background and Aim—The natural history of KRAS mutations in mucinous pancreatic cysts (MPCs) over time remains to be fully understood. The aim of this study was to examine the performance of DNA markers and assess changes of KRAS mutations over time.

Methods—Patients who underwent EUS-FNA of pancreatic cysts with at least two separate molecular analysis results were included in the study. We assessed the baseline patient and cyst characteristics, and DNA fluid analysis. The presence of either a KRAS mutation, or a CEA > 192 ng/ml was used as the diagnostic standard for mucinous cysts when surgical pathology was not available.

Results—A total of 933 pancreatic cyst fluid samples were collected, including 117 with≥2 FNAs. Examinations were performed over a median of 30 months (range 1–115 months). Forty-three (36%) had a mutant KRAS on the index analysis out of which 26 had a change in their KRAS status to the wild-type. Eighty-one (64%) had a wild-type KRAS on the index analysis out of which 18 had change in their KRAS status to mutant type. There was no significant difference in the index cyst characteristics, presence of symptoms, or main duct involvement based on KRAS status change. Increasing age was associated with a changing KRAS mutation status ($p = 0.023$).

Conclusion—KRAS mutations gain and loss in pancreatic cyst fluid appears to occur frequently during long-term surveillance of MPCs. Age appears to be the only predictor for KRAS change over time.

Keywords

EUS-FNA; IPMN (Intraductal Papillary Mucinous Neoplasm); CEA (Carcinoembryonic Antigen); KRAS

Mohammad Al-Haddad, moalhadd@iu.edu.

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Introduction

Pancreatic cysts are often detected on abdominal imaging performed for non-pancreatic indications. The detection of pancreatic cysts has increased over time due to the increased awareness and prevalent use of radiologic imaging modalities [1, 2]. Pancreatic cysts can be broadly categorized as neoplastic or non-neoplastic based on pathology. The neoplastic group includes mucinous cysts (MPCs) where two main pathologies are dominant: intraductal papillary mucinous neoplasms (IPMNs) or mucinous cystic neoplasms (MCNs) [3]. IPMNs and MCNs have malignant potential. Endoscopic ultrasound with fine needle aspiration (EUS-FNA) to accrue pancreatic cyst fluid for cytology and tumor markers is commonly performed to assist in clinical decision making [4, 5]. Given the very limited yield of cytology, carcinoembryonic antigen levels (CEA) and KRAS mutational status in cyst fluid are often used to differentiate between non-mucinous and mucinous cysts [6, 7]. In the case of mucinous cysts, subsequent surveillance using MRI imaging or EUS is recommended due to their potential of malignant progression [3]. CEA levels have previously been shown to be relatively stable with some variation on sequential EUS-FNA, but the behavior of KRAS mutations, and its potential association with phenotypical changes in MPCs over time is poorly described [8]. The aim of this study was to assess the changes of CEA and KRAS mutations in pancreatic cysts fluid over time and assess their impact on patient outcomes. We also report on the performance of molecular markers in combination with CEA for the diagnosis of MPCs in a large surgically-resected cohort.

Methods

Patients

This is a single center retrospective study of prospectively collected data between November 2007 and February 2018 for patients with clinically suspected pancreatic cysts who underwent EUS-FNA with cyst fluid analysis. The study was approved by the Indiana University Institutional Review Board. Data were retrieved from a large prospectively collected institutional pancreatic cyst registry, and it included patient demographics, recent history of pancreatitis, the presence of gastrointestinal symptoms attributed to the MPC, imaging findings (location of cyst, cyst characteristics, and involvement of the main pancreatic duct), cytology and cyst fluid analysis. Patients underwent DNA-based molecular testing of pancreatic cyst fluid as part of their clinical care.

All patients who had molecular analysis of pancreatic cyst fluid and were at least 18 years of age at the time of their index EUS were eligible for inclusion. Patients with at least 2 years of follow-up were included. Clinical data in addition to all available imaging reports (EUS, magnetic resonance imaging, and/or CT) were reviewed. High-risk morphological features including mural nodules, solid component, and main duct dilation (6 mm or larger) were confirmed by EUS imaging review and other imaging modalities. These parameters were based on the 2012 Fukuoka recommendations [9]. We continued to adopt the 2012 Fukuoka guidelines for the identification of high-risk lesions throughout the study despite the publication of several other guidelines to maintain consistency.

EUS-FNA

Patients were referred to have EUS-FNA for further evaluation of pancreatic cyst lesions found on imaging based on symptoms or incidentally. EUS-FNA was performed under propofol sedation by one of six expert endosonog-raphers. Diagnostic cyst fluid aspiration was performed using standard 19-, 22-, or 25- gauge needles at the discretion of the endosonographer. Color doppler imaging was utilized prior to needle puncture to confirm a lack of significant vascular structures within the needle path. The following EUS findings were documented: pancreatic cyst characteristics (location, size, number, mural nodules or mass, the presence of septations, the presence of connection to pancreatic duct). Post EUS-FNA intravenous antibiotics (ciprofloxacin or amoxicillin-clavulanate) were administered as per protocol followed by a three-day oral course. Repeat EUS-FNA was performed as part of pancreatic cyst surveillance or based on subsequent imaging/clinical findings. Decision for repeat EUS-FNA was discussed between endoscopist and patient. Several factors were considered in repeat sampling including patients with family history of pancreas cancer in first degree relative; patients with interval development of symptoms related to the cyst; cyst increasing in size by 30% compared to prior surveillance, and cysts with interval development of high-risk morphological changes (upstream main duct dilation, mural nodule, thickening of the wall or development of thick septations).

Cytological and Molecular Fluid Analysis

Cyst fluid obtained by EUS-FNA was sent for cytologic examination by our own pathologists, and for CEA and molecular analysis at an outside commercial laboratory (PancraGEN; Interpace Diagnostics, Pittsburgh, PA). A minimum of 0.2 ml was allocated for molecular and biochemical markers as per the outside laboratory requirements and cytological exams were conducted on the residual fluid. The fluid was tested for individual point mutations in KRAS and GNAS, tumor suppressor gene (TSG) loss of heterozygosity (LOH), and quantity of DNA as per the company protocol. In this study, we focused on KRAS oncogene mutation (on codons 12 and 13 in this particular assay) as the main determinant of mucinous pathology given its increasing utilization and high specificity for mucinous pathology, as we have previously demonstrated [4]. The ability to check GNAS mutations became feasible in 2017 and therefore was not available on all samples obtained. The presence of oncogene mutation was determined by standard sequencing methodologies as previously described [10].

The presence of a KRAS mutation or a CEA ≥ 192 ng/ml were adopted as standards of a mucinous cyst [10]. Patient outcomes were categorized as “malignant” at follow-up based on surgical pathology in resected patients or on unequivocal malignant cytopathology in non-resected patients. The threshold of stability in cyst size on EUS or pancreas imaging (CT and MR) was established as < 20% of changes in any single dimension over time as we have previously reported [11]. To assess temporal changes in KRAS and CEA, we studied the status of both biomarkers in patients when at least two separate molecular analyses from the same cyst were available over the course of the study. Robust clinical and radiological follow-up was facilitated by our multidisciplinary Pancreas Cyst Program, which has been in place since 2007. All cysts with highly suspicious or malignant cytology in surgically fit patients were considered for surgery. In addition, cysts with high-risk stigmata based on

the International Consensus Guide-lines including main pancreatic duct (MPD) size dilation (10 mm or higher), jaundice or enhancing solid nodule/mass were referred to surgery. In addition, we adopted worrisome features such as MPD size 6–10 mm, or cysts over 3 cm in size as s basis for referral to a surgeon, but not absolute surgical indication criteria.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve (AUC) of molecular analysis in the diagnosis of MPCs were calculated using the criterion standard of surgical pathology in resected cysts. Baseline EUS-FNA findings and imaging morphological features of the cysts were assessed and compared to subsequent surveillance studies to assess the impact of KRAS changes over time. CEA cut-off of 192 and KRAS mutational status results were compared for inter-test agreement.

The categorical variables were measured as the count and percentage while continuous variables were measured as the mean and standard deviation. Statistical comparisons were performed with a chi-square test or Fischer exact test for categorical variable. The t-test or the Wilcoxon rank-sum test was used for continuous variables. Inter-test agreement was derived using Cohen's kappa (k) statistic. A p value <0.05 was considered statistically significant. Statistical analysis was carried out using IBM SPSS Statistics, version 24.0 (SPSS, Chicago, Illinois, USA).

Results

Patients

Our study included 933 patients who underwent EUS-FNA and pancreatic cyst fluid molecular analysis. Of these patients, 117 (13%) underwent two or more EUS-guided cyst fluid aspirations from the same lesion.

Index Biomarker and DNA analysis

The median age of the 933 patients was 66 years (range 13–91) on initial cyst sampling by EUS. Cyst fluid CEA was available in 646 patients (median 171 ng/ml, range 0–392,000) while KRAS mutation status was available on all 933 patients and was mutated in 242 (26%) of patients.

Surgical Outcomes

Surgical resection was performed in 118 patients (15%) with mucinous pathology confirmed in 86 (73%). The median time from EUS-FNA to surgery was 16 months (range 1–45). Patients with mucinous cysts were found to be older than those with non-mucinous cysts (63 vs. 54 years, p value = 0.002). Mucinous lesions were associated with main duct involvement and mural nodules (Table 1). There was no significant difference in the cyst size, location, and presence of cyst-related symptoms or pancreatitis between patients diagnosed with mucinous vs. non-mucinous pathology (Table 1).

Out of the 86 lesions with confirmed mucinous pathology, 76 were found to have IPMN and 10 patients had MCNs (Table 2). On surgical pathology, seventy patients (81%) had

low/intermediate-grade dysplasia, nine (10%) had high-grade dysplasia, and seven (8%) had IPMN-based invasive adenocarcinoma (Fig. 1). All seven patients with invasive disease had elevated CEA levels (median: 1870 ng/ml, range: 834–93,000), 6 out of 7 (86%) had positive KRAS and 5 out of 7 (71%) had at least one TSG-LOH mutation.

Performance Characteristics of CEA and Molecular (DNA) Analysis

CEA level (> 192 ng/ml) had a sensitivity of 66% (95% CI: 54–77%) and specificity of 83% (67–98%) for the diagnosis of MPCs. KRAS and GNAS had sensitivities of 45% (25–56%) and 36% (8–65%), and specificities of 94% (85—100%) and 100%, respectively. The sensitivity of KRAS increased to 77.1% and 51.2% after combining it with CEA level (> 192) and at least one TSG-LOH, respectively. KRAS had a positive predictive value of 95% and negative predictive value of 38.2% in diagnosing mucinous pancreatic cysts. There was no statistical difference in the AUC after combining KRAS with CEA compared to KRAS alone (Tables 3 and 4).

Serial KRAS and CEA Changes

In the EUS-FNA surveillance cohort, 117 patients underwent two or more fluid aspirations for DNA analysis from the same lesion: 92 had two, 22 had three, and 3 had four. The median age of this group was 67 years (range 21–90 years), and the examinations were performed over a median of 30 months apart (range 1–115 months). Baseline mean cyst size was 19 ± 11 mm and 65/121 (54%) were symptomatic on presentation. Forty-four patients (38%) had change in their KRAS mutation status during surveillance. For comparison purposes, we have classified patients in four groups: 26 (22%) lost KRAS mutation, 18 (15%) gained KRAS mutation compared to the index fluid analysis, 17 patients (14%) maintained mutant KRAS status, and 56 patients (48%) had consistently wild-type KRAS status (Fig. 2). Ten patients out the 117 cohort had documented surgery of which nine (90%) had low grade IPMN and one patient had IPMN-invasive adenocarcinoma. The patient with adenocarcinoma lost their KRAS mutation during subsequent surveillance. For the nine patients diagnosed with IPMN, seven had consistently positive KRAS mutation, one had consistently negative KRAS mutation, and another one had a gain in KRAS mutation.

There was no significant difference in the index cyst size or morphology (including development of high-risk stigmata (HRS) or worrisome features (WF) per the 2012 International consensus guidelines), location, presence of symptoms, or main duct involvement between the four different groups of patients [9]. The only significant difference was age, as younger patients tend to remain more consistently wild-type over time ($p = 0.023$). There was no evidence of malignancy or high-grade dysplasia on FNA cytology from patients who underwent two or more pancreas cyst fluid aspirations.

Among our overall cohort of 933 patients, a total of 555 patients had both CEA and KRAS analysis available from the index examination. Out of those, 268 patients (48%) had a CEA> 192 and 161 patients (29%) had a KRAS mutation. Concordance between CEA and KRAS status for a mucinous pathology was found in 112 patients (20%) where CEA was > 192 in the presence of a KRAS mutation, while concordance for non-mucinous pathology was found in 240 (43%) where CEA was < 192 with no KRAS mutation detected. There was

discordance between CEA and KRAS mutational status in remaining 203 patients, resulting in a fair agreement overall ($k = 0.26$).

We have noticed a similar trend during the second and third cyst fluid sampling where both biomarkers were available (n = 55 and 9, respectively), where the fair agreement persisted ($k = 0.38$ and $k = 0.23$, respectively).

Discussion

Our retrospective analysis of serial EUS-FNA in surveillance of pancreatic cysts demonstrates frequent changes in KRAS mutation status. However, we report no clear association with new clinical or phenotypic changes in lesions that acquire or lose KRAS mutations over time.

The natural history of MPCs continues to be of high interest due to their variable malignant potential and the high morbidity associated with surgical resection. Since most patients with pancreatic cysts do not undergo surgery and yield from FNA cytology is low, classifying cysts as mucinous and committing to long-term surveillance depends heavily on cyst fluid analysis of biomarkers like CEA and DNA mutations. Based on variable level evidence, gastrointestinal and radiological societies have published several practice guidelines addressing the management of asymptomatic pancreatic cysts. Changes in cyst morphology (development of solid component, dilation of pancreatic duct and/ or cyst size > 3 cm) are generally endorsed as indications for EUS-FNA. [3, 9, 12]. Thus, EUS-FNA is an important checkpoint and a significant contributor in shaping the clinical decision to resect or observe a MPC.

To date, the evidence linking phenotypical and molecular changes in MPCs continues to evolve. It is widely accepted that KRAS mutations occur early in the course of and are necessary for the malignant progression of MPCs [13, 14]. Based on data by Omori et al., malignant progression of IPMN models rely on the persistence of KRAS mutations followed by the sequential accumulation of TSG-LOH mutations that are necessary for carcinogenesis [15, 16].

Incorporating pancreatic cyst fluid biomarkers in the long-term management of pancreatic cysts, however, remains controversial. The lack of adoption of biomarkers in the various cyst management guidelines in the last decade is an attestation to this controversy. CEA levels > 192 ng/ml have been shown in several studies to be > 60% sensitive and > 80% specific in differentiating mucinous from non-mucinous pancreatic cyst [4, 6, 17-20]. Our study shows that when gold standard histopathologic diagnosis is available, a CEA level > 192 correctly identifies 66% of mucinous pancreatic cysts and is 83% specific in doing so. The presence of KRAS mutation OR elevated CEA increases the sensitivity to 77% but at a cost of decreased specificity to 92%. Prior studies have reported similar results where the sensitivity of CEA increases by combining KRAS and GNAS results [6, 7, 18, 20]. Despite the fair agreement we report between CEA and DNA markers, we find a real and practical value in KRAS and GNAS analysis when cyst fluid is too thick to be aspirated and/or is insufficient to run a CEA assay, which typically requires 0.5 ml or more in mainstream

hospital-based laboratories. In patients with pancreatic cysts who undergo serial surveillance with EUS-FNA, there is very limited literature on the trends in molecular analysis. As reported by Nakai et al., serial EUS-FNA has previously shown stable CEA levels in the majority of patients during repeat measurements [8]. In that study, fluctuations of CEA without significant changes in EUS imaging were observed in the minority of patients (approximately 20%).

Reduction in cell-free DNA and KRAS levels in plasma have been reported in colorectal cancer patients undergoing chemotherapy [21]. However, very few studies have reported changes in KRAS mutation on repeated analysis during surveillance in patients with pancreatic cysts [18, 22, 23]. Winner et al. in a small study of 40 patients, reported that CEA levels did not change significantly between serial measurements; however. KRAS mutation changes were frequent in 16 patients [23]. Nikiforova et al. repeated KRAS analysis in follow-up visits for 41 patients and reported 27% of cysts (9/33), who had a wild-type KRAS in index aspiration, had acquired a mutant-type KRAS [18]. Similarly, 12% of (1/8) cysts with mutant KRAS on index EUS-FNA had changed to wild-type KRAS on follow-up analysis. Dura-tion between and frequency of follow-up serial examinations were not reported in this study. Our study aimed to explore the occurrence of changes in KRAS through serial EUS-FNA examinations in a larger sample size and over a longer period. In 121 patients, 37% had a change in status in KRAS mutation status from index examination. The change in status was independent of CEA level >/ = 192 on a univariate regression analysis. Loss or gain of mutation, independent of other factors, could indicate a dynamically changing molecular environment within pancreatic cysts.

One potential explanation to changing KRAS mutational status could be the platform used to detect this mutation. By using the same external laboratory, which relied on the same methodology (Sanger sequencing) for the entire duration of the study, we have eliminated the possibility of change due to enhanced sensitivity, nowadays brought along by next generation sequencing NGS. It is plausible that fluctuating concentrations of the cell-free DNA could have led to this outcome. Whether or not the amount of KRAS in cyst fluid follows any stress or reparative processes in the cell is not clear and could be investigated in future. [15, 24] KRAS NGS has been proven to more sensitive and more specific compared to Sanger sequencing, which is important in the interpretation of our results. The increasing accessibility to NGS-based assays calls for the revalidation of our study findings using this technique. Another potential explanation could be the heterogeneity of mutations throughout IPMNs and the variable presence KRAS mutations within different locations of an IPMN lesion [25].

Our study is the first large cohort to explore the consistency of molecular analysis in surveillance of patients with pancreatic cysts. KRAS mutation has been reported as a key area of research in molecular analysis of pancreatic cyst fluid and we report the performance of KRAS and changes in mutational status in a large group of patients over time. We also report the performance of molecular analysis in differentiating mucinous vs. non-mucinous cysts in pathology confirmed classification on a large cohort with surgical pathology. The availability of pathologic diagnosis strengthens our results significantly. Thus, our study is hypothesis-generating for large-scale prospective studies needed to define and predict such

changes in mutations during surveillance. These findings can have a wide impact on the management of pancreatic cysts, particularly as it relates to being able to classify a cyst as mucinous or non-mucinous based on molecular profile, and the effect this could have on continuing or discontinuing imaging surveillance.

Our cohort study has a few limitations. Selection bias could not be avoided because of the cohort design. By including all consecutive patients who underwent pancreas cyst fluid sampling over the course of a decade, we captured pancreatic cysts with various risk profiles. NGS has been shown to increase sensitivity of identifying low concentrations of mutant KRAS compared to Sanger sequencing or allele specific locked nucleic acid PCR techniques and has been shown to influence clinical management in pancreatic cysts (15, 23, 24). Adhering to Sanger technique during the course of our study for molecular analysis could affect the accuracy of the results. We also noted that a significant portion of patients with non-mucinous cysts reported symptoms which might have been non-specific in this group of patients.

Conclusion

KRAS gain and loss in cyst fluid appears to occur frequently during long-term surveillance of suspected mucinous cysts, leading to lesions oscillating above and below the diagnostic threshold for a mucinous cyst over time. Whether this reflects sampling limitations or is a true reflection of a dynamic cyst fluid milieu is not clear. Age appears to be the only predictor for KRAS change over time, with no other clear baseline predictors for mutational gain.

Abbreviations

EUS-FNA	Endoscopic ultrasound-fine needle aspiration
IPMN	Intraductal papillary mucinous neoplasm
MCN	Mucinous cystic neoplasm
MPC	Mucinous pancreatic cyst
CEA	Carcinoembryonic antigen
KRAS	Kirsten RAt sarcoma gene
TSG-LOH	Tumor suppressor gene-loss of heterozygosity
HRS	High-risk stigmata
WF	Worrisome features
NGS	Next generation sequencing

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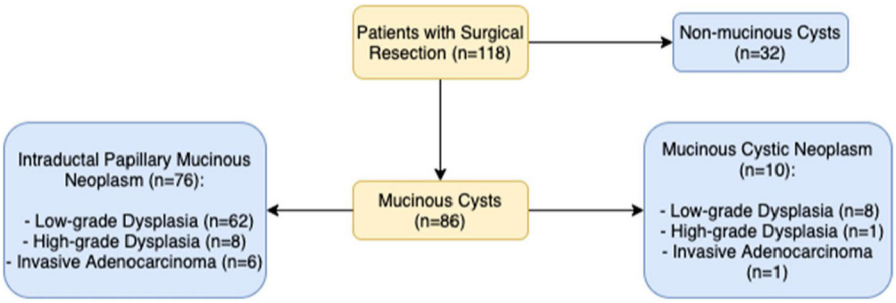

Fig. 1.
Study flowchart for our surgical cohort ($n = 118$)

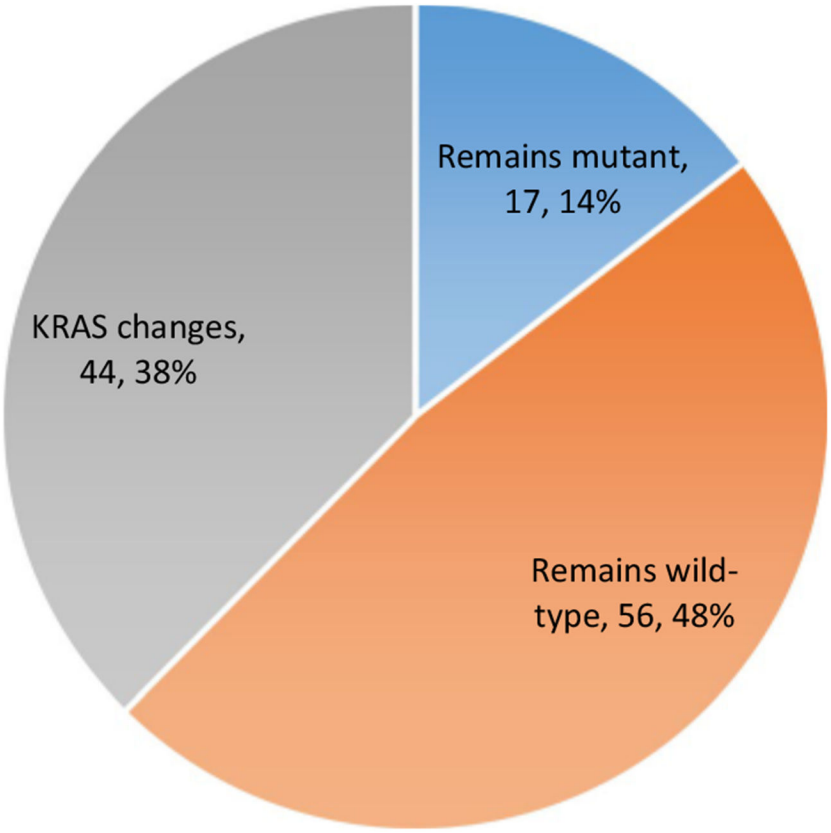

Fig. 2.
Pie chart representing KRAS variations in pancreatic cyst longitudinal surveillance

Table 1

Baseline characteristics of surgical cohort patients (n = 118)

	Mucinous cysts (n = 86)	**Non-mucinous cysts (n = 32)**	***P* value**
Age, mean (SD)	63 (14)	54 (14)	0.002
Location, n (%)			0.318
Head	18 (21)	8 (25)	
Tail	29 (34)	11 (34)	
Body	19 (22)	11 (34)	
Neck	5 (6)	0	
Uncinate Process	6 (7)	0	
Pancreatitis, n (%)	23 (28)	9 (28)	0.977
Symptoms, n (%)	51 (62)	20 (65)	0.82
Cyst characteristics			
Long axis (mm), mean (SD)	24 (17)	32 (19)	0.24
Short axis (mm), mean (SD)	20 (15)	24 (12)	0.253
Presence of thick septation, n (%)	27 (32)	16 (50)	0.075
Presence of nodule, n (%)	9 (11)	8 (25)	0.049
Duct Involved			0.044
Main duct involved, n (%)	15 (17)	10 (31)	
Branch duct involved, n (%)	30 (35)	5 (16)	
Mixed main and branch ducts, n (%)	11 (13)	1 (3)	

Table 2

Pathological Findings for surgical patients (n = 118)

Pathology	Number (%)
Mucinous Cysts	86 (73)
IPMN	69 (58)
MCN	10 (8)
Adenocarcinoma (IPMN-derived)	7 (6)
Non-mucinous Cysts	32 (23)
Pancreatic Intraepithelial Neoplasia	7 (6)
Neuroendocrine Tumor	7 (6)
Serous Cystadenoma	6 (5)
Chronic Pancreatitis	7 (6)
Pseudocyst	3 (3)
Other benign pathologies	2 (2)

Table 3

Performance of molecular (DNA) markers for our surgical cohort of 118 patients for the diagnosis of a mucinous lesion

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
KRAS	45.3	93.5	95	38.2	0.694
CEA (≥ 192)	65.6	82.6	91.3	46.3	0.741
GNAS	36.4	100	100	50	0.682
LOH	18.8	96.7	94.1	29.6	0.577
KRAS OR CEA	77.1	91.5	78.3	52.9	0.777
KRAS OR TSG-LOH	51.2	90.3	93.6	40.0	0.707

PPV—Positive Predictive Value; *NPV*—Negative Predictive Value; *AUC*—Area Under Curve; *LOH*—Loss of Heterozygosity

Table 4

Baseline clinical characteristics between different patient groups based on the behavior of KRAS in pancreatic cysts over time

	Consistent wild-type (*n* = 56)	Consistent mutant (*n* = 17)	Change from wild type to mutant (*n* = 18)	Change from mutant to wild type (*n* = 26)	p value
Age, mean ± SD	62 ± 14	67 ± 10	70 ± 7	69 ± 9	0.023
Location, n (%)					0.84
Head and neck	27 (54)	6 (40)	8 (50)	12 (50)	
Body and tail	23 (46)	9 (60)	8 (50)	12 (50)	
Pancreatitis, n (%)	10 (18)	1 (6)	4 (24)	4 (15)	0.550
Symptoms, n(%)	31 (55)	9 (53)	7 (41)	18 (70)	0.329
Cyst characteristics					
Long axis (mm), mean ± SD	22 ± 16	15 ± 7	18 ± 8	18 ± 10	0.163
Short axis (mm), mean ± SD	18 ± 15	10 ± 6	13 ± 8	13 ± 6	0.161
Presence of thick septations, n (%)	19 (34)	5 (29)	7 (41)	11 (42)	0.785
Presence of nodule, n (%)	4 (7)	0 (0)	2 (12)	0 (0)	0.231
Duct Involved					0.574
Main duct involved, n (%)	7 (13)	2 (12)	3 (18)	3 (18)	
Branch duct involved, n (%)	16 (29)	9 (53)	6 (35)	6 (35)	
Mixed main and branch ducts, n (%)	7 (13)	3 (18)	2 (12)	2 (12)	
DNA characteristics					
CEA ≥ 192, n (%)	20 (47)	8 (73)	9 (64)	11 (45)	0.333