

# World Journal of *Gastrointestinal Oncology*

*World J Gastrointest Oncol* 2020 September 15; 12(9): 942-1079



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**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Jia-Hui Li; Production Department Director: Xiang Li; Editorial Office Director: Jin-Lei Wang.

**NAME OF JOURNAL**

*World Journal of Gastrointestinal Oncology*

**ISSN**

ISSN 1948-5204 (online)

**LAUNCH DATE**

February 15, 2009

**FREQUENCY**

Monthly

**EDITORS-IN-CHIEF**

Rosa M Jimenez Rodriguez, Pashtoon Kasi, Monjur Ahmed

**EDITORIAL BOARD MEMBERS**

<https://www.wjgnet.com/1948-5204/editorialboard.htm>

**PUBLICATION DATE**

September 15, 2020

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<https://www.wjgnet.com/bpg/gerinfo/242>

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## Observational Study

# Methylation changes at the *GNAS* imprinted locus in pancreatic cystic neoplasms are important for the diagnosis of malignant cysts

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**Author contributions:** All authors have contributed to the paper concept and design and agreed on the final content of the manuscript; Faias S, Duarte M, and Albuquerque C acquired and interpreted the data; Pereira L performed the statistical analysis; Faias S, Pereira L, Chaves P, Cravo M, and Albuquerque C drafted the manuscript under the supervision of both senior authors, Albuquerque C and Pereira AD; all authors critically revised the manuscript and approved the final version of the manuscript.

**Supported by** a Research Grant from Sociedade Portuguesa de Endoscopia Digestiva in 2018.

**Institutional review board**

**statement:** The study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1143).

**Informed consent statement:** All

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## Abstract

### BACKGROUND

Guanine nucleotide-binding protein, alpha stimulating (*GNAS*) mutations are characteristic of intraductal papillary mucinous neoplasms (IPMNs). Pancreatic ductal adenocarcinomas (PDACs) harboring *GNAS* mutations originate in IPMNs. *GNAS* is a complex imprinted locus that produces five transcripts regulated by differential methylated regions, *NESP55*, *GNASAS*, *GNASXL*, *GNAS1A*, and *GNAS*.

### AIM

To evaluate if methylation changes in the differential methylated regions of *GNAS* locus contributed to malignant progression of pancreatic cysts.

### METHODS

patients gave informed consent.

**Conflict-of-interest statement:** The authors have no conflicts of interest to disclose.

**Data sharing statement:** No additional data are available.

**STROBE statement:** Guidelines of STROBE statement have been adopted.

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**Manuscript source:** Unsolicited manuscript

**Received:** April 15, 2020

**Peer-review started:** April 15, 2020

**First decision:** July 5, 2020

**Revised:** July 18, 2020

**Accepted:** August 1, 2020

**Article in press:** August 1, 2020

**Published online:** September 15, 2020

**P-Reviewer:** Cheng J, Hori T

**S-Editor:** Gong ZM

**L-Editor:** Filipodia

**P-Editor:** Li JH



GNAS locus methylation was analyzed in archival pancreatic cyst fluid (PCF) obtained by endoscopic ultrasound with fine-needle aspiration by methylation specific-multiplex ligation dependent probe amplification. Results were normalized and analyzed using Coffalyser.Net software.

## RESULTS

Fifty-two PCF samples obtained by endoscopic ultrasound with fine-needle aspiration and previously characterized for *KRAS* and *GNAS* mutations were studied. The final diagnoses were surgical (11) and clinicopathological (41), including 30 benign cysts, 14 pre-malignant cyst, and eight malignant cysts. Methylation changes at *NESP55*, *GNASAS*, *GNAS1A*, and especially *GNASXL* were more frequent in malignant cysts, and *NESP55* and *GNASAS* were useful for diagnosis. A combined variable defined as “GNAS locus methylation changes” was significantly associated with malignancy (6/8 malignant cysts and only 2/20 benign cysts) and improved classification. Hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters was found in 3/3 PDACs.

## CONCLUSION

This is the first study to identify methylation changes in the *GNAS* locus, improving the diagnosis of malignant pancreatic cysts and suggesting a role in progression to PDAC.

**Key Words:** Intraductal papillary mucinous neoplasms; Pancreas cyst; Methylation; Biomarker; *GNAS* locus; Pancreatic neoplasm

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**Core Tip:** Pancreatic cystic lesions are a clinical dilemma due to risk of malignancy. Somatic mutations of guanine nucleotide-binding protein, alpha stimulating (*GNAS*) are characteristic of intraductal papillary mucinous neoplasms. We found methylation changes in differentially methylated regions at the *GNAS* locus in pancreatic cyst fluid predominantly of malignant cysts. Methylation changes in *GNAS* locus may improve the diagnosis of malignant cysts and shed light on the development of novel therapeutic approaches for pancreatic cancer.

**Citation:** Faias S, Duarte M, Pereira L, Chaves P, Cravo M, Dias Pereira A, Albuquerque C. Methylation changes at the *GNAS* imprinted locus in pancreatic cystic neoplasms are important for the diagnosis of malignant cysts. *World J Gastrointest Oncol* 2020; 12(9): 1056-1064

**URL:** <https://www.wjgnet.com/1948-5204/full/v12/i9/1056.htm>

**DOI:** <https://dx.doi.org/10.4251/wjgo.v12.i9.1056>

## INTRODUCTION

Pancreatic cystic lesions (PCLs) constitute a clinical dilemma due to indeterminate risk of malignancy, including benign cysts (BCs), pre-malignant cysts (PMCs), and malignant cysts (MCs)<sup>[1]</sup>. Intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are cystic precursors of pancreatic ductal adenocarcinoma (PDAC), allowing early diagnosis<sup>[2]</sup>.

Somatic mutations in guanine nucleotide-binding protein, alpha stimulating (*GNAS*) are characteristic of IPMNs<sup>[3,4]</sup>, but their role in carcinogenesis is unclear, with early occurrence precluding prediction of dysplasia<sup>[5,6]</sup>. However, if detected in PDACs, somatic mutations in *GNAS* are specific for an IPMN origin<sup>[3]</sup>.

*GNAS* is a complex imprinted locus in the long arm of chromosome 20 (20q13.32)<sup>[7]</sup>, which encodes the  $\alpha$ -subunit of the stimulatory heterotrimeric G protein (*G $\alpha$* ), a ubiquitous signaling protein translated from *GNAS* exons 1-13. This locus encodes four monoallelic (*NESP55*, *AS*, *XL*, *1A*) and one biallelic (*G $\alpha$* ) transcript, due to differentially methylated regions (DMRs) in paternal and maternal alleles, denominated imprinting<sup>[8,9]</sup>. Paternal methylation of *NESP55* and maternal methylation of *AS*, *XL*, and *1A* lead, respectively, to maternal and paternal allele expressions, with *G $\alpha$*  biallelically expressed in most tissues, due to absent

methylation<sup>[10]</sup>.

Epigenetic alterations in the *GNAS* locus have not been previously evaluated in PCLs. Methylation of DMRs may occur at the somatic level and modulate *Gsa* expression<sup>[10,11]</sup>, leading us to hypothesize that methylation changes in DMRs at the *GNAS* locus could contribute to tumor progression of PCLs. To test our hypothesis, we performed a longitudinal cohort pilot study of PCLs and analyzed *GNAS* locus methylation in pancreatic cyst fluid (PCF) samples.

## MATERIALS AND METHODS

### Case selection

All patients gave informed consent, and the study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1143).

For this study we performed molecular analysis in samples of 52 patients with more than 1 mL of PCF stored in the biorepository of our hospital, with sample processing and storage described in a previous publication<sup>[12]</sup>. Clinical data, including demographics, cyst characteristics, and treatment decision, have been prospectively registered.

After undergoing endoscopic ultrasound with fine needle aspiration, patients were evaluated in clinics, and referred for surgery (surgical cohort, surgical pathology diagnosis) or imaging surveillance, palliation, or endoscopic drainage (clinical cohort, clinico-cytological diagnosis) when surgery was not clinically indicated and a surgical pathology specimen was not available for diagnosis. The diagnostic criteria for the clinical cohort were determined *a priori* by one of the investigators (SF) after reviewing imaging features, PCF levels of CEA, and cytology analysis of PCLs, all with a prolonged imaging and clinical follow-up (of at least 24 mo). To evaluate *GNAS* locus methylation distribution and the performance of methylation analysis for cyst diagnosis, PCLs were further classified into one of three groups: Group 1) Benign cysts (BCs), including neoplastic benign and inflammatory cysts (serous cystadenomas (SCAs), pseudocysts, and lymphangiomas); Group 2) Mucinous pre-malignant cysts (PMCs), including IPMNs and MCNs with low grade atypia (LG); Group 3) High-risk/malignant cysts (MCs), including cystic PDACs, IPMNs with adenocarcinoma (ADC) or high grade atypia (HG), MCN-HG, and neuroendocrine cystic tumors (NETs).

### Patients and specimens

The samples studied were predominantly from female patients (35/52, 67%) with a mean age of  $59 \pm 15$  years (29-91); 22 PCLs were in the head, 20 in the body, nine in the tail, and one case of multiple pancreatic locations. The mean cyst size was  $3.9 \pm 2.3$  cm (1-10), CEA level in PCF was  $> 192$  ng/mL in 17/52 (33%), and malignant/atypical cytology was present in 11/52 (21%) PCF samples, as shown in [Table 1](#).

These 52 PCF samples obtained by endoscopic ultrasound with fine-needle aspiration (EUS-FNA) have been previously characterized for *KRAS* and *GNAS* mutations<sup>[12]</sup>, which were present in nine and two samples, respectively.

The final diagnoses, 11 surgical and 41 clinicopathological, encompassed 30 BCs (SCAs, pseudocysts, and lymphangiomas), 14 PMCs (IPMNs and MCNs), and eight MCs (one cystic PDAC, one IPMN-ADC, one NET, and five mucinous-malignant).

### Methylation analysis and categorization

For this study, DNA was extracted from 0.250 mL of archival PCF. Methylation analysis of the *GNAS* locus was performed by methylation specific-multiplex ligation dependent probe amplification (MS-MLPA) (SALSA MS-MLPA ME031-B1, MRC-Holland®, Amsterdam, The Netherlands), according with the manufacturer's instructions. MS-MLPA fragments were analyzed on the Applied Biosystems® 3130 Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, United States) using the GeneMapper® software. Results were normalized and analyzed using Coffalyser.Net software (MRC-Holland®).

We studied methylation in four DMRs, *NESP55*, *GNASAS*, *GNASXL*, and *GNAS1A*, and in the biallelic expressed *Gsa*, including two exonic regions. DMRs were classified as hypermethylated or hypomethylated, according to the percentage of methylation obtained using the Coffalyser.net software recommended by the manufacturer, if methylation percentage was, respectively, above or below the reference values plus or minus twice the standard deviation (SD). The normal methylation of *NESP55* is approximately 50%, as only the paternal allele is methylated, similarly to the

**Table 1 Demographics and clinical characteristics of the study population**

Characteristics	Value
Female gender, <i>n</i> (%) ( <i>n</i> = 52)	35 (67.3)
Mean age at EUS-FNA, <i>y</i> , mean ± SD (interval)	59.1 ± 14.8 (29-91)
Cyst location, <i>n</i> (%) ( <i>n</i> = 52)	
Head	22 (42.3)
Body	20 (38.5)
Tail	9 (17.3)
Multiple cyst locations	1 (1.9)
Cyst size, cm, mean ± SD (interval)	3.9 ± 2.3 (1-10)
Cyst size > 3 cm, <i>n</i> (%)	29 (55.8)
Cyst with nodule/mass, <i>n</i> (%)	18 (34.6)
EUS imaging, <i>n</i> (%) ( <i>n</i> = 52) <sup>1</sup>	
No high risk features	13 (25)
1 high risk feature	29 (55.8)
≥ 2 risk features	10 (19.2)
PCF CEA, <i>n</i> (%) ( <i>n</i> = 52)	
CEA < 192 ng/mL	31 (59.6)
CEA ≥ 192 ng/mL	17 (32.7)
No result available	4 (7.7)
PCF cytology, <i>n</i> (%) ( <i>n</i> = 52)	
Non-diagnostic	27 (51.9)
Negative for malignancy	14 (26.9)
Suspicious/malignant	10 (19.2)
NET	1 (2)
Treatment decision, <i>n</i> (%) ( <i>n</i> = 52)	
Follow up	34 (65.4)
Surgery	11 (21.2)
Endoscopic drainage	1 (1.9)
Palliation (symptomatic or chemotherapy)	6 (11.5)

<sup>1</sup>High-risk features: cyst size ≥ 3 and solid component or thick wall or dilated Wirsung (> 10 mm). CEA: Carcinoembryonic antigen; EUS-FNA: Endoscopic ultrasound with fine needle aspiration; NET: Neuroendocrine tumor; PCF: Pancreatic cyst fluid; SD: standard deviation.

percentage of methylation in *GNASXL*, *GNASAS*, and *GNAS1A*, as only the maternal alleles are expected to be methylated. The methylation of *Gsa* exon 1 is usually absent, as neither maternal nor paternal allele is methylated. Methylation of *Gsa* exonic regions (exons 9 and 13) is usually near 100%, as both maternal and paternal alleles are methylated. The MS-MLPA kit comprised three methylation sensitive probes for *NESP55*, three for *GNASAS*, five for *GNASXL*, two for *GNAS1A*, and four for *Gsa* methylation evaluation.

### Statistical analysis

The methylation levels obtained for each of the individual DMRs and for each individual MS-MLPA probe were calculated and converted into a categorical variable defined as: (1) Hypomethylated if methylation level obtained was below the cut-off level minus twice the SD; (2) Hypermethylated if the methylation level obtained was above the cut-off level plus twice the SD; and (3) Normally methylated if neither criteria (1) or (2) were met. A combined variable, including hypermethylation at

upstream DMRs or intragenic hypomethylation of *GNAS* locus, defining “*GNAS* locus methylation changes” pattern was created. For (epi)genotype-phenotype associations, Fisher’s exact test and chi-square test were performed as well as Kendall’s rank correlation adjusted for age and gender, using partial correlation. Methylation analysis in mucinous and malignant cysts was also represented by boxplot, and Mann-Whitney was used to assess the difference of median methylation values. The diagnostic accuracy of PCF biomarkers was assessed by receiver operating characteristics curve analysis. Statistics were performed using SPSS Statistical software, version 23 (Armonk, NY, United States), with a *P* value < 0.05 considered as statistically significant.

## RESULTS

*GNAS* locus methylation was informative in 38/52 (73%) PCF samples, with the remaining (14/52) non-informative due to inadequate quality/quantity of DNA and rarely, to copy-number variation (probe ratios below 0.7 or above 1.3, regarded as indicative of heterozygous deletion or duplication, respectively, according with the manufacturer (Coffalyser.Net software, MRC-Holland®). Methylation changes at *NESP55*, *GNASAS*, *GNAS1A*, and especially *GNASXL* were more frequent in MCs (Table 2), presenting wider methylation levels of these DMRs compared to non-malignant cysts, which showed methylation levels around 50% in imprinted alleles (Figure 1).

Based on the influence of methylation changes at DMRs in the modulation of *GNAS* transcription<sup>[10,11]</sup> and on the suggested role for hypomethylated exons in transcription regulation and its overlap with predicted enhancers<sup>[13]</sup>, we defined a combined variable documenting “*GNAS* locus methylation changes”: (1) Presence of hypermethylation in at least two DMRs or in one DMR for all MLPA probes; or (2) Presence of intragenic hypomethylation of *GNAS* in at least two exonic regions. Notably, “*GNAS* locus methylation changes” was significantly associated with malignancy (6/8 MCs and only 2/20 BCs) (Table 2), and it is of note that one of these two BCs was later diagnosed as pancreatic cancer.

We further analyzed the correlation between methylation changes and malignancy, while controlling for gender and age. We found a strong significant positive rank correlation between malignancy and *GNAS* methylation changes ( $r = 0.837$ ,  $P < 0.001$ ) and a moderate rank correlation with *GNASAS* hypermethylation ( $r = 0.431$ ,  $P = 0.015$ ), *GNASXL* hypermethylation ( $r = 0.434$ ,  $P = 0.011$ ), and *NESP55* hypermethylation ( $r = 0.539$ ,  $P = 0.003$ ), which was sustained after controlling for gender and age using partial correlation analysis (Table 3).

Moreover, the “*GNAS* locus methylation changes” variable improved MCs classification in samples with clinicopathological diagnosis (possible diagnostic uncertainty) as well as surgical diagnosis (definitive diagnosis but limited number of cases), further supporting our results.

Interestingly, simultaneous hypermethylation in *NESP55* and *GNASXL* DMRs was detected exclusively in 3/3 PDACs. Hypomethylation in two exonic *GNAS* regions (exons 9 and 13) was detected in the only NET in this series.

Additionally, “*GNAS* locus methylation changes” was associated with symptoms, *KRAS*/*GNAS* mutations, and malignant/atypical cytology but not with patient gender, age, or CEA level in PCF (Table 4), with the area under the curve analysis revealing better performance than cytology for diagnosis of MCs (Table 5).

## DISCUSSION

Aberrant DNA methylation in PCF of IPMNs progressing to high-grade dysplasia and carcinoma has been described<sup>[14]</sup>, but *GNAS* locus methylation was not studied therein. We report for the first time methylation changes in the *GNAS* locus, namely hypermethylation of *GNASXL*, *NESP55*, *GNASAS*, and *GNAS1A* in PCLs. Notably, hypermethylation of *GNASXL*, and especially the combined variable “*GNAS* locus methylation changes”, was associated with malignancy, suggesting the potential to be used for diagnosis of MCs and for monitoring cancer progression, if confirmed in larger series. Indeed, hypermethylation of *GNASXL* has been associated to *GNAS* locus gain of function<sup>[10]</sup>, and although its possible association with malignant progression remains poorly understood, *GNAS* oncogenic potential appears to be unquestionable<sup>[3-5,10,15]</sup>. Moreover, somatic DNA methylation has been shown to drive

**Table 2 Frequency of GNAS locus methylation changes in malignant, mucinous, and benign cysts, n (%)**

Informative cyst fluid methylation analysis, 38 samples	Malignant, n = 8	Mucinous pre-malignant, n = 10	Benign, n = 20	P value
<i>NESP55</i> hypermethylation	3 (37.5)	0 (0.0)	1 (5.0)	0.053
<i>GNASAS</i> hypermethylation	3 (37.5)	1 (10.0)	3 (15.0)	0.065
<i>GNASXL</i> hypermethylation	4 (50)	0 (0.0)	2 (10.0)	0.004
<i>GNAS1A</i> hypermethylation	1 (12.5)	0 (0.0)	0 (0)	0.0355
<i>GNAS</i> locus methylation changes	6 (75.0)	0 (0.0)	2 (6.7)	0.000

GNAS locus methylation changes: DMR hypermethylation or *GNAS* intragenic hypomethylation. *GNAS*: Guanine nucleotide-binding protein, alpha stimulating.

**Table 3 Correlation between methylation status and malignancy with partial correlation controlling for patients' gender and age**

Rank correlation	Kendall		Partial	
	Possible confounders		Gender	Age
Malignant cysts	Correlation	P value	Correlation	Correlation
<i>NESP55</i> hypermethylation	0.539	0.003	0.545	0.519
<i>GNASAS</i> hypermethylation	0.431	0.015	0.459	0.584
<i>GNASXL</i> hypermethylation	0.434	0.011	0.461	0.356
<i>GNAS1A</i> hypermethylation	0.160	0.361	0.191	0.147
<i>GNAS</i> locus methylation changes	0.837	<0.001	0.870	0.825

GNAS: Guanine nucleotide-binding protein, alpha stimulating.

**Table 4 Frequencies of distinct clinical features and pancreatic cystic fluid analysis in the two groups, with or without GNAS locus methylation changes**

Cyst fluid samples	GNAS locus methylation changes	No GNAS locus methylation changes	P value
Female	63%	75%	0.486
Age > 65 yr	50%	40%	0.216
Symptoms	63%	17%	0.008
CEA > 192 ng/mL	63%	25%	0.133
<i>KRAS</i> / <i>GNAS</i> mutation	63%	11%	0.008
Cytology, malignant/atypical	63%	7%	0.003

GNAS locus methylation changes: DMR hypermethylation or *GNAS* intragenic hypomethylation. CEA: Carcinoembryonic antigen; *GNAS*: Guanine nucleotide-binding protein, alpha stimulating.

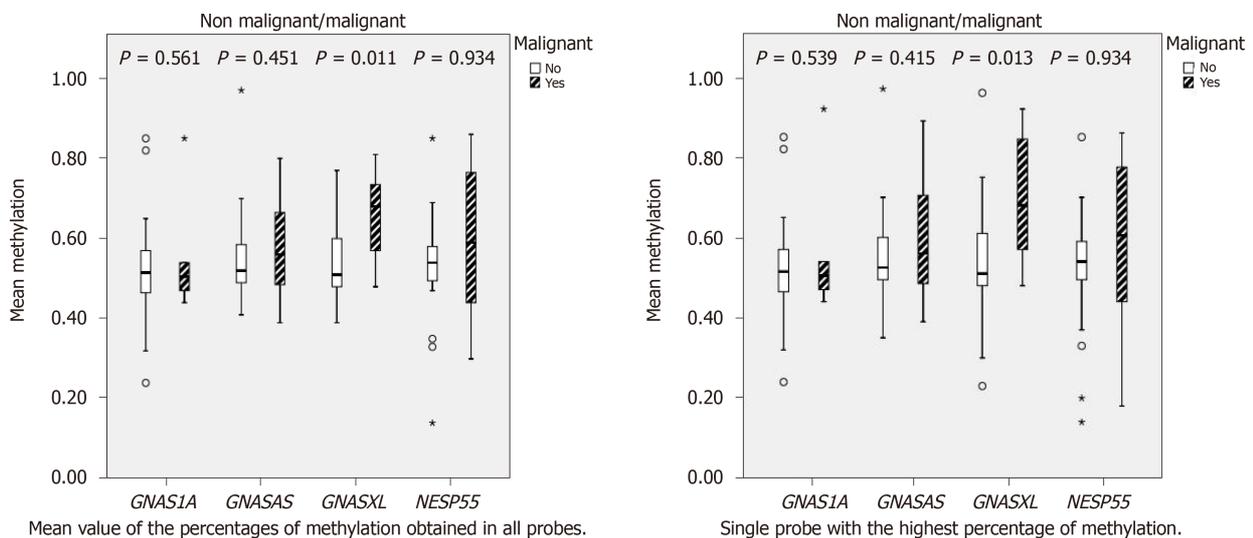
transcription within the imprinted *GNAS* cluster<sup>[11]</sup>, further supporting our results. *NESP55* also appears to regulate imprinting at the *GNAS* complex locus, and its hypermethylation in the maternal allele may lead, similarly to maternal deletion, as previously described, to subsequent modulation of *GNAS*<sup>[10]</sup>.

Herein, hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters, and therefore overall increase of methylation in these two DMRs, was detected exclusively in PDAC, further suggesting a role of *GNAS* in malignant progression of PCL. Interestingly, the detection of exonic *GNAS* hypomethylation in the pancreatic NET is in agreement with the recent findings showing that pancreatic NETs are genetically and phenotypically related to pancreatic ductal adenocarcinoma, having a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors

**Table 5** Area under the curve for diagnosis of mucinous and malignant cysts

Variables	Mucinous cysts				Malignant cysts			
	AUC	P value	Confidence interval		AUC	P value	Confidence interval	
			Lower limit	Upper limit			Lower limit	Upper limit
CEA in mg/dL	0.889	0.002	0.720	1.000	0.812	0.038	0.579	1.000
Cytology	0.598	0.443	0.349	0.847	0.771	0.072	0.571	0.970
Mutation (KRAS/GNAS)	0.833	0.009	0.634	1.000	0.841	0.023	0.615	1.000
Met_NESP55	0.620	0.35	0.370	0.869	0.759	0.085	0.481	1.000
Met_AS	0.590	0.483	0.339	0.841	0.741	0.108	0.461	1.000
Met_XL	0.474	0.841	0.228	0.721	0.629	0.389	0.357	0.902
Met_1A	0.513	0.92	0.262	0.764	0.565	0.667	0.261	0.868
GNAS locus methylation changes	0.645	0.256	0.400	0.891	0.971	0.002	0.901	1.000

AUC: Area under the curve; CEA: Carcinoembryonic antigen; GNAS: Guanine nucleotide-binding protein, alpha stimulating; Met: Methylation changes.



**Figure 1** Methylation analysis of non-malignant and malignant cysts. GNAS: Guanine nucleotide-binding protein, alpha stimulating.

G3<sup>[16]</sup>. In agreement with the role of GNAS in the progression to PDAC is also the recent finding that overexpression of mutant GNAS, resulting in constitutive activation of Gα, in a mouse model of *Kras*<sup>G12D</sup>-driven pancreatic cancer, led to the formation of moderately differentiated PDAC that were locally invasive and increased mitogen-activated protein kinase activation<sup>[17]</sup>.

Although copy-number alterations, which could in part explain some of the methylation changes found, were detected in only one case, we cannot exclude the presence of uniparental disomy (UPD) associated copy-neutral loss of heterozygosity (LOH), as previously described by Bastepe *et al*<sup>[18]</sup> to explain GNAS methylation changes. An analysis of LOH in the GNAS locus would be needed to evaluate uniparental disomy (UPD) associated copy-neutral LOH (which can often be segmental) and investigate if some of these methylation alterations may indeed reflect epigenetic alterations or could instead be explained (at least in part) by acquired UPD. Nevertheless, independent of their cause (epigenetic or acquired UPD), the resulting methylation alterations detected in the GNAS locus DMRs appear to be related to malignant progression and may improve MCs diagnosis. Our study may contribute to the current epigenetic landscape of PCs, similar to recent studies documenting a role for methylation markers in discriminating pancreatic neoplasia<sup>[19,20]</sup>, possibly offering an opportunity for early diagnosis for pancreatic cancer.

Ultimately, the significant association of GNAS locus methylation changes to

malignant behavior suggests a role for modulation of *GNAS* expression in the malignant progression of PCs, which may be relevant for the development of novel therapeutic approaches for pancreatic cancer. Due to small sample size and poor DNA yield, the final analysis was based on eight samples with HGD/cancer. Although the small sample size and lack of validation in an independent sample are significant limits regarding the present study, our pilot data may be the basis for exploring *GNAS* methylation in larger, well-characterized sets of samples that may represent future validation studies. Finally, as gene methylation may affect gene expression, additional evaluation of *GNAS* transcripts in PCF may elucidate their function in PCLs.

## ARTICLE HIGHLIGHTS

### Research background

Pancreatic cystic lesions (PCLs) constitute a clinical dilemma due to indeterminate risk of malignancy. Intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms are cystic precursors of pancreatic ductal adenocarcinoma (PDAC), possibly allowing early diagnosis. Somatic mutations in *GNAS* are characteristic of IPMNs, but their role in carcinogenesis is unclear. *GNAS* is a complex imprinted locus that encodes the  $\alpha$ -subunit of the stimulatory heterotrimeric G protein ( $G\alpha$ ), an ubiquitous signaling protein. This locus encodes four monoallelic (*NESP55*, *AS*, *XL*, *1A*) and one biallelic (*G $\alpha$* ) transcript(s), due to differentially methylated regions (DMRs) in paternal and maternal alleles, denominated imprinting. Paternal methylation of *NESP55* and maternal methylation of *AS*, *XL*, and *1A* lead, respectively, to maternal and paternal allele expressions, with *G $\alpha$*  biallelically expressed in most tissues, due to absent methylation.

### Research motivation

*GNAS* somatic mutations are characteristic of IPMNs, although epigenetic alterations in the *GNAS* locus have not been previously evaluated in PCLs. Methylation of DMRs at the *GNAS* locus may occur at the somatic level and modulate *G $\alpha$*  expression.

### Research objectives

In this study, we evaluate if methylation changes in DMRs at the *GNAS* locus could contribute to tumor progression of PCLs.

### Research methods

We performed a longitudinal cohort study of PCLs with *GNAS* locus methylation analysis performed in PCF samples obtained by endoscopic ultrasound with fine needle aspiration.

### Research results

Fifty-two PCF samples obtained by endoscopic ultrasound with fine needle aspiration and previously characterized for *KRAS* and *GNAS* mutations were studied. The final diagnoses were surgical (11) and clinicopathological (41), including 30 benign cysts, 14 pre-malignant cyst, and eight malignant cysts. Methylation changes at *NESP55*, *GNASAS*, *GNAS1A*, and especially *GNASXL* were more frequent in malignant cysts and were useful for their diagnosis. A combined variable defined as “*GNAS* locus methylation changes” was significantly associated with malignancy (6/8 malignant cysts and only 2/20 benign cysts) and improved classification. Hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters was found in 3/3 PDACs.

### Research conclusions

This is the first study to identify methylation changes in the *GNAS* locus that improved the diagnosis of malignant PCs and suggest a role in progression to PDAC.

### Research perspectives

Although the small sample size and lack of validation in an independent sample are significant limits regarding the present study, our pilot data may be the basis for exploring *GNAS* methylation in larger, well-characterized sets of samples. As methylation status may impact gene expression, additional evaluation of *GNAS* transcripts in PCF may elucidate their function in pancreatic cystic neoplasms.

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