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# *APC* promoter is frequently methylated in pancreatic juice of patients with pancreatic carcinomas or periampullary tumors

MIREIA M. GINESTA[1], ZAMIRA VANESSA DIAZ-RIASCOS[1], JULI BUSQUETS[2], NÚRIA PELAEZ[2], TERESA SERRANO[3], MIQUEL ÀNGEL PEINADO[4], ROSA JORBA[5], FRANCISCO JAVIER GARCÍA-BOROBIA[6], GABRIEL CAPELLA[1] and JOAN FABREGAT[2]

[1]Translational Research Laboratory, Catalan Institute of Oncology, Bellvitge Biomedical Research Institute, Duran i Reynals Hospital; Departments of [2]General and Digestive Surgery, and [3]Pathology, Bellvitge University Hospital, 08907 Barcelona; [4]Department of Mechanism of Tumor Progression, Institute of Predictive and Personalized Cancer Medicine, 08916 Barcelona; [5]Department of Surgery, Joan XXIII Hospital, 43005 Tarragona; [6]Department of Surgery, Hepatopancreatic Unit, Parc Taulí University Hospital, 08208 Barcelona, Spain

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**Abstract.** Early detection of pancreatic and periampullary neoplasms is critical to improve their clinical outcome. The present authors previously demonstrated that DNA hypermethylation of *adenomatous polyposis coli (APC)*, *histamine receptor H2 (HRH2)*, *cadherin 13 (CDH13)*, *secreted protein acidic and cysteine rich (SPARC)* and *engrailed-1 (EN-1)* promoters is frequently detected in pancreatic tumor cells. The aim of the present study was to assess their prevalence in pancreatic juice of carcinomas of the pancreas and periampullary area. A total of 135 pancreatic juices obtained from 85 pancreatic cancer (PC), 26 ampullary carcinoma (AC), 10 intraductal papillary mucinous neoplasm (IPMN) and 14 chronic pancreatitis (CP) patients were analyzed. The methylation status of the *APC, HRH2, CDH13, SPARC* and *EN-1* promoters was analyzed using methylation specific-melting curve analysis (MS-MCA). *Kirsten rat sarcoma viral oncogene homolog* (*KRAS)* mutations were also tested with allele-specific quantitative polymerase chain reaction amplification. Out of the 5 promoters analyzed, *APC* (71%) and *HRH2* (65%) were the most frequently methylated in PC juice. *APC* methylation was also detected at a high frequency in AC (76%) and IPMN (80%), but only occasionally observed in CP (7%). *APC* methylation had a high sensitivity (71-80%) for all types of cancer analyzed. The panel (where a sample scored as positive when ≥2 markers were methylated) did not outperform *APC* as a single marker. Finally, *KRAS* detection in pancreatic juice offered a lower sensitivity (50%) and specificity (71%) for detection of any cancer. *APC* hypermethylation in pancreatic juice, as assessed by MS-MCA, is a frequent event of potential clinical usefulness in the diagnosis of pancreatic and periampullary neoplasms.

*Correspondence to:* Dr Gabriel Capella, Translational Research Laboratory, Catalan Institute of Oncology, Bellvitge Biomedical Research Institute, Duran i Reynals Hospital, Avenue Gran Via de l'Hospitalet 199-03, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
E-mail: gcapella@iconcologia.net

*Abbreviations:* FNA, fine-needle aspiration; qPCR, quantitative polymerase chain reaction; MCA, melting curve analysis; PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasm; CP, chronic pancreatitis; PPJ, pure pancreatic juice; *APC, adenomatous polyposis coli*

*Key words: APC*, methylation, pancreatic cancer, IPMN, AC, PC, oncology, cancer genetics, molecular pathology

## Introduction

Carcinoma of the exocrine pancreas or pancreatic cancer (PC) is one of the most aggressive solid tumors (1,2). At diagnosis, only 10-15% of patients are amenable for surgical resection, and even for this selected group of patients, prognosis is poor, with a 5-year survival of 15-40% (1,2). Ampullary tumors are also aggressive, although to a lesser extent (3). Intraductal papillary mucinous neoplasms (IPMNs) are a distinct subset of tumors that are increasingly recognized and may be cured if they are timely detected (3).

Early detection of PC is the best and currently only option to improve its dismal prognosis (4). The high frequency (75-100%) of *Kirsten rat sarcoma viral oncogene homolog (KRAS)* mutations in PC (5,6), has lead to extensive research aimed at establishing their role as diagnostic markers for this disease. However, when assessing pancreatic juice, its clinical utility is hampered by a lack of specificity (7-9) linked to the detection of *KRAS* mutations in a significant proportion of patients with chronic pancreatitis (CP) (8,10). In fact, *KRAS* mutations have been detected in variable proportion (0-60%) in ductal lesions in CP samples and even in normal appearing ducts (10).

Pancreatic juice can be considered a good surrogate of the status of the pancreatic duct epithelium, since it contains exfoliated cells from all areas of the pancreas (10). In this regard, genetic alterations can be more easily detected in DNA extracted

from pancreatic juice compared with tissue blocks (10). This may well reflect a field effect within the pancreas or, when the tumor is already evident, the possibility that exfoliated cells only represent part of a tumor that is intrinsically heterogeneous (11).

Aberrant promoter methylation of tumor suppressor genes is a frequent and early event in multiple tumors, including carcinomas of the pancreas and the periampullary area (12,13). The use of epigenetic abnormalities as biomarkers is based on their relative high frequency and the development of methodologies that can sensitively detect methylation even when cancer cells are a minority of the cells analyzed (14). Candidate genes for these purposes should ideally have a high prevalence of hypermethylation in tumors cells not being present in the absence of disease. The present authors previously reported that promoter hypermethylation of *engrailed-1 (EN1-), histamine receptor H2 (HRH2), cadherin 13 (CDH13), adenomatous polyposis coli (APC)* and *secreted protein acidic and cysteine rich (SPARC)* genes are frequent events in PC, where they may aid the clinical assessment of fine-needle aspiration (FNA) biopsies of pancreatic masses (15).

The aim of the present study was to assess the prevalence of the promoter methylation detection of the above panel of genes in pancreatic juice using methylation specific-melting curve analysis (MS-MCA), a sensitive and robust technique for the analysis of promoter methylation status (15). The present study evaluated the performance of each gene separately or as a panel, and compared it with *KRAS* mutation detection in patients with CP and with carcinomas of the pancreas and the periampullary area.

## Materials and methods

*Patients and samples*. Between January 2004 and December 2010, a total of 135 patients undergoing surgical resection at the Hospital Universitari de Bellvitge (L'Hospitalet de LLobregat, Barcelona) due to pancreatic disease were prospectively included in a study aimed to identify novel biomarkers in pancreatic diseases. The diagnosis was as follows: 85 PCs (9.4% of which were well differentiated, 75.3% moderately differentiated and 15.3% poorly differentiated), 26 ampullary carcinomas (ACs) (21 of the pancreaticobiliary subtype and 5 of the intestinal subtype), 10 IPMNs (2 with invasive carcinoma and 1 with carcinoma *in situ*) and 14 CPs. Table I summarizes the main characteristics of the patients included in the study. One of the inclusion criteria was obtaining a minimum of 200 ml retrograde pancreatic juice from the main pancreatic duct during the surgical procedure. In a subset of 20 cases (16 PCs and 4 ACs), paired biopsies were also analyzed. All patients provided written informed consent to participate in the study and to have their biological specimens analyzed. The present study was approved by the Ethical Committee of the University Hospital of Bellvitge (Barcelona, Spain).

*Sample processing and DNA extraction*. The intraoperative pure pancreatic juice (PPJ) was snap frozen immediately after collection. DNA extraction was directly performed from the PPJ with no further processing. A modified standard phenol-chloroform method was used to optimize DNA extraction (16). DNA extraction from NP9 and NP18 cell lines (previously generated from pancreatic ductal adenocarcinoma biopsies in the Translational Research Laboratory, Duran i Reynals Hospital, Barcelona, Spain) used as controls and biopsies was performed using the saline method (17), and the DNA was precipitated with 2-propanol after mechanical disgregation and overnight digestion with proteinase K at 53˚C.

Table I. Main characteristics of the study population.

| Characteristics | PC | AC | IPMN | CP |
|---|---|---|---|---|
| Patients (n) | 85 | 26 | 10 | 14 |
| Age (years) | 62±12 | 71±8 | 66±14 | 47±12 |
| Gender (M/F) | 44/41 | 17/9 | 8/2 | 12/2 |
| Not applicable | | | 7 | 14 |
| Not available | 1 | | | |
| TNM stage | | | | |
| T1N0 | 2 | 3 | | |
| T2N0 | | 9 | | |
| T2N1 | | 6 | | |
| T3N0 | 14 | 3 | 2 | |
| T3N1M0 | 60 | 4 | | |
| T3N1M1 | 3 | | | |
| T4N1M0 | 4 | | | |
| T4N1M1 | 1 | | | |
| *In situ* | | 1 | 1 | |

PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasm; CP, chronic pancreatitis; M, male; F, female; TNM, tumor-node-metastasis.

*Methylation assessment*. MS-MCA was used (15,18) to assess the methylation status of *EN-1, HRH2, SPARC, APC* and *CDH13* gene promoters. A total of 800 ng genomic DNA were treated with sodium bisulfite according to the manufacturer's protocol (EZ-DNA Methylation-Gold™ kit; Zymo Research Corporation, Irvine, CA, USA). Quantitative polymerase chain reaction (PCR) with temperature dissociation or MCA was used to assess the difference in melting temperatures between methylated and unmethylated samples. For *APC* and *CDH13,* direct amplification analysis was used, while for the remaining three genes, a nested PCR approach was selected. PCR was performed using IMMOLASE™ DNA Polymerase (Bioline, London, UK). For qPCR, either 25 ng bisulfite-modified DNA or 1 $\mu$l pre-amplified DNA was used as a template, using a LightCycler® 486 (Roche Applied Science, Penzberg, Germany). Primer sequences, annealing temperature and CpG residues targeted in each reaction are detailed in Table II. Whole lymphocyte DNA amplified with the REPLI-g kit (Qiagen, Inc., Valencia, CA, USA) was used as unmethylated control. NP18 or NP9 pancreatic cell lines were used as positive controls. The primers used did not target CpG residues amenable for methylation, thus resulting in unbiased amplification.

The analytical sensitivity and robustness of the method were assessed using serial dilutions of methylated DNA in increasing amounts of unmethylated DNA (15). For all selected genes, the technique was optimized to have an analytical sensitivity of 5% (Fig. 1). MS-MCA results were compared with those of direct sequencing of the bisulfite-treated DNA as

Table II. DNA hypermethylation and *KRAS* mutations analyses.

A, Primer sequences and PCR conditions for DNA hypermethylation analysis

| Gen | PCR | Annealing temperature (˚C) | CpG (n) | Primers sequence (5'→3') |
|---|---|---|---|---|
| *EN-1* | External | 52 | 28 | F: ACTATCCTACTTATAAACTC<br>R: AGAATAATAAAGATAAGAGAT |
| | Nested | 54 | | F: CTACTTATAAACTCAACCAA<br>R: GTTTTAGGGATTTAGAGTTT |
| *HRH2* | External | 56 | 18 | F: GGGTGGATTTGGAAAGTGT<br>R: TTACTCTACTCATCCCACAA |
| | Nested | 62 | | F: GGGTGGATTTGGAAAGTGT<br>R: TCCAAATATCCCCAACAAAA |
| *SPARC* | External | 54 | 16 | F: TGGAGGGGAGATAGATTTAGTT<br>R:AACCAAAAACAAACACAAAAAA |
| | Nested | 58 | | F: TTTTGAGTGGTTTTTTGTTGTT<br>R:ATCCACCTTCTAAAAAACAACAA |
| *APC* | | 64 | 16 | F: GGTTAGGGTTAGGTAGGTTG<br>R: CTACACCAATACAACCAC |
| *CDH13* | | 65 | 23 | F: TGATTTGTGAGGTTGAGTTTTAA<br>R: ACCCCTCTTCCCTACCTAAAA |

B, *KRAS* mutations analyzed, and probe and primer sequences

| Codon[a] | Aminoacid | WT | Mutation | Control cell line | Probe sequence (5'→3') |
|---|---|---|---|---|---|
| 12 | - | GGT | - | NP18 | TTGGAGCTGGTGGCGTA |
| 12 | G12C | GGT | TGT | MIA PaCa-2 | TTGGAGCT**T**GTGGCGTA |
| 12 | G12V | GGT | GTT | SW480 | TTGGAGCTG**T**TGGCGTA |
| 12 | G12D | GGT | GAT | NP9 | TTGGAGCTG**A**TGGCGTA |
| 12 | G12A | GGT | GCT | SW1116 | TTGGAGCTG**C**TGGCGTA |
| 12 | G12S | GGT | AGT | A549 | TAGTTGGAGCT**A**GTGGCGTA |
| 12 | G12R | GGT | CGT | CAL-62 | TTGGAGCT**C**GTGGCGTA |
| 13 | - | GGC | - | NP18 | CTTGCCTACG**C**CACCAG |
| 13 | G13D | GGC | GAC | DLD-1 | CTTGCCTACG**T**CACCAG |

[a]Forward primer codons 12 and 13, 5'-GCCTGCTGAAAATGACTGAATATAAACT-3'; reverse primer codon 12, 5'-GCTGTATCGTCAAGG CACTCTT-3'; reverse primer codon 13, 5'-GAATTAGCTGTATCGTCAAGGCACT-3'. PCR, polymerase chain reaction; *APC*, *adenomatous polyposis coli*; *HRH2*, *histamine receptor H2*; *CDH13*, *cadherin 13*; *EN-1*, *engrailed-1*; *SPARC*, *secreted protein acidic and cysteine rich*; *KRAS*, *Kirsten rat sarcoma viral oncogene homolog*; WT, wild-type.

previously described (19). All results were blindly evaluated by M.M.G. and G.C. with 100% of concordance with the analysis. All analysis depicting the presence of 5-10% of methylated alleles were repeated, and only when the two tests yielded the same results, the samples were scored as methylated.

*KRAS mutation detection*. *KRAS* mutation analysis was based on an allele-specific qPCR assay performed on a Light Cycler® 480 (Roche Applied Science). PCR primers were designed to amplify the target region, and TaqMan® MGB probes (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to screen 7 common mutations in *KRAS* codons 12 and 13 (Table II). For each allele, one probe targeted the wild-type variant (tagged with the VIC® fluorophore; Applied Biosystems; Thermo Fisher Scientific, Inc.), while the other probe targeted the mutant variant (tagged with the fluorescein fluorophore). The Light Cycler® 480 software version 1.5 (Roche Applied Science) was used to determine the genotype of the sample by measuring the intensity distribution of the used dyes following PCR amplification. This technique has an analytical sensitivity of 5% in a background of wild-type DNA (11).

*Statistical analysis*. The Fisher's exact test for categorical data was used to evaluate the association between each methylation marker and diagnosis. Differences in the prevalence of markers among groups were assessed by the exact McNemar test. Diagnostic classifiers from methylation panels were estimated using the random forests test. Software R version 3.2.0 (https://www.r-project.org/), a language and environment for statistical computing, was used for all statistical calculations.

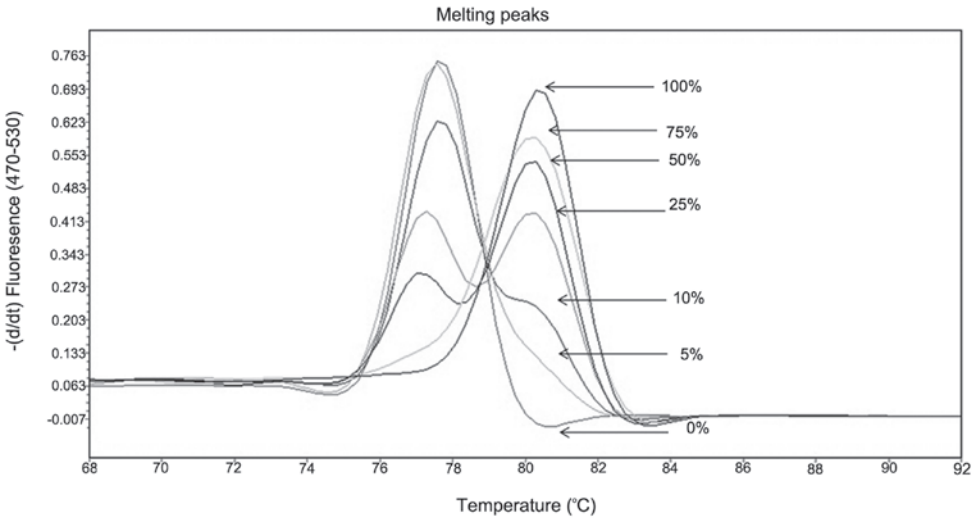


Figure 1. Analytical sensitivity of the detection of methylated alleles using methylation specific-melting curve analysis of the methylation status of the *adenomatous polyposis coli* promoter.

## Results

*Methylation pattern differs among the different tumor types.* Promoter analysis was informative in the majority of cases [*EN-1,* 92% (125/136); *HRH2,* 95% (129/135); *SPARC* 86% (116/135); *APC,* 98% (132/135); and *CDH13,* 90% (122/135)]. Non-informative cases were not considered. The frequencies of methylation for each marker and tumor type are detailed in Fig. 2 and Table III. Out of the 5 markers analyzed, both *APC* and *HRH2* exhibited the highest frequency of methylation (71 and 65%, respectively) in PC, while *CDH13*, *SPARC* and *EN-1* methylation was detected at significant albeit lower frequency (57, 49 and 37%, respectively). In IPMN or AC, *APC* methylation was identified at a similar frequency. *HRH2* and *CDH13* promoters were less often methylated in AC (P=0.03 and P=0.02, respectively) (Fig. 2 and Table III).

The prevalence of methylation in CP was low for all analyzed markers (0-14%), being only negative for the *CDH13* promoter (Fig. 2). In total, 3 samples had 1 marker methylated, while 1 sample had 2 methylated markers. In spite of the differences observed in methylation frequencies among the distinct tumor types, it was not possible to create a classifier that discriminated among them (out-of-bag estimate of error rate, 34%), indicating that the pattern of promoter hypermethylation does not predict the site of origin of the tumor.

As an individual marker, *APC* yielded the best performance, with a remarkable sensitivity of 71% and a specificity of 93% for PC (Table IV). When all tumors were considered, sensitivity remained high (73%). When all markers were considered and a threshold of ≥2 markers was established to score a sample as positive, the panel did not outperform *APC* hypermethylation as a single marker (Fig. 3) both for PC only or when all tumor types were considered.

*KRAS.* The mutation analysis of *KRAS* codon 12 was informative in 134 of 136 (99%) samples, and mutations were detected in a similar proportion in all tumors (52% PC, 42% AC and 45% IPMN), while the prevalence of mutations in CP was also high (33%; P>0.05) (Table III). No mutation was detected at the codon 13 of the *KRAS* gene. As expected, the combination of *KRAS* mutations did not improve the performance of any individual methylation marker or panel (Table IV).

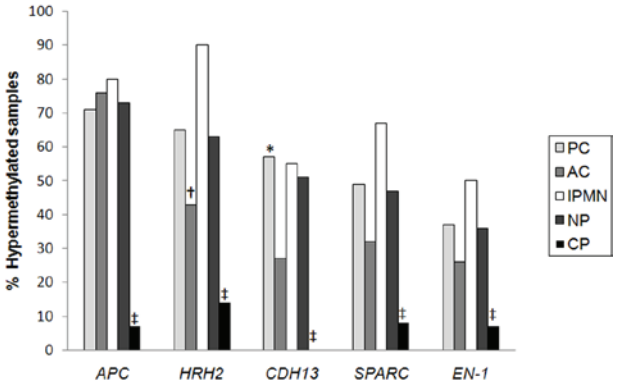


Figure 2. Proportion of methylated samples according to sample type. *P<0.05 PC vs. AC; †P<0.05 AC vs. IPMN; ‡P<0.05 CP vs. PC, AC and IPMN. PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasm; NP, neoplasm of pancreatic area (all types of tumors together); CP, chronic pancreatitis; *APC*, *adenomatous polyposis coli*; *HRH2*, *histamine receptor H2*; *CDH13*, *cadherin 13*; *EN-1*, *engrailed-1*; *SPARC*, *secreted protein acidic and cysteine rich*.

*APC hypermethylation and KRAS mutation in paired samples.* In order to assess the impact of sampling in the performance of the test in pancreatic juice, the *APC* promoter methylation status was assessed in paired biopsies in a subset of 20 cases. Regarding the *APC* methylation status, 11 of 20 (55%) of cases showed concordant results (7 methylated and 4 unmethylated), and in 9 cases, discrepancies were observed. In 6 of them (30%), the pancreatic juice was methylated, whereas the corresponding biopsy was not, and in the other 3 cases (15%), the pancreatic juice was negative in spite of having detected tissue methylation (data not shown). In all, pancreatic juice analysis correctly identified 65% of this subset of PCs, which is moderately higher than the yield of biopsies analyzed (50%). These results highlight the relevance of sampling when assessing genetic or epigenetic aberrations in pancreatic juice.

Table III. Prevalence of all methylation markers analyzed and *KRAS* codons 12 and 13 mutations depending on tumor type[a].

| Cancer | Met *APC* | Met *HRH2* | Met *CDH13* | Met *SPARC* | Met *EN-1* | ≥2 Met markers | Mut KRAS |
|---|---|---|---|---|---|---|---|
| PC | 71% (59/83) | 65% (53/82) | 57% (44/77) | 49% (35/72) | 37% (29/78) | 72% (61/83) | 50% (41/82) |
| AC | 76% (19/25) | 43% (10/23) | 27% (6/22) | 32% (7/22) | 26% (6/23) | 56% (14/25) | 42% (11/26) |
| IPMN | 80% (8/10) | 90% (9/10) | 55% (5/9) | 67% (6/9) | 50% (5/10) | 80% (8/10) | 55% (5/9) |
| NP | 73% (86/118) | 63% (72/115) | 51% (55/108) | 47% (48/103) | 36% (40/111) | 70% (83/118) | 47% (57/117) |
| CP | 7% (1/14) | 14% (2/14) | 0% (0/14) | 8% (1/13) | 7% (1/14) | 7% (1/14) | 33% (4/14) |

[a]Percentages are expressed on the total number of informative amplified samples. Met, methylated; Mut, mutated; PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasms; NP, neoplasm of pancreatic area (all types of tumors together); CP, chronic pancreatitis; *APC*, *adenomatous polyposis coli*; *HRH2*, *histamine receptor H2*; *CDH13*, *cadherin 13*; *EN-1*, *engrailed-1*; *SPARC*, *secreted protein acidic and cysteine rich*; *KRAS, Kirsten rat sarcoma viral oncogene homolog*.

Table IV. Sensitivity and specificity of each methylation marker separately and combined (panel), and sensitivity and specificity of *KRAS* mutations detection separately and in combination with the panel of methylation markers.

| | Sensitivity % (95% CI) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Comparison | *APC* | *HRH2* | *CDH13* | *SPARC* | *EN-1* | Panel | *KRAS* | *KRAS*+Panel |
| PC vs. CP | 71% (61-80) | 65% (54-74) | 57% (46-67) | 49% (37-60) | 37% (27-48) | 72% (61-80) | 52% (41-62)[a] | 78% (68-85) |
| AC vs. CP | 76% (57-88) | 43% (26-63) | 27% (13- 48) | 32% (16-53) | 26% (12-46 ) | 54% (35-71) | 44% (26-63) | 69% ( 50-83) |
| IPMN vs. CP | 80% (49-94) | 90% (59-98) | 56% (26-81) | 67% (35-88) | 50% (23-76) | 80% (49-94) | 56% (26-81) | 80% (49-94) |
| NP vs. CP | 73% (64-80) | 63% (53-70) | 51% (41-60) | 47% (37-56) | 36% (27-45) | 69% (60-76) | 50% (41-59)[a] | 76% (68-83)[a] |
| Specificity (95% CI) | 93% (69-99) | 86% (60- 96) | 100% (78-100) | 92% (67-99) | 93% (68-98) | 93% (68-99) | 71% (45-88) | 79% (52%-92) |

[a]P<0.05 panel vs. *KRAS*. PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasm; NP, neoplasm of pancreatic area (all types of tumors together); CP, chronic pancreatitis; CI, confidence interval; *APC*, *adenomatous polyposis coli*; *HRH2*, *histamine receptor H2*; *CDH13*, *cadherin 13*; *EN-1*, *engrailed-1*; *SPARC*, *secreted protein acidic and cysteine rich*; *KRAS, Kirsten rat sarcoma viral oncogene homolog*.

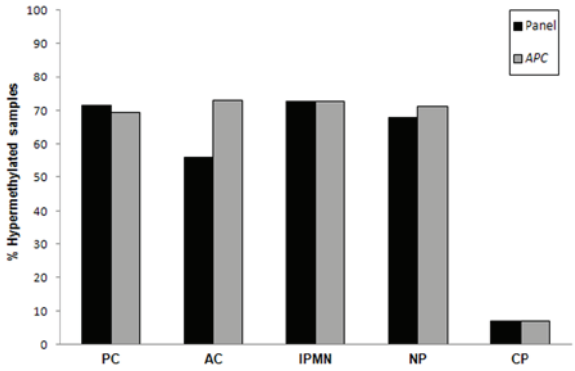


Figure 3. Proportion of methylated samples when considering all markers as a panel (≥2 hypermethylated markers) and proportion of samples with methylated *adenomatous polyposis coli*. PC, pancreatic cancer; AC, ampullary carcinoma; IPMN, intraductal papillary mucinous neoplasm; NP, neoplasm of pancreatic area (all types of tumors together); CP, chronic pancreatitis; *APC, adenomatous polyposis coli*.

## Discussion

The present study has identified *APC* promoter hypermethylation as a relevant biomarker for pancreatic juice assessment when pancreatic and periampullary tumors are suspected. Previously, APC promoter hypermethylation had been detected at high frequency in PC biopsies (33-60%) (20-22) or FNA biopsies (15). In fact, promoter hypermethylation is likely the most prevalent *APC* gene aberration in PC, as loss of heterozygosity and mutations have been only occasionally detected (23) [Catalogue of Somatic Mutations in Cancer (COSMIC) database; http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=APC]. Of note, *APC* hypermethylation in pancreatic juice yields a high sensitivity (71-80%) and specificity (93%) for the identification of PC in a clinical context where more promising alterations such as *KRAS* mutations have failed. The yield is also good for other periampullary and pancreatic neoplasms, thus increasing its potential usefulness. In line with the present observations, *APC* hypermethylation has been reported in ≤50% of infiltrative IPMNs (21,24). No previous data are available for *APC* promoter methylation in AC, where, in contrast to PC, *APC* gene mutations have been detected in ≤42% of cases (COSMIC database).

Regarding the other markers analyzed in the present study, *HRH2* methylation has also shown a high sensitivity (65%), which is slightly higher than the 50% observed in the evaluation of FNA biopsies of pancreatic masses (15). This frequency is similar to that detected in colorectal carcinoma tissues (25). However, its lower prevalence in AC diminishes its potential clinical usefulness. According to the present results, *CDH13* was also methylated in ≤50% of PC juices, in line with the results obtained by Sakai *et al* (13). However, while *CDH13* methylation was the only marker not being detected in CP, its relatively low prevalence in AC and IPMN was not high enough to add diagnostic value. *SPARC* hypermethylation have been previously reported at high frequency in PC, IPMN and CP. In the present study, the prevalence of methylation was still high but markedly lower (40%) than previously reported (3). Among the markers tested in the present study, the *EN-1* promoter methylation status was the one with the lowest sensitivity (37%), which is well below the 60% sensitivity observed in FNA biopsies (15).

*EN-1*, *HRH2, SPARC* and *CDH13* promoter methylation effectively distinguished between neoplasia and CP, although, in line with previous observations, 1 of 14 pancreatic juices analyzed exhibited hypermethylation (26). It must be emphasized that hypermethylation may be detected in normal pancreatic parenchyma and in non-neoplastic pancreatic juice.

When assessing pancreatic juice, the present panel did not add information to *APC* as a single marker. The high prevalence of *APC* hypermethylation in all tested tumor types, together with a low proportion of false positives, account for the excellent performance of *APC*. This is in contrast with the previous study by the present authors evaluating FNA biopsies of pancreatic masses (15). In that setting, the panel outperformed any single marker, as previously described for DNA stool testing for colorectal cancer (27). *APC* and the other markers analyzed are methylated not only in PC, but in other tumors arising in the area (mainly AC or IPMN), thus expanding its potential utility. However, while certain differences depending on tumor type have been observed, no evident classifier could be established for a particular histology.

*KRAS* mutations at codons 12 and 13 have been considered good diagnostic markers for PC (5,6) (COSMIC database; http://cancer.sanger.ac.uk/cosmic/search?q=KRAS). However, their utility in the assessment of pancreatic juices is limited due to the relative low prevalence of *KRAS* mutations in pancreatic juice and its frequent detection in CP (8-10). Accordingly, in the present study, *KRAS* mutations assessment did not add diagnostic value to *APC* hypermethylation.

The pancreatic juice provides information from cells shed from all areas of the pancreatic epithelium, thus yielding a comprehensive sampling of the target organ (11). However, this may or may not reflect what occurs in the biopsies of selected areas of the tumor. The study of paired pancreatic juice-biopsies have revealed a relatively low degree of concordance between *APC* methylation in juice compared with biopsies. Notably, discrepancies go in both directions (positive in biopsy-negative in juice or vice versa). Limited representation of pancreatic tumor cells in the juice (as tumors may not directly shed cells into the juice) is widely accepted as responsible for false-negative results in pancreatic juice, which occurred in 3 cases in the present series, whereas 5 cases exhibited reverse results (positive juice-negative biopsy). Intratumoral heterogeneity of *APC* status or the presence of these alterations in non-neoplastic pancreatic ducts may account for this observation. Irrespective of the cause, in the present study, *APC* methylation status still offered a very good yield in the diagnosis of pancreatic neoplasms. This is in contrast to the results obtained with highly sensitive *KRAS* mutation techniques, where its clinical utility was not obvious (11) due to the high number of KRAS mutations in CP.

As discussed above, sampling may be a relevant issue when assessing genetic or epigenetic aberrations in pancreatic juice. However, the present authors do not consider that the use of anterograde collection during surgery is worse than retrograde collection of pancreatic juice during endoscopic retrograde cholangiopancreatography (ERCP) or collection of duodenal juice after secretin stimulation. The high yield of *APC* hypermethylation and the excellent performance of markers observed for AC indicate that their relative impact may be less relevant than anticipated. In fact, the present authors anticipate an even better performance after secretin stimulation during ERCP, as the volume collected will certainly increase (26).

Age and chronic inflammation may affect DNA methylation (28). The well established association between ageing and hypermeyhylation for specific genes and tissues makes it necessary to consider age as a confounder (28). In addition, persistent inflammation is considered to result in an accelerate ageing of the affected tissues with increased methylation of specific markers (29). In the present limited series, younger cancer patients exhibited higher methylation levels than those with CP, suggesting that neither factor is influencing in a significant manner the present results.

The robustness of the present results may be partially attributable to the use of MS-MCA, a reliable technique that assesses the hypermethylation status of all CpG included in the amplicon analyzed. This technique was selected due to its simplicity, reproducibility and analytical sensitivity, which enables the detection of ≤5% of methylated alleles (15). Unlike the often used standard or quantitative MS-PCR, MS-MCA is dependable (as it uses methylation-independent primers) and less prone to false positives, and allows the simultaneous analysis of multiple CpG,

thus providing a more comprehensive picture of the methylation present in a heterogeneous cell population than MS-PCR.

In conclusion, the present study has observed that *APC* promoter hypermethylation is a frequent event in pancreatic and periampullary neoplasms that may be useful when assessing pancreatic juice. In the present study, *APC* promoter hypermethylation outperformed the other genetic and epigenetic markers analyzed, based on its high prevalence in all tumor types evaluated and its great specificity. Differences in the prevalence of the methylation markers depending on tumor types may be envisioned, although further studies are required to assess whether specific methylation profiles may occur in AC and IPMN. Additionally, the results of the present study highlight the strengths and weaknesses of pancreatic juice as a surrogate of tumor biopsies. The present study opens the door to explore whether *APC* hypermethylation could be used as a biomarker of pancreatic and periampullary tumors in less invasive samples such as duodenal juice or circulating DNA.

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