Next Generation Sequencing Improves the Accuracy of 
KRAS Mutation Analysis in Endoscopic Ultrasound Fine 
Needle Aspiration Pancreatic Lesions

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Abstract
The use of endoscopic ultrasonography has allowed for improved detection and pathologic analysis of fine needle aspirate material for pancreatic lesion diagnosis. The molecular analysis of KRAS has further improved the clinical sensitivity of preoperative analysis. For this reason, the use of highly analytical sensitive and specific molecular tests in the analysis of material from fine needle aspirate specimens has become of great importance. In the present study, 60 specimens from endoscopic ultrasonography fine needle aspirate were analyzed for KRAS exon 2 and exon 3 mutations, using three different techniques: Sanger sequencing, allele specific locked nucleic acid PCR and Next Generation sequencing (454 GS-Junior, Roche). Moreover, KRAS was also tested in wild-type samples, starting from DNA obtained from cytological smears after pathological evaluation. Sanger sequencing showed a clinical sensitivity for the detection of the KRAS mutation of 42.1%, allele specific locked nucleic acid of 52.8% and Next Generation of 73.7%. In two wild-type cases the re-sequencing starting from selected material allowed to detect a KRAS mutation, increasing the clinical sensitivity of next generation sequencing to 78.95%. The present study demonstrated that the performance of molecular analysis could be improved by using highly analytical sensitive techniques. The Next Generation Sequencing allowed to increase the clinical sensitivity of the test without decreasing the specificity of the analysis. Moreover we observed that it could be useful to repeat the analysis starting from selectable material, such as cytological smears to avoid false negative results.

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Introduction
Pancreatic ductal adenocarcinoma (PDAC) represents the fourth-highest cause of cancer death in the United States with the lowest survival rate among the most common cancers (~6%) [1]. Several imaging techniques have been developed to improve early diagnosis of pancreatic masses, such as multi-detector-row computed tomography (MDCT), transcutaneous ultrasonography (TUS), magnetic resonance imaging (MRI), endoscopic ultrasonography (EUS), endoscopic retrograde cholangiopancreatography (ERCP) and positron emission tomography (PET) scanning [2–4]. Among these techniques, endoscopic ultrasonography guarantees the highest-resolution imaging of the pancreas, allowing for the detection of small masses [3], of lymph node involvement [2] and of vascular tumor infiltration [3]. The introduction of the EUS-guided fine needle aspiration (EUS-FNA) in the clinical practice has supported clinicians in the preoperative diagnosis of pancreatic tumors helping to correctly and promptly selecting patients eligible for a curative surgical intervention or for other treatment [4,6,7].

Although EUS-FNA shows high diagnostic clinical sensitivity and specificity, a subset of cases are characterized by limited cellularity or inadequate material for cytologic evaluation [8]. Other than these “unsatisfactory” specimens, inconclusive cytologic cases include those samples described as “suspicious of malignancy” or with “presence of atypical cells” which also represent a significant problem for clinicians and pathologists. The combination of cytologic evaluation and molecular analysis, especially in inconclusive cases, has enhanced the diagnostic power of the EUS-FNA technique [9–12].

Mutant KRAS has been reported in >90% of cases of pancreatic ductal adenocarcinoma [13] and in 30 to 45% of cases of intraductal papillary mucinous neoplasm (IPMN), a pre-malignant distinct pathological entity which is thought to be a precursor of PDAC [14–17]. KRAS mutations were not detected in acinar
carcinomas of the pancreas, in pancreatic neuroendocrine tumors (pNET) or in solid pseudopapillary tumors (SPPT) [18–20].

*KRAS* mutations represent an early genetic event in PDAC pathogenesis and, as regards solid lesions, it is considered a tumor marker for pancreatic adenocarcinoma [21–23]. The detection of *KRAS* mutations in a pancreactic lesion sample is useful to confirm the preoperative diagnosis or to suggest the presence of malignancy in those cases where EUS-FNA cytology is inconclusive [11,22,24,25]. Moreover it has been observed that *KRAS* point mutations could also occur in chronic pancreatitis and are associated with evolution towards pancreatic cancer [26,27]. Several techniques could be used for *KRAS* mutation analysis, including Single-Strand Conformation Polymorphism (SSCP) [9], Restriction Fragment Length Polymorphism (RFLP) assays [28,29], Enriched-PCR and enzyme Linked Mini-sequence Assay (ELMA-PCR) [30], clamping Peptide Nucleic Acids PCR (PNA-PCR) [31], Allele Specific Locked Nucleic Acid PCR (ASLNAqPCR) [32] and Sanger sequencing [15,28]. Considering that cytological material obtained from EUS-FNA is often composed of heterogeneous cell populations, it is crucial to make use of accurate and high analytical sensitive molecular tests to detect even a small proportion of mutated cells in a background of wild-type ones [33].

In this work we analyzed the *KRAS* gene mutational status in 60 consecutive cases of pancreatic lesions starting from material directly collected with EUS-FNA and using three different molecular techniques. We compared Sanger sequencing (considered the gold standard technique for DNA sequence analysis) with 454 NGS and ASLNAqPCR.

### Table 1. Percentage of mutated *KRAS* samples according to preoperative cytology evaluation.

| Cytology Diagnosis (number of cases) | *KRAS* mutated samples |
|-------------------------------------|------------------------|
|                                     | 454 NGS (%) | End-point of mutated samples | ASLNA (%) | End-point of mutated samples | Sanger sequencing (%) | End-point of mutated samples |
| C1 (20)                             | 8 (40.0)     | 6 IPMN (4 BD, 2 MD), 2 NA   | 4 (20.0)   | 2 IPMN (BD), 2 NA           | 4 (20.0)               | 3 IPMN (2 BD, 1 MD), 1 NA   |
| C1c (17)                            | 8 (47.1)     | 4 (23.5)                    |            |                           | 4 (23.5)               |                           |
| C1s (4)                             | 0 (0)        | 0 (0)                       |            |                           | 0 (0)                  |                           |
| C2 (4)                              | 0 (0)        | 0 (0)                       |            |                           | 0 (0)                  |                           |
| C3 (2)                              | 1 (50.0)     | Mal. Inop. Neoplasia        | 1 (50.0)   | 1 Mal. Inop. Neoplasia    | 1 (50.0)               | 1 Mal. Inop. Neoplasia    |
| C4 (9)                              | 7 (77.8)     | 5 PDAC, 1 IPMN (BD), 1 Mal. Inop. Neoplasia | 6 (66.7)   | 4 PDAC, 1 Mal. Inop. Neoplasia, 1 IPMN (BD) | 5 (55.6)               | 3 PDAC, 1 Mal. Inop. Neoplasia, 1 IPMN (BD) |
| C5 (20)                             | 11 (55.0)    | 9 PDAC, 2 Mal. Inop. Neoplasia | 10 (50.0)   | 8 PDAC, 2 Mal. Inop. Neoplasia | 6 (30.0)               | 5 PDAC, 1 Mal. Inop. Neoplasia |
| C5 PDAC (13)                        | 11 (84.6)    | 10 (76.9)                   |            |                           | 6 (46.2)               |                           |
| C5 Not PDAC. (7)                    | 0 (0)        | 0 (0)                       |            |                           | 0 (0)                  |                           |
| NA (5)                              | 4 (80.0)     | 3 IPMN (2 BD, 1 MD), 1 NA   | 3 (60.0)   | 2 IPMN (1 BD, 1 MD), 1 NA | 1 (20.0)               | 1 IPMN (BD)               |
| TOTAL (60)                          | 31 (51.7)    | 24 (40.0)                   |            |                           | 17 (28.3)              |                           |

ASLNAqPCR, Allele Specific Locked Nucleic Acid qPCR; NGS, Next Generation Sequencing; PDAC, Pancreatic Ductal AdenoCarcinoma; Not PDAC, malignant neoplasia but not Pancreatic Ductal Adenocarcinoma; NA, cytology not available.

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### Table 2. Percentage of mutated *KRAS* samples according to different techniques per final end-point.

| Final End-Point | Number of *KRAS* mutated samples using: | 454 NGS (%) | ASLNAqPCR (%) | Sanger (%) |
|----------------|-----------------------------------------|-------------|---------------|------------|
| Adenocarcinomatous and pre-neoplastic lesions (n = 38) | 28 (73.7) | 21 (55.3) | 16 (42.1) |
| PDAC (n = 20) | 14 (70) | 12 (60) | 8 (40) |
| IPMN (n = 12) | 10 (83.3) | 5 (41.7) | 5 (41.7) |
| Inop. Neoplasia (n = 6) | 4 (66.7) | 4 (66.7) | 3 (50) |
| Not-adenocarcinomatous lesions (n = 7) | 0 (0) | 0 (0) | 0 (0) |
| pNET (n = 5) | 0 (0) | 0 (0) | 0 (0) |
| SPPT (n = 2) | 0 (0) | 0 (0) | 0 (0) |
| Benign Lesions (n = 12) | 0 (0) | 0 (0) | 0 (0) |
| NA (n = 3) | 3 (100) | 3 (100) | 1 (33.3) |

PDAC, Pancreatic Ductal AdenoCarcinoma; IPMN, Intraducatal Pancreatc Mucinous Neoplasia; Inop. Neoplasia, Malignant inoperable neoplasia; pNET, pancreatic NeuroEndocrine Tumor; SPPT, Solid PseudoPapillary Tumor; NA, end-point not available.

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with two highly analytical sensitive and semi-quantitative techniques: ASLNAqPCR [32] and 454 Next Generation Sequencing (454 GS-Junior platform, Roche). The aim of the present study was to evaluate if a highly analytical sensitive technique could provide more accurate results (meaning fewer false negative and fewer false positive results) in the routine analysis of \textit{KRAS} in pancreatic lesions. Moreover, considering that usually in pancreatic specimens only mutations in \textit{KRAS} exon 2 are investigated [15,20,28,34], we tested if it could be useful to analyze also \textit{KRAS} exon 3 mutations. Finally, taking into consideration that evaluation of cellular composition is not possible from EUS-FNA material directly collected into a tube (“direct” EUS-FNA), we re-tested \textit{KRAS} starting from cytologic smears and compared the two results, one obtained by targeting selected cells with cytologic atypia and the other directly obtained from EUS-FNA specimens.

**Materials and Methods**

**Selection of Cases**

Sixty samples of EUS-FNA obtained from pancreatic lesions were analyzed. According to ecoendoscopy they were classified as solid (31 cases) or cystic (29 lesions) lesions of the pancreas. Patients were 23 male and 37 female, ages ranging from 17 to 84 (mean 66 yrs).

EUS-FNA was performed using a linear echoendoscope (Fujinon, Inc., Saitama, Japan), the aspirated material was smeared on microscope slides for on-site examination or immediately fixed in 95% ethanol for Papanicolaou staining; the remaining material was partly placed in a tube containing 4% formaldehyde solution for cell block preparation and partly in a tube containing 100% ethanol for \textit{KRAS} analysis (“direct” EUS-FNA material). Cases were diagnosed on preoperative evaluation according to standard criteria as unsatisfactory (C1), negative for malignancy (C2), atypical cells present (C3), suspicious for malignancy (C4) or positive for malignancy (C5) [35]. Regarding the unsatisfactory samples, we decided to distinguish them as C1c if the EUS-FNA was performed on a cystic lesion or C1s if EUS-
FNA was performed on solid lesion. We considered C1s, C3 and C4 as inconclusive diagnoses.

Since \textit{KRAS} mutational analysis is part of the routine diagnostic workup of patients with pancreatic lesions the need for ethic committee’s approval was not necessary for this study, in accordance with medical ethical guidelines of the Azienda Unità Sanitaria Locale di Bologna. Accordingly to these guidelines, a comprehensive written informed consent was signed for the procedure (endoscopic ultrasound fine needle aspiration) that produced the tissue samples. All information regarding the human material was managed using anonymous numerical codes. All samples were handled in compliance with the Helsinki declaration (http://www.wma.net/en/30publications/10policies/b3/).

**DNA Extraction and \textit{KRAS} Analysis**

DNA from direct EUS-FNA material or cytological smears was extracted using MasterPure DNA Purification Kit (Epincentre, Madison WI, USA) according to manufacturer’s instruction. DNA from cytoblocks was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostic, Manheim, Germany). Cytological smears were considered evaluable for analysis if at least a hundred of neoplastic cells were present in the slide. The smears were scanned as virtual slides for archiving (ScanScope CS2 Digital Slide Scanner, Aperio, CA, USA) prior to dissecting.

\textit{KRAS} mutational analysis was performed using three different techniques: Sanger sequencing, Allele Specific Locked Nucleic Acid PCR (ASLNAqPCR) and 454 Next Generation Sequencing (454-NGS).

**Sanger sequencing.** DNA was amplified using previously described primers [32], purified and sequenced for \textit{KRAS} exon 2 and exon 3, according manufacturer’s protocol. Sequencing was carried out according to standard procedures using a CEQ2000 XL automatic DNA sequencer (Beckman Coulter, Inc., Fullerton,
Mutated alleles was calculated according to the following formula:

\[ R = 2^{- \frac{(Ct_{Mut} - Ct_{WT})}{\text{Ct}_{Q61H}}} \]

where R is the “Ratio”, Ct refers to the threshold cycle and Mut and WT refer to mutated and wild-type alleles, respectively. The analytical sensitivity of ASLNaqPCR is below 1%, as previously described [32]. Comparisons between clinical sensitivities were performed according to recommendations previously described [37]. Results with a p-value of <0.05 were considered to be statistically significant.

### Results

Cytologic results and features, final end-point and KRAS molecular analysis are summarized in Tables 1 and 2.

### Cytologic Evaluation (Table 1)

Material for cytologic evaluation was available for 55 cases. According to preoperative cytology diagnosis, specimens were classified as follows: unsatisfactory (C1, 20 cases), negative for malignancy (C2, 4 cases), atypical cells present (C3, 2 cases), suspicious for malignancy (C4, 9 cases), positive for malignancy (C5, 20 cases). Among the latter, 13 were PDAC, 5 were pNET. TN were cases in which a mutation was found but with a “benign” endpoint or else diagnosed as SPPT or pNET. FN resulted wild-type and with a “benign” endpoint or else diagnosed as PDAC/MD-IPMN.

### Statistical Measures of Performance

We considered a result as true positive (TP), false positive (FP), true negative (TN) or false negative (FN) as follows. TP were cases when a mutation in KRAS and which were PDAC, IPMN, both Branch Duct - BD-IPMN - and Main Duct - MD-IPMN were considered as adenocarcinomatous/preneoplastic lesions; neuroendocrine (pNET) and solid pseudopapillary tumors (SPPT) were considered as not adenocarcinomatous lesions.

### Follow Up and Final Diagnosis

To determine the performance of the three different techniques, sequencing data were compared with the histological diagnosis for patients that underwent surgery. For patients that were not operated on, sequencing data were compared with a final endpoint based on a combination of clinicopathologic features and follow-up information.

According to final end-point, we distinguished three different categories of lesions: i) benign lesions; ii) adenocarcinomatous lesions (including precursor lesions of adenocarcinoma); iii) non-adenocarcinomatous lesions.

We considered non-neoplastic cysts, pseudocysts and pancreatic cysts as benign lesions; the inoperable neoplasias with poor progression, PDAC and intraductal papillary mucinous neoplasms (IPMN, both Branch Duct - BD-IPMN - and Main Duct - MD-IPMN) were considered as adenocarcinomatous/preneoplastic lesions; neuroendocrine (pNET) and solid pseudopapillary tumors (SPPT) were considered as not adenocarcinomatous lesions.

### Multiple KRAS mutations according to different techniques

| Technique (#sample) | Mutations | Final End-Point |
|--------------------|-----------|----------------|
| Sanger sequencing   |           |                |
| #4                 | G12D/G12V | PDAC           |
| #53                | G12D/G12V | PDAC           |
| ASLNaqPCR           |           |                |
| #4                 | G12D/G12V | PDAC           |
| #5                 | G12D/G12V | PDAC           |
| #22                | G12D/G12V | IPMN           |
| #26                | G12C/G12V | NA             |
| #53                | G12C/G12D/G12R/G12V | PDAC |
| 454 NGS             |           |                |
| #4                 | G12D/G12V | PDAC           |
| #5                 | G12D/G12V | PDAC           |
| #6                 | G12V/Q61H | PDAC           |
| #22                | G12D/G12V | IPMN           |
| #26                | G12C/G12V | NA             |
| #53                | G12C/G12D/G12R/G12V | Q61H |
| #54                | G12D/Q61H | PDAC           |

CA, U.S.A. Strands were analyzed using forward and reverse primers.

### Allele specific locked nucleic acid PCR

Mutations in exon 2 were analyzed using ASLNaqPCR optimized for the 7 most common KRAS mutations (G12A, G12C, G12D, G12R, G12S, G12V and G12D) as previously described [32]. The percentage of mutated cell lines (OCUT-1) (data not shown).
which were respectively a BD-IPMN with low-grade dysplasia and a PDAC upon follow-up. In two cases, double mutations in KRAS exon 2 were observed (see “Multiple KRAS mutations” paragraph).

As summarized in Table 1, KRAS mutations were found in the 20.0% of inadequate samples (C1), in one of the two cases (50.0%) with atypical cells (C3), in the 55.6% of the cases suspect for malignant neoplasia (C4) and in the 30.0% of samples diagnosed as C5. All C2 cases showed no mutations in the KRAS gene and they were benign cysts (3 cases) or pancreatitis (one case) on follow-up. One of five cases with no available material for cytological evaluation was mutated for KRAS. Upon follow-up two were IPMN, one BD-IPMN and one MD-IPMN with moderate dysplasia. No further information was available in the third case.

Considering the final endpoint, using Sanger sequencing we detected a KRAS mutation in 42.1% of adenocarcinomatous and pre-neoplastic lesions (in 40% of PDAC, 41.7% of IPMNs and in the 50% of inoperable neoplasms), while no KRAS mutations were observed in not adenocarcinomatous or in benign lesions (Table 2).

454 Next-generation sequencing. Using 454-NGS, 31 of 60 samples (51.7%) showed a mutation in KRAS exon 2 (24 of 60, 40%) and/or in KRAS exon 3 (10 of 60, 16.7%) (Figures 1–2). Raw data are available upon request. All mutations in KRAS exon 2 detected by Sanger sequencing and ALSNAqPCR were also detected using 454-NGS (Figures 1A–C).

In 7 cases (four C1c, one C4, one C5; in one case no material was available for cytologic evaluation) only a KRAS exon 3 (codon 61) substitution was found, while in 3 cases (two C4 and one C5) a KRAS exon 3 substitution was found in association with a mutation in KRAS exon 2 (see “Multiple KRAS mutations” paragraph). All mutations in KRAS exon 3 detected by Sanger sequencing were also detected using 454-NGS.

| Table 4. Discrepant results obtained with the three techniques. |
|---------------------------------------------------------------|
| Cytological (preoperative) Diagnosis (# of consecutive case)   | KRAS mutational status | 454 NGS (% of mutated reads) | ASLNaqPCR (Ratio) | Sanger sequencing | Final End-Point |
|---------------------------------------------------------------|------------------------|-------------------------------|--------------------|-------------------|----------------|
| C1                                                            |                        |                               |                    |                   |                |
| #31                                                          | Q61H (4.3)             | WT                            | WT                 | BD-IPMN           |                |
| #37                                                          | Q61H (31.0)            | WT                            | Q61H               | MD-IPMN           |                |
| #42                                                          | G12V (2.7)             | G12V (0.02)                    | WT                 | NA                |                |
| #44                                                          | Q61L (3.0)             | WT                            | WT                 | BD-IPMN           |                |
| #46                                                          | Q61R (1.1)             | WT                            | WT                 | MD-IPMN           |                |
| C4                                                            |                        |                               |                    |                   |                |
| #52                                                          | Q61L (22.0)            | WT                            | Q61L               | PDAC              |                |
| #57                                                          | G12D (6.5)             | G12D (0.03)                    | WT                 | PDAC              |                |
| #60                                                          | G12R (12.0)            | G12R (0.06)                    | WT                 | PDAC              |                |
| C5                                                            |                        |                               |                    |                   |                |
| #8                                                           | G12D (19.3)            | G12D (0.01)                    | WT                 | PDAC              |                |
| #9                                                           | Q61H (15)              | WT                            | WT                 | PDAC              |                |
| #10                                                          | G12V (1.5)             | G12V (0.08)                    | WT                 | PDAC              |                |
| #11                                                          | G12V (1.0)             | G12V (0.02)                    | WT                 | PDAC              |                |
| #14                                                          | G12D (3.0)             | G12D (0.02)                    | WT                 | Malignant Inop. Neop1 |                |
| NA                                                            |                        |                               |                    |                   |                |
| #21                                                          | G12V (3.7)             | G12V (0.02)                    | WT                 | MD-IPMN           |                |
| #25                                                          | Q61H (1.4)             | WT                            | WT                 | BD-IPMN           |                |
| #26                                                          | G12C&G12V (2.6&2.0)    | G12C&G12V (0.02&0.01)          | WT                 | NA                |                |

1 Malignant Inop. Neop, Malignant Inoperable Neoplasia; patient did not undergo surgery, FU determined according to clinical data. FU, Follow-Up; NGS, Next Generation Sequencing; ASLNaqPCR, Allele Specific Locked Nucleic Acid qPCR; WT, Wild-Type; PDAC, Pancreatic Ductal AdenoCarcinoma; IPMN, Intraductal papillary mucinous neoplasm; BD, Branch Duct; MD, Main Duct; NA, follow-up not available.

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KRAS mutations were found in 40.0% of inadequate samples (C1), in one of the two cases (50.0%) with atypical cells (C3), in the 77.8% of cases suspect for malignant neoplasia (C4) and in eleven cases diagnosed as malignant neoplasia (C5) (Table 1). All the C2 cases showed no mutations in the KRAS gene. Four cases with no available material for cytologic evaluation were mutated for KRAS and upon follow-up three were IPMN (two BD-IPMN and one MD-IPMN with moderate dysplasia). No further information was available in the third case (Table 1).

Considering the final endpoint, using 454-NGS we detected a KRAS mutation in the 75.7% of adenocarcinomatous and pre-neoplastic lesions (in the 70% of PDAC cases, in the 83.3% of IPMNs and in the 66.7% of inoperable neoplasias). No KRAS mutations were observed in not adenocarcinomatous or in benign lesions (Table 2).

Multiple KRAS Mutations

Using Sanger sequencing double mutations of the KRAS gene exon 2 were observed in two cases (one C4 and one C5) that were PDAC upon follow-up (Table 3). ASLNaqPCR allowed to detect multiple mutations in 5 of 24 mutated cases (one C4 and two C5; in two cases cytologic evaluation was not available). These five cases were one IPMN and five PDAC upon follow-up (Table 3). Finally, next generation sequencing analysis allowed to observe multiple mutation of KRAS gene in 7 of 31 mutated cases (two C4 and three C5; in two cases no cytology material was available). In 3 of these 31 mutated cases, mutations in KRAS exon 2 and in KRAS exon 3 were observed. Upon follow-up, these seven cases were one IPMN and six PDAC (Table 3).

Discrepant KRAS Results between Sanger Sequencing, ASLNaqPCR and 454-NGS

Results of discrepant cases are summarized in Table 4. In 16 cases discordant results in KRAS mutational status were obtained using at least one of the three different techniques (Table 4). Upon cytologic evaluation, 5 of 16 cases were diagnosed as malignant (C5) and 3 as suspect for malignancy (C4). In 5 cases the samples were cyst content material considerate inadequate for

![Figure 3. Example of molecular results in a KRAS exon 2 mutated sample (case #57, Table 4) with discordant results between the three techniques. A) Electropherogram obtained using Sanger sequencing. The mutation is not identified. B) Using ASLNaqPCR the KRAS G12D mutation is identified by the right curve (G12D). The left curve indicates the wild-type allele (WT). The ratio between the two curves corresponds to ~6% of mutated alleles. C) Profile obtained using 454-NGS, the KRAS G12D mutation is identified by the vertical green bar. The percentage of mutated alleles is indicated on the left y axis while the total number of reads on the right one.

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All cases were pancreatic ductal adenocarcinoma (PDAC) after ASLNAqPCR, but not with Sanger sequencing (Figures 3 A–C). No follow up was available for this case. In one sample a mutation in KRAS exon 2 gene was detected using 454-NGS and ASLNAqPCR, but not with Sanger sequencing (#42, Table 4). No follow up was available for this case. In one sample a mutation in KRAS exon 3 was detected both with 454-NGS and Sanger sequencing (#37, Table 4); it was a MD-IPMN (with low-grade dysplasia) according to histological evaluation. In the three remaining cases (#31, #44 and #46, Table 4) a mutation in KRAS exon 3 was detected only using 454-NGS and all three cases were IPMN (two BD-IPMN and one MD-IPMN) after post-operative histologic evaluation (Figures 2 A–C).

C4 cases. In two C4 samples (#57 and #60, Table 4) a mutation in KRAS exon 2 gene was detected using 454-NGS and ASLNAqPCR, but not with Sanger sequencing (Figures 3 A–C). All cases were pancreatic ductal adenocarcinoma (PDAC) after post-operative histologic evaluation. In one case (#52, Table 4) a mutation in KRAS exon 3 was detected both with 454-NGS and Sanger sequencing but not using ASLNAqPCR. It was a PDAC according to histological evaluation.

C5 cases. In four C5 cases (#8, #10, #11 and #14, Table 4) a mutation in KRAS exon 2 gene was detected using 454-NGS and ASLNAqPCR, but not with Sanger sequencing. All cases were malignant carcinoma upon follow-up: three were diagnosed as pancreatic ductal adenocarcinoma (PDAC) after post-operative histologic evaluation, one case did not undergo surgery and was considered a malignant primary pancreatic neoplasm, based on clinical findings. In one case (#9, Table 4) a mutation in KRAS exon 3 was detected only with 454-NGS and was found to be a PDAC after post-operative histologic evaluation.

Cases with cytologic evaluation not available. All three samples with no cytologic evaluation and discrepant KRAS results were from cystic lesions. In two cases (#21 and #26, Table 4) at least one mutation in KRAS exon 2 gene was detected using 454-NGS and ASLNAqPCR, but not using Sanger sequencing. According to follow-up, one case (#21, Table 4) was a MD-IPMN (with moderate dysplasia). In the other no follow-up information was available. In the third case (#25, Table 4) a mutation in KRAS exon 3 was detected only with 454-NGS and the lesion was diagnosed as BD-IPMN after post-operative histologic evaluation.

Considering that it was not possible to determine the proportion or the presence of neoplastic cells in EUS-FNA material directly collected into a tube, we decided to re-analyze the samples not yet processed in our laboratory using a new technique (directed PCR).

### Table 5. Molecular results of KRAS analysis in material obtained from cytological smears.

| Number of consecutive analyzed Cases | Pre-operative Diagnosis | KRAS status on FNA material | KRAS status on cytological smear/cytoblock (% of mutated reads) | Final End-Point |
|-------------------------------------|-------------------------|-----------------------------|---------------------------------------------------------------|----------------|
| #1 #2                               | C5                      | WT                          | G12C (1.3)                                                   | PDAC           |
| #3                                  | C5                      | WT                          | WT                                                           | Malignant Inop. Neop.¹ |
| #7                                  | C5                      | WT                          | WT                                                           | pNET           |
| #15                                 | C5                      | WT                          | WT                                                           | pNET           |
| #16                                 | C5                      | WT                          | WT                                                           | pNET           |
| #17                                 | C5                      | WT                          | WT                                                           | pNET           |
| #50                                 | C5                      | WT                          | WT                                                           | SPPT           |
| #51                                 | C5                      | WT                          | WT                                                           | SPPT           |
| #19                                 | C4                      | WT                          | WT                                                           | PDAC           |
| #55                                 | C4                      | WT                          | WT                                                           | Malignant Inop. Neop.¹ |
| #43²                               | C3                      | WT                          | G12V (1.7)                                                   | PDAC           |

FNA, Fine Needle Aspiration; FU, Follow-Up; PDAC, Pancreatic Ductal Adenocarcinoma; pNET, pancreatic NeuroEndocrine Tumor; SPPT, Solid PseudoPapillary Tumor. ¹Malignant Inop. Neop, Malignant Inoperable Neoplasm, no histological evaluation was possible.

*¹* A digital slide of this sample is available at the following address [http://vetrinodigitale.ausl.bo.it/spectrum_Login.php](http://vetrinodigitale.ausl.bo.it/spectrum_Login.php).

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### Table 6. Statistical performance of KRAS molecular analysis using the three different techniques.

|                        | 454 NGS       | ASLNAqPCR     | Sanger        | 454 NGS       | ASLNAqPCR     | Sanger        |
|------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| **Performance**        |               |               |               |               |               |               |
| SPEC (%)               | 100.00        | 100.00        | 100.00        | 100.00        | 100.00        | 100.00        |
| SENSIT (%)             | 52.78         | 52.78         | 44.19         | 73.68         | 52.78         | 42.11         |
| PPV (%)                | 100.00        | 100.00        | 100.00        | 100.00        | 100.00        | 100.00        |
| NPV (%)                | 55.26         | 55.26         | 36.84         | 65.52         | 55.26         | 46.34         |
| ACC (%)                | 70.18         | 70.18         | 57.89         | 82.46         | 70.18         | 70.18         |
| FDR (%)                | 0.00          | 0.00          | 0.00          | 0.00          | 0.00          | 0.00          |

Ex, Exon; NGS, Next Generation Sequencing; ASLNAqPCR, Allele Specific Locked Nucleic Acid qPCR; Sanger, Sanger sequencing; SPEC, Specificity; SENS, Clinical Sensitivity; PPV, Positive Predictive Value; NPV, Negative Predictive Value; ACC, Accuracy; FDR, False Discovery Rate. In bold the higher value per each parameter.

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KRAS Mutational Status in Cytologic Smears

Considering that it was not possible to determine the proportion or the presence of neoplastic cells in EUS-FNA material directly collected into a tube, we decided to re-analyze the samples not yet processed in our laboratory using a new technique (directed PCR).
mutated for KRAS starting from cytological smears or cyto-blocks. The slides suitable for the manual dissection of neoplastic cells were selected by a pathologist and the representative area was marked. Residual material for molecular analysis was available for 24 of 29 patients that were wild-type for KRAS according all three techniques. On cytologic evaluation, these 24 samples were diagnosed as: C1 in ten cases, C2 in 3 cases, C3 in one case, C4 in two cases and C5 in 8 cases (Table 5). In the 13 cases evaluated as unsatisfactory (C1c or C1s) or negative for malignancy (C2) the re-analysis was not performed. Due to the higher clinical sensitivity of NGS (see “Statistical measures of performance” paragraph) the analysis was repeated only using 454-NGS.

Two further mutations in the KRAS gene were observed in material obtained from cytologic smears. A KRAS G12V mutation was observed in a sample with “atypical cells” (C3) upon cytologic evaluation (#43 Table 5). This case was a PDAC according to post-operative histologic evaluation. A KRAS G12C mutation was detected in a sample with a “malignant” cytologic diagnosis (C5) (#1 Table 5) that was an adenocarcinoma after post-operative histologic evaluation. The remaining cases were wild-type for KRAS, even after the analysis was repeated on material dissected from the cytology specimen. According to follow-up these cases were PDAC (one case), pNET (4 cases), SPPT (2 cases). Two were inoperable malignant neoplasms (Table 5).

### Statistical Measures of Performance
As shown in Table 6, when the analysis of KRAS (exon 2 and exon 3) was performed on direct FNA the 454-NGS, ASL-NAqPCR and Sanger sequencing had 100% specificity. If only KRAS exon 2 was analyzed, the clinical sensitivity of 454-NGS evaluation (#43 Table 5).

| 454 NGS performances | KRAS analysis only on direct EUS-FNA sample | KRAS analysis performed also on cells scraped from the cytologic smears or cytoblocks |
|----------------------|-------------------------------------------|----------------------------------------------------------------------------------|
| SPEC (%)             | 100.00                                    | 100.00                                                                           |
| SENS (%)             | 73.68                                     | 78.95                                                                            |
| PPV (%)              | 100.00                                    | 100.00                                                                           |
| NPV (%)              | 65.52                                     | 70.37                                                                            |
| ACC (%)              | 82.46                                     | 85.96                                                                            |
| FDR (%)              | 0.00                                      | 0.00                                                                             |

SPEC, Specificity; SENS, Clinical Sensitivity; PPV, Positive Predictive Value; NPV, Negative Predictive Value; ACC, Accuracy; FDR, False Discovery Rate.

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Figure 4. Proposed algorithm for the detection of KRAS mutations in EUS-FNA material from pancreatic lesions. Ex, Exon; WT, Wild-Type.

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(52.78%) was higher (p<0.001) than Sanger sequencing (44.19%) and comparable with that of ASLNaqPCR (52.78%). Clinical sensitivity (73.68%, p<0.001), negative predicted value (63.52%) and accuracy (82.46%) of 454-NGS were higher if KRAS exon 3 was also analyzed (Table 6).

The repeated analysis using 454-NGS of KRAS wild-type samples starting from DNA obtained from cytologic specimens led to increases in clinical sensitivity (70.95%, p<0.05), negative predictive value (70.37%) and accuracy (83.96%) (Table 7).

Discussion

Detection of KRAS mutations can be used to improve the preoperative diagnosis of pancreatic EUS-FNA samples. For this reason it is of crucial importance to perform the analysis with highly specific and analytical sensitive techniques. Sanger sequencing has been widely used for KRAS analysis, but the low analytical sensitivity (~20% of mutated alleles) of the method can lead to false negative results [32] if compared with highly sensitive methods. Mutation specific assays, as ASLNaqPCR, notably improve the analytical sensitivity of KRAS molecular analysis, allowing to recognize 1% of mutated alleles [32]. However, these assays are designed only for particular “hot-spot” KRAS mutations (e.g. in KRAS exon 2). They can therefore underestimate the number of mutated alleles, an example being those specimens with substitutions in KRAS exon 3 that are not usually targeted by mutation specific assays. Next generation sequencing merges the high analytical sensitivity of mutation specific assays with the broader capabilities of direct sequencing methods like Sanger, that not being mutation specific also allows the detection of unusual or unexpected mutations. Our data demonstrate that 454-NGS has a higher clinical sensitivity (73.68%) than ASLNaqPCR (52.78%, p<0.001) and Sanger sequencing (42.11%, p<0.001) in KRAS mutation detection of pancreatic lesions starting from EUS-FNA material. Mutations in specimens with a low proportion of mutated alleles (<20%, corresponding to <40% of cells considering the mutation heterozygous) were detected only using 454-NGS and ASLNaqPCR (if the mutation was present in exon 2) or only using 454-NGS (if the mutation was in KRAS exon 3). These results are fully compatible with the fact that that Sanger sequencing can detect a mutation only if it is present in more than 40% of the cells.

Our analysis of KRAS using highly analytical sensitive techniques such as ASLNaqPCR or 454-NGS not only increases the clinical sensitivity of the test but also maintains a very high level of specificity (100%).

Even if KRAS exon 2 mutations were still the majority (40% of all analyzed cases using NGS), mutations of KRAS exon 3 were also observed many samples (16.7% using NGS). Using Sanger sequencing our percentage of KRAS exon 3 mutated cases was about 3%, similar to that generally reported in the literature [38]. In three cases the KRAS exon 3 mutation would not have affected the molecular diagnosis, considering that the mutation was found in association with another KRAS mutation in exon 2. However, in seven cases the mutation in KRAS exon 3 was the only alteration found. All these seven cases would have been considered wild-type for KRAS although, on follow-up, five were IPMN and two were PDAC. For this reason, the detection of additional mutation in KRAS exon 3 provides very useful diagnostic information. Moreover, when both KRAS exon 2 and exon 3 were analyzed, the clinical sensitivity of 454-NGS was higher (73.68%) than when only KRAS exon 2 was investigated (52.78%).

IPMN is a spectrum of neoplasms in the pancreatic duct epithelium characterized by cystic dilatation of the main pancreatic ducts and/or of their branches. IPMN is considered to be a precursor of PDAC. It has been proposed that the process of IPMN follows an adenoma - carcinoma sequence and that the time of progression to malignancy is about 5 years [39]. Since the process is slow, a correct diagnosis of IPMN provides the opportunity to cure the patient, before an invasive preoperative adenocarcinoma develops.

The results presented here indicate not only that IPMN are frequently mutated for KRAS (83.3% in our series, consistent with other studies that analyze only KRAS exon 2 in IPMN [14,15,40]), but also that they are commonly mutated in KRAS exon 3 (41.7%).

Analysis of KRAS starting from direct EUS-FNA material allows to obtain good quality DNA for molecular analysis that is therefore available at the same time of the cytologic evaluation. In samples with unsatisfactory cytology (e.g. cyst content material, C1c), the analysis of KRAS from EUS-FNA material directly collected into a tube is the only way to evaluate the mutational status of the gene. However, it is important to note that the direct analysis of EUS-FNA does not allow for the determination of the presence or the proportion of neoplastic cells in the specimen. Since this type of analysis is “blind”, DNA could originate from a population of cells not representative of the lesion, resulting in false negative results.

For this reason, in samples directly collected in a tube with a wild-type KRAS result it is important to repeat the analysis after the dissection of diagnostic material from the corresponding cytology specimen (smear or cytoblock) if there are atypical cells in the cytology preparation. In fact, in our series two cases found to be wild-type for KRAS starting from direct EUS-FNA material resulted mutated after the analysis was repeated on cells scraped from the cytologic smears. Both cases were diagnosed as adenocarcinoma after postoperative histologic evaluation.

We propose an algorithm for KRAS analysis of pancreatic lesions (Figure 4). Next generation sequencing is more labor intensive and time-consuming that mutation specific techniques, as ASLNaqPCR. The turn-around time of 454-NGS depends on the throughput of the laboratory and batches of at least 100 amplicons (targeting the same or different exons) have to be run to lower the cost of the sequence to ~20€ per amplicon. One hundred amplicons correspond to the analysis of 50 cases if both KRAS exons 2 and 3 are analyzed, for a total cost of ~40€ per patient.

To reduce turnaround time, the KRAS analysis could be performed by initially studying only KRAS exon 2, using a highly analytical sensitive mutation-specific method that is not time-consuming (less than 2 working days, including DNA extraction). This molecular analysis is carried out in conjunction and at the same time of the cytologic evaluation. If the sample is not mutated, KRAS analysis should be expanded to include KRAS exon 3, using a highly analytical sensitive direct sequencing technique such as pyrosequencing or Next Generation sequencing. If the sample is still not mutated for KRAS and the cytologic smear shows atypical, suspicious or malignant cells, KRAS analysis should be repeated starting from cells dissected from the cytologic smear or cytoblock (Figure 4).

The algorithm we propose allowed us to reach a clinical sensitivity of ~80% and a remarkable negative predictive value of ~70%, if cases that were wild type for KRAS starting from material directly collected with EUS-FNA were re-analyzed on selected material dissected from the cytologic smear or cytoblock. We want to point out that this high clinical sensitivity was achieved without any false positive result, with a 100% of specificity.

Four routine clinical test it is desirable, if not mandatory, to perform DNA analysis only after careful morphologic evaluation of the cytologic or histologic material submitted for molecular diagnosis. In fact, a negative KRAS result on a sample that consists
of non-neoplastic cells or that is inadequate must be considered not-informative for clinical purposes. The results obtained in the present study stress the importance of morphologic evaluation of the material analyzed to detect KRAS mutations.

Finally, the analysis of KRAS with a semi-quantitative method (454-NGS or ASLNaqPCR), performed on material of well-known cellular composition, allows to clarify if mutations (single or multiple) are present in a small percentage of tumor cells (subclones) or in the vast majority of them as in the case of all “driver” mutations [33]. This type of evaluation is obviously not possible starting from direct EUS-FNA material.

In conclusion, our study underlines the importance of using a highly analytical sensitive technique for KRAS—as well as for any other molecular marker - mutation analysis to support the pathologist in the diagnosis of pancreatic lesions, as also recently shown in a meta-analysis by Fuccio et al. [42]. In this series Next Generation Sequencing has allowed us to reach a very high clinical sensitivity without getting false positive results. Highly

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