High methylation levels of PCDH10 predict poor prognosis in patients with pancreatic ductal adenocarcinoma

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies and is not a clinically homogeneous disease, but subsets of patients with distinct prognosis and response to therapy can be identified by genome-wide analyses. Mutations in major PDAC driver genes were associated with poor survival. By bioinformatics analysis, we identified protocadherins among the most frequently mutated genes in PDAC suggesting an important role of these genes in the biology of this tumor. Promoter methylation of protocadherins has been suggested as a prognostic marker in different tumors, but in PDAC this epigenetic modification has not been extensively studied. Thus, we evaluated whether promoter methylation of three frequently mutated protocadherins, PCDHAC2, PCDHGCS and PCDH10 could be used as survival predictors in PDAC patients.

Methods: DNA extracted from 23 PDACs and adjacent non-neoplastic pancreatic tissues were bisulfite treated. Combined Bisulfite Restriction Analysis (COBRA) coupled to denaturing high-performance liquid chromatography (dHPLC) detection and bisulfite genomic sequencing (BGS) were used to determine the presence of methylated CpG dinucleotides in the promoter amplicons analyzed.

Results: In an exploratory analysis, two protocadherins showed the same pattern of CpG methylation in PDAC and adjacent non-neoplastic pancreatic tissues: lack of methylation for PCDHAC2, complete methylation for PCDHGCS. Conversely, the third protocadherin analyzed, PCDH10, showed a variable degree of CpG methylation in PDAC and absence of methylation in adjacent non-neoplastic pancreatic tissues. At Kaplan–Meier analysis, high levels of PCDH10 methylation defined according to the receiver operating characteristic (ROC) curve analysis were significantly associated with worse progression-free survival (PFS) rates ($P = 0.008$), but not with overall survival (OS). High levels of PCDH10 methylation were a prognostic factor influencing PFS (HR = 4.0; 95% CI, 1.3–12.3; $P = 0.016$), but not the OS.

Conclusions: In this study, we show for the first time that the methylation status of PCDH10 can predict prognosis in PDAC patients with a significant impact on the outcome in terms of progression-free survival. High levels of PCDH10 promoter methylation could be useful to identify patients at high risk of disease progression, contributing to a more accurate stratification of PDAC patients for personalized clinical management.

Keywords: Pancreatic ductal adenocarcinoma, Epigenetics, Protocadherins, PCDH10, DNA methylation, Survival, Combined bisulfite restriction analysis (COBRA), mRNA expression
Background
Pancreatic ductal adenocarcinomas (PDAC) arise from the exocrine pancreas, account for 95% of pancreatic cancers and, due to the poor survival rate, represent the seventh leading cause of cancer-related deaths worldwide and the third in the United States [1]. PDAC are typically diagnosed at advanced stages when the only available treatments are palliative. The poor clinical outcome of PDAC is attributable to early local spread, the high trend of distant metastasis, and resistance to radio- and chemotherapy [2]. A better understanding of molecular and epigenetic events affecting progression and response to therapy has the potential to improve early diagnosis, prognostic evaluations, and to provide new elements for rational therapeutic approaches.

Some studies analyzed the mutational landscape of PDAC using state of the art genomic sequencing [3–6]. Conversely, the characterization of epigenetic changes occurring in PDAC has not been extensively studied. A comprehensive study analyzed genome-wide promoter methylation in pancreatic cell lines with the aim to improve the diagnosis of PDAC and to identify key regulatory genes and pathways that merit therapeutic targeting [7]. A subset of CpG island showing aberrant methylation in cell lines was also investigated in PDAC tumor specimens, but the levels of methylation often differed from that observed in cell lines [7]. Considering the importance of epigenetic changes in malignant transformation, further characterization of these alterations in PDAC tumor specimens is needed.

Genes that are frequently mutated in PDAC are likely to play an essential role in the biology of this tumor, and they might also be a target of epigenetic dysregulation. Therefore, studying epigenetic changes in these genes may provide complementary evidence of their role in PDAC malignant transformation.

Protocadherins were included in the homophilic cell adhesion gene set that was shown to be subject to frequent alterations in an early study on transcriptome sequencing pancreatic cancers [8], but this observation was not highlighted in subsequent genome-wide studies [3–6]. These genes are among those showing aberrant methylation in pancreatic cancer cell lines [7], suggesting their relevant role in PDAC carcinogenesis. Protocadherins represent a major subfamily of the cadherin superfamily [9, 10] and more than seventy coding genes for protocadherins have been identified. Based on their organization, their protein products can be divided into two large groups: “clustered” and “non clustered” protocadherins [11]. The clustered protocadherins constitute the largest group. Unlike the clustered, the non clustered protocadherins are so named because their genes are not located in a single gene locus, but in three different chromosomal loci. They contain six extracellular cadherin domains, a transmembrane domain and a cytoplasmic tail differing from that of the classical cadherins [10]. Protocadherins exhibit cell-to-cell adhesion activities, but distinct from that of classical cadherins, and are believed to possess other important functions such as signal transduction and growth control, although the exact mechanisms of action have not been fully elucidated. Different studies indicated a potential role as tumor suppressors for some of them [12]. The onset and the malignant progression of different cancers are often associated with the lack of expression of protocadherins caused by an epigenetic silencing event that involves hypermethylation of specific chromosomal regions [13]. Promoter methylation of protocadherins has been suggested as a prognostic marker in different tumors, including prostate, gastric, colorectal, bladder and clear cell renal cell carcinoma [13], but in PDAC this epigenetic modification has not been extensively studied. In particular, only PCDH10 had been previously studied in PDAC primary tumors, but that study failed to find any correlation between PCDH10 methylation status and tumor staging [14].

Considering that protocadherins are frequently mutated in PDAC [8] and could play a crucial role in the biology of this tumor, but little is known about their epigenetic modifications, we analyzed promoter methylation of three protocadherins. In particular we analyzed promoter CpG methylation of PCDH10, PCDHAC2 and PCDHGC5 that in our query of The Cancer Genome Atlas database resulted among the most frequently mutated in PDAC. Notably, PCDH10 promoter methylation had been previously suggested as a prognostic marker in prostate, gastric and colorectal cancer [13]. In our study, PCDH10 methylation was identified as a factor associated with PDAC progression-free survival and, consequently, we suggest its possible role as a prognostic marker that might be useful for personalized treatment.

Methods
Patients samples
Samples from surgically resected primary PDAC were collected from a series of 23 patients recruited at the Department of Surgery of “Casa Sollievo della Sofferenza” Hospital, IRCCS San Giovanni Rotondo. Only patients with histologically proven primary PDAC were enrolled in the study. Exclusion criteria for patients were a previous diagnosis for PDAC and neoadjuvant treatment before surgery. Tumors were staged in accordance with the TNM classification [15]. Clinical features and tumor characteristics were reported in Table 1. Patients gave informed written consent and approval from the ethical committee of the “Casa
Sollievo della Sofferenza” IRCCS, San Giovanni Rotondo was obtained. In DNA methylation analyses Capan-2 human pancreatic cancer cell line was used as a control fully methylated for PCDH10 [7]. For PCDH10 mRNA expression analysis we used PCDH10 fully methylated pancreatic (Capan-2, AsPC-1) and gastric (AGS) cancer cell lines, as well as PCDH10 unmethylated breast cancer cell line (MB-231) [16, 17].

Promoter methylation analysis

DNA extraction and bisulfite modification of DNA

Resected PDACs and adjacent non-neoplastic tissues from the same patients were taken separately, immediately frozen in liquid nitrogen and stored at −80 °C until the nucleic acid extraction. These control tissues were verified as tumor-free by a pathologist.

Genomic DNA was isolated using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA concentration and purity were controlled by NanoDrop Spectrophotometer (Thermo Fisher, Waltham, MA, USA).

Bisulfite treatment was performed according to the manufacturer’s protocol (EpiTect Bisulfite Kit, Qiagen). The bisulfite-treated DNA was amplified with primers designed according to MethPrimer [18]. Primer sequences and PCR conditions are available in Table 2.

Combined bisulfite restriction analysis (COBRA)

COBRA is a technique to semiquantitate the methylated and unmethylated DNA after sodium bisulfite treatment by using restriction enzyme cutting sites. PCR products containing CpG dinucleotides and at least 1 BstUI restriction site were digested with BstUI (New England BioLabs) that recognizes the sequence 5′-CGCG-3′, retained in the bisulfite-treated methylated DNA, but not in the unmethylated DNA. The DNA digests were separated by denaturing high-performance liquid chromatography (dHPLC) (Wave 1100, Transgenomic, Omaha, NE).

In case of methylated CpG dinucleotides, after enzymatic digestion, the 235 bp PCDHAC2 PCR product, encompassing the promoter region −43 to +192 bp from the transcription start site, provides two fragments of 210 and 25 bp, respectively; the 290 bp PCDHGCS PCR product, encompassing the promoter region −3287 to −2997 bp upstream from the transcription start site, provides two fragments of 200 and 90 bp, respectively; the 196 bp PCDH10 PCR product, encompassing the promoter region −1204 to −418 bp upstream from the transcription start site, has two cutting sites for BstUI and provides three fragments of 113, 52 and 31 base pairs, respectively. For PCDH10, the presence of two cutting sites for BstUI restriction enzyme hampered the interpretation of the analysis in case of partial methylation of the analyzed CpG islands. For this reason, we used BGS for this gene in all cases analyzed. Also for PCDHAC2 and PCDHGCS DNA from a representative tumor and non-neoplastic sample were subjected to bisulfite genomic sequencing (BGS) to verify COBRA results independently.

| Variable                        | Value (%) |
|---------------------------------|-----------|
| Age at diagnosis (yr)           |           |
| Median                          | 67.0      |
| Range                           | 38–78     |
| Gender                          |           |
| Male                            | 11 (47.8) |
| Female                          | 12 (52.2) |
| Tumor location                  |           |
| Head                            | 21 (91.3) |
| Body                            | 1 (4.3)   |
| Tail                            | 1 (4.3)   |
| Tumor stage                     |           |
| I                               | 0 (0.0)   |
| II                              | 6 (28.6)  |
| III                             | 15 (71.4) |
| IV                              | 0 (0.0)   |
| LN metastasis                   |           |
| No                              | 6 (26.1)  |
| Yes                             | 17 (73.9) |
| PCDH10 methylation status       |           |
| Low                             | 16 (69.6) |
| High                            | 7 (30.4)  |
| Tumor progression               |           |
| No                              | 8 (34.8)  |
| Yes                             | 15 (65.2) |
| Occurrence of death             |           |
| No                              | 5 (21.7)  |
| Yes                             | 18 (78.3) |

| Table 2 Sequences of primers employed for PCR amplification of bisulfite-treated DNA |
|---------------------------------|--------|--------|----------------|--------|
| gene               | Amplicon (bp) | CpG n. | Sequence 5′- 3′ |
| PCDHAC2           | 235     | 11     | f.aggggttgatgtgattttttaagat r.accaacaatgcatcctaactc |
| PCDHGCS           | 290     | 14     | f.ggttagttgtttttaagat r.ccaactatcaatttaaat |
| PCDH10            | 196     | 16     | f. gtgtggattggtggtgattaagat r.cccacctatctaataat |

Curia et al. BMC Cancer (2019) 19:452 Page 3 of 11
BGS
We directly sequenced the PCR products generated from bisulfite-treated templates with the same primers used for amplification (Table 2).

Sequencing analysis was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Methylation status was expressed as the percentage of CpG methylated over the total number of CpG included in the sequence analyzed. In some cases, sequence analysis of bisulphite-treated DNA showed the simultaneous presence of both peaks (T and C), but in these cases there was always a major peak accounting for at least 70% of the total signal. This major peak was considered to call the island as methylated (C major peak) or unmethylated (T major peak) in subsequent analyses. For PCDH10, in cases showing CpG dinucleotides with the simultaneous presence of both peaks (T and C), we also analyzed data taking into account the relative height of the two peaks. The inclusion of this information in the analyses introduced marginal variations (2–3%) in the percentage of methylation status, and the subsequent analyses of the association between methylation and prognosis yielded virtually identical results.

Analysis of PCDH10 expression by RT-PCR in cancer cell lines
Total RNA was extracted from Capan-2, AsPC-1, AGS, MB-231 cancer cell lines using Trizol reagent (Invitrogen Corp., Carlsbad, California, USA). Complementary DNA (cDNA) was synthesised as previously described [19] and amplified for PCDH10 gene with previously published primers [20]. PCDH10 cDNA RT-PCR amplified fragments were separated by dHPLC.

Statistical analysis
A cut-off of 52% was chosen to dichotomize PCDH10 methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characteristic (ROC) curve analysis. Consequently, the methylation levels according to the receiver operating characteristic (ROC) curve analysis. Consequently, the tumor was identified as characterist

Results

Querying public database for genes most frequently mutated in PDAC
To select genes that may play a key role in PDAC, we analyzed PDAC data in The Cancer Genome Atlas (TCGA) provisional database (accessed January 17, 2014) to identify functionally related gene groups frequently mutated in this tumor. Protocadherins were among the most frequently mutated genes in PDAC samples analyzed by TCGA (Table 3). We then used DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov) to identify enriched biological themes and function-related gene groups among the top 43 genes with > 10 mutations in PDAC according to TCGA. This analysis indicated that “Cadherins” including PCDH10, PCDHGCS, PCDH15, PCDHAC2, CDH10 were among functional-related gene groups statistically enriched after Bonferroni (P = 0.014) and Benjamini (P = 0.004) corrections (Table 4). Notably, the enrichment of the term “Cadherins” was confirmed in a more recent analysis (April 18, 2018) in which we included the top 424 genes with > 10 mutations in PDAC, merging mutational data from different databases, including TCGA, International Cancer Genome Consortium (ICGC), Queensland Centre for Medical Genomics (QCMG), UTSouthwestern Medical Center (UTSW) (UP_SEQ_FEATURE domain: Cadherin 5; Bonferroni P = 1.8 $10^{-8}$; Benjamini P = 8.1 $10^{-10}$). Based on the above results, in this study we analyzed CpG methylation for three genes that appeared to be frequently mutated in PDAC, including PCDHGCS and PCDHAC2 that had not been studied before for epigenetic modifications and PCDH10, whose promoter methylation had been previously suggested as a prognostic marker in other cancers [13].

Methylation analysis of PCDHAC2, PCDHGCS and PCDH10
An exploratory study of methylation analysis on PCDHAC2, PCDHGCS and PCDH10 was carried out in 11 pancreatic adenocarcinomas.

For PCDHAC2, dHPLC analysis of COBRA showed in all cases only the presence of the full-length fragment in tumors, indicating that the fragment was not cut because the C in the cutting site was unmethylated and thus converted to T by bisulfite treatment abolishing the cutting site (Fig. 1a, left panel). The same pattern indicating the absence of CpG methylation was observed in the non-neoplastic pancreatic tissues analyzed. BGS showed the presence of a minor C peak, indicating modest methylation, in three of nine CpG dinucleotides sequenced both in tumor and non-neoplastic tissue (Fig. 1a, right panel). These results confirmed the monomorphic pattern of methylation in tumor and non-neoplastic tissues and the lack of relevant CpG methylation indicated by COBRA. As
far as PCDHG5, analysis by COBRA indicated that CpG dinucleotides were methylated in the amplicon analyzed both in tumor (Fig. 1b, left panel) and non-neoplastic tissues. BGS agreed with this finding (Fig. 1b, right panel). For PCDH10, in the exploratory BGS analysis, the pattern of methylation in tumors among cases was different, with six cases showing lack of methylation and five cases showing >50% methylation of CpG dinucleotides, whereas non-neoplastic pancreatic tissues resulted unmethylated. Overall, in the exploratory analysis, both for PCDHAC2 and PCDHG5 all cases had similar patterns of methylation (Table 5) providing an indicator that could be related to clinicopathological features. Conversely, for PCDH10 the 11 cases analyzed in the exploratory study had different patterns of methylation, providing an indicator that could be related to clinicopathological features. Therefore, we extended BGS analysis of PCDH10 to the whole series of 23 pancreatic adenocarcinomas available (Table 5). This extended analysis revealed that tumors derived from nine cases resulted not methylated and 14 methylated, with a percentage of methylation ranging from 8 to 91%, with a mean ± SE of 55.0 ± 7.8 (Fig. 1c, left panel, Tables 5 and 6). In all non-neoplastic pancreatic tissues analyzed PCDH10 resulted unmethylated. Sequencing of the human pancreatic carcinoma cell line Capan-2 showed complete methylation of PCDH10 CpG dinucleotides, as expected for this control cell line (Fig. 1c, right panel).

Since RNA samples from tissues of the patients analyzed were not available, we analyzed cDNA from pancreatic (Capan-2, AsPC-1) and gastric (AGS) cancer cell lines fully methylated for PCDH10, as well from a breast cancer cell line (MB-231) unmethylated for PCDH10, to assess whether methylation status of PCDH10 CpG dinucleotides was associated with effects on the expression of the corresponding transcript. In line with methylation status, Capan-2, AsPC-1 and AGS cell lines fully methylated for PCDH10 did not express the corresponding mRNA, whereas the cell line MB-231 unmethylated for PCDH10 expressed the corresponding transcript (Fig. 2).

**Table 3** Top genes with > 10 mutations in PDAC tumors from TCGA (provisional, accessed January 17, 2014)

| Gene   | Cytoband | Gene size (Nucleotides) | n. Mutations |
|--------|----------|-------------------------|--------------|
| TP53   | 17p13.1  | 3924                    | 37           |
| PCDHG5 | 5q31     | 4641                    | 36           |
| KRAS   | 12p12.1  | 7302                    | 33           |
| ZFHX3  | 16q22.3  | 17,503                  | 26           |
| PCDHAC2| 5q31     | 5970                    | 26           |
| TCF20  | 22q13.3  | 7548                    | 24           |
| CHD3   | 17p13.1  | 9758                    | 16           |
| PCDH15 | 10q21.1  | 14,967                  | 16           |
| GIGYF1 | 7q22     | 6709                    | 15           |
| PCDH10 | 4q28.3   | 5516                    | 15           |
| ANK3   | 10q21    | 22,521                  | 14           |
| MED15  | 22q11.2  | 10,374                  | 14           |
| GZF1   | 20p11.21 | 5495                    | 14           |
| KIAA007| 1q22     | 5635                    | 14           |
| TTBK2  | 15q15.2  | 11,642                  | 13           |
| SUPT6H | 17q11.2  | 10,615                  | 13           |
| GRM1   | 6q24     | 7272                    | 13           |
| CDKN2A | 9p21     | 4400                    | 13           |
| RANGAP1| 22q13    | 5463                    | 13           |
| CHD4   | 12p13    | 7474                    | 12           |
| ZFCH51 | 12q21.1  | 9670                    | 12           |
| TAO2   | 16p11.2  | 9058                    | 12           |
| SIPA1L1| 1q42.4   | 12,412                  | 12           |
| HOXA1  | 7p15.3   | 2539                    | 12           |
| PAS2D1 | Xq28     | 4429                    | 12           |
| NOS1AP | 1q23.3   | 8449                    | 12           |
| ZMIZ1  | 10q22.3  | 10,735                  | 12           |
| TMCC1  | 3q22.1   | 7912                    | 12           |
| MAML2  | Xq28     | 5958                    | 12           |
| SMARC2 | 12q13.2  | 8555                    | 11           |
| CDH10  | 5p14.2   | 3660                    | 11           |
| FTSJ3  | 17q23.3  | 5213                    | 11           |
| MUC4   | 3q29     | 21,325                  | 11           |
| MED12L | 3q25.1   | 12,619                  | 11           |
| TCHH   | 1q21.3   | 6900                    | 11           |
| MAGEC1 | Xq26     | 4270                    | 11           |
| NAV2   | 11p15.1  | 14,577                  | 11           |
| RSPH6A | 19q13.3  | 2547                    | 11           |
| FUZ    | 19q13.33 | 2951                    | 11           |
| SF3A1  | 22q12.2  | 6327                    | 11           |
| CDC27  | 17q21.32 | 6823                    | 11           |
| FOXN3  | 14q31.3  | 11,033                  | 11           |
| CXXC1  | 18q12    | 4319                    | 11           |

**Hypermethylation of PCDH10 correlates with poor prognosis in PDAC patients**

Fourteen out of 23 (60.9%) tumors showed PCDH10 methylation. In these cases, the percentages of methylation ranged from 8 to 91%, with a mean ± SE of 55.0 ± 7.8. The box-and-whisker diagram shows the PCDH10 methylation levels registered among 23 PDAC cases (Fig. 3).

By ROC curve analysis, cases were dichotomized according to PCDH10 methylation status: tumors with...
methylation levels above 52% (n = 7) were considered \( PCDH10^{High} \), and those with methylation levels below the cut-off value were considered \( PCDH10^{Low} \) (n = 16). By chi-square test, \( PCDH10 \) methylation status was found inversely correlated with the clinical presentation of jaundice (\( P = 0.036 \)) (Table 7).

A disease progression was observed in 85.7% (6/7) of patients with \( PCDH10^{High} \) and 56.3% (9/16) of those with \( PCDH10^{Low} \) tumors. Death rates were 85.7 and 75.0% for patients with high and low methylation of \( PCDH10 \), respectively. At Kaplan–Meier analysis, \( PCDH10^{High} \) was significantly associated with worse PFS rates (\( P = 0.008 \)), but not with OS (Fig. 4).

**Discussion**

PDAC is one of the worst malignant tumors, which commonly has an unfavourable prognosis. Currently, the most important clinical prognostic indicators of disease outcome are the PDAC staging based on the size and

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**Table 4** Most significant enriched biological themes and functional-related gene groups identified by DAVID in PDAC tumors from TCGA

| Category          | Term                                      | Count | Genes                                                                 | PValue | Bonferroni | Benjamini |
|-------------------|-------------------------------------------|-------|----------------------------------------------------------------------|--------|------------|-----------|
| UP_SEQ_FEATURE    | compositionally biased region:Poly-Pro    | 11    | TCF20, MED15, ANK3, NAV2, MAML1, PCDH195, SFA31, ZFHX3, CHD4, GIGYF1, CHD3 | 1.20E-08 | 3.53E-06   | 3.53E-06  |
| UP_SEQ_FEATURE    | compositionally biased region:Poly-Clin   | 8     | TCF20, NOS1AP, MED15, SMARCC2, MAML1, SFA31, ZFHX3, GIGYF1             | 3.22E-08 | 9.48E-06   | 4.74E-06  |
| UP_KEYWORDS       | Coiled coil                               | 21    | TCHH, TAO2K, NOS1AP, PCDH15, RANGAP1, TMCC1, CXXC1, ZFC3H1, HOXA1, TCF20, KRAS, CXXN2A, TBBK2, ANK3, PAS01, CHD4, CHD3, ZFHX3, CHD4, GIGYF1, FTS13, CHD3, SUPT6H | 4.29E-07 | 4.85E-05   | 4.85E-05  |
| UP_KEYWORDS       | Alternative splicing                      | 37    | PCDHG5C, MAGEC1, PCDHAC2, TMCC1, Fuz, MED12L, CXXC1, ZFCH3H1, HOXA1, TCF20, KRAS, CXXN2A, TBBK2, ANK3, PAS01, CHD4, CHD3, ZFHX3, CHD4, GIGYF1, FTS13, CHD3, SUPT6H | 3.27E-06 | 3.69E-04   | 1.85E-04  |
| UP_SEQ_FEATURE    | compositionally biased region:Ser-rich    | 9     | ZFC3H1, TCF20, TBBK2, TAO2K, ANK3, NAV2, SFA31, GRM1, MUC4            | 3.13E-06 | 9.21E-04   | 3.07E-04  |
| UP_KEYWORDS       | Transcription regulation                  | 16    | TPS3, FOXN3, GZF1, MED12L, CXXC1, HOXA1, TCF20, CXXN2A, MED15, ZMIZ1, SMARCC2, MAML1, ZFHX3, CHD4, CHD3, SUPT6H | 3.09E-05 | 0.003488449 | 0.001164171 |
| UP_KEYWORDS       | Phosphoprotein                            | 31    | RANGAP1, MAGEC1, TMCC1, MED12L, CXXC1, ZFCH3H1, TCF20, DCXN2A, TBBK2, ANK3, CHD4, GIGYF1, FTS13, CHD3, ZFHX3, CHD4, CHD3, SUPT6H | 3.77E-05 | 0.004251235 | 0.001064507 |
| UP_KEYWORDS       | Transcription                             | 16    | TPS3, FOXN3, GZF1, MED12L, CXXC1, HOXA1, TCF20, CXXN2A, MED15, ZMIZ1, SMARCC2, MAML1, ZFHX3, CHD4, CHD3, SUPT6H | 4.30E-05 | 0.004850515 | 9.72E-04  |
| UP_KEYWORDS       | Nucleus                                   | 24    | TAO2K, TPS3, RANGAP1, CDC27, SFA31, FOXN3, GZF1, CXXC1, MED12L, HOXA1, TCF20, DCXN2A, TBBK2, MED15, NAV2, ZMIZ1, SMARCC2, PAS01, MAML1, ZFHX3, CHD4, FTS13, CHD3, SUPT6H | 4.90E-05 | 0.005518068 | 9.22E-04  |
| UP_KEYWORDS       | Isopeptide bond                           | 11    | TCF20, ANK3, ZMIZ1, SMARCC2, TPS3, RANGAP1, SFA31, ZFHX3, CHD4, CHD3, CXXC1 | 7.13E-05 | 0.008026619 | 0.001150624 |
| UP_KEYWORDS       | UbI conjugation                           | 13    | TCF20, CXXN2A, MED15, ZMIZ1, SFA31, SMARCC2, TPS3, RANGAP1, SFA31, ZFHX3, CHD4, CHD3, ZFHX3 | 1.06E-04 | 0.011912431 | 0.001496873 |
| SMART             | SM00112:CA cadherin repeats               | 5     | PCDH10, PCDHG5C, PCDH15, PCDHAC2, CHD10 | 3.65E-04 | 0.014140837 | 0.014140837 |
| INTERPRO          | IPR020894:Cadherin conserved site         | 5     | PCDH10, PCDHG5C, PCDH15, PCDHAC2, CHD10 | 1.22E-04 | 0.016216219 | 0.016216219 |
| INTERPRO          | IPR002126:Cadherin domain:Cadherin 5     | 5     | PCDH10, PCDHG5C, PCDH15, PCDHAC2, CHD10 | 1.44E-04 | 0.019141272 | 0.009616878 |
| UP_SEQ_FEATURE    |                                          | 5     | PCDH10, PCDHG5C, PCDH15, PCDHAC2, CHD10 | 6.80E-05 | 0.019802839 | 0.004978905 |

List of most significant enriched biological themes and functional-related gene groups identified by DAVID (http://david.abcc.ncifcrf.gov/) among the top 43 mutated genes with > 10 mutations in 66 PDAC tumors from The Cancer Genome Atlas database (TCGA, provisional, accessed January 17, 2014). The top terms included “SM00112:CA cadherin repeats (SMART),” “IPR020894:Cadherin conserved site” (INTERPRO), “IPR002126:Cadherin” (INTERPRO), “domain:Cadherin 5” (UP_SEQ_FEATURE). The table is ordered according to Bonferroni correction and all included terms were statistically significant after Bonferroni and Benjamini corrections. Protocadherin genes recurring in the table are in bold.
extent of the primary tumor and presence and extent of metastasis. Beyond the parameters used in the stage grouping (i.e., TNM classification), no additional prognostic factors are recommended for clinical care of PDAC patients. Thus, additional prognostic biomarkers are needed to provide a better risk assessment.

Recent studies showed that PDAC is not a clinically homogeneous disease, but molecularly defined subsets of patients with distinct clinical features, including prognosis and response to therapy, can be identified by integrated genome-wide analyses [4–6]. Among the four major PDAC driver genes (KRAS, CDKN2A, TP53,
SMAD4), mutations in SMAD4 were associated with poor survival, whereas mutations in KRAS, CDKN2A and TP53, or the presence of multiple (> 4) mutations or homozygous deletions among the most frequently mutated genes were not associated with survival [21].

In addition to mutations, epigenetic modifications may play an important role in PDAC as suggested by the observation that aberrant CpG island methylation of repromo, a gene involved in p53-induced G2 cell cycle arrest, was shown to associate with worse prognosis [22]. However, the characterization of epigenetic changes occurring in PDAC has not been extensively studied, and the only genome-wide study of promoter methylation in PDAC analyzed primarily cell lines [7].

Since genes that are frequently mutated in PDAC may be crucial for the biology of this tumor, and they might also be a target of epigenetic dysregulation, we searched for genes frequently mutated in PDAC by querying The Cancer Genome Atlas (TCGA) provisional database. The bioinformatics analysis identified protocadherins among the most mutated genes in PDAC.

| Table 5 | PCDHAC2, PCDHGCS and PCDH10 methylation status according to clinicopathological features of patients (n = 23) |
| --- | --- |
| Sample ID | Age range (yr) | Tumor stage | Methylation status (%) | PCDHAC2 | PCDHGCS | PCDH10 |
| PKCH20212 T | 65–70 | II | 0 | 100 | 0 |
| PKCH20212 T | 55–60 | II | 0 | 100 | 0 |
| PKCH20212 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH21913 T | 60–65 | III | 0 | 100 | 0 |
| PKCH5309 T | 50–55 | II | 0 | | |
| PKCH20212 T | 55–60 | III | 0 | | |
| PKCH14511 T | 75–80 | III | 0 | 100 | 50 |
| PKCH20712 T | 70–75 | II | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH21913 T | 60–65 | III | 0 | | |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH21913 T | 60–65 | III | 0 | | |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH21913 T | 60–65 | III | 0 | | |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH21913 T | 60–65 | III | 0 | | |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |
| PKCH14511 T | 70–75 | III | 0 | 100 | 50 |
| PKCH3408 T | 75–80 | III | 0 | 100 | 55 |

| Table 6 | PCDH10 methylation status according to clinicopathological features of patients (n = 23) |
| --- | --- |
| Variable | Low: | High: | P* |
| n (%) | n (%) |
| Gender | Male | 7 (43.8) | 4 (57.1) | 0.554 |
| Female | 9 (56.3) | 3 (42.9) | |
| Jaundice | No | 4 (25.0) | 5 (71.4) | 0.036* |
| Yes | 12 (75.0) | 2 (28.6) | |
| Vascular invasion | No | 15 (93.8) | 7 (100.0) | 0.499 |
| Yes | 1 (6.3) | 0 (0.0) | |
| Neural invasion | No | 13 (81.3) | 5 (71.4) | 0.621 |
| Yes | 3 (18.8) | 2 (28.6) | |
| LN metastasis | No | 4 (25.0) | 2 (28.6) | 1.000 |
| Yes | 12 (75.0) | 5 (71.4) | |
| Stage | II | 4 (28.6) | 2 (28.6) | 1.000 |
| III | 10 (71.4) | 5 (71.4) | |

*Pearson’s χ2 test
*Statistically significant

Therefore, we evaluated whether the epigenetic differences in terms of promoter methylation of protocadherins between the tumor and non-tumor tissue samples could be used as survival predictors in PDAC patients. In particular, we studied the promoter methylation of PCDHAC2, PCDHGCS and PCDH10 because they emerged among the most mutated genes in PDAC through the aforementioned unbiased in silico approach. Notably, the methylation status of PCDHAC2 and PCDHGCS were never analyzed before in PDAC, while PCDH10 had been previously studied in PDAC cancer cell lines [7] and one study analyzed this gene in PDAC primary tumors [14].

In our study PCDHAC2 resulted hypomethylated, whereas PCDHGCS was hypermethylated in all PDAC samples and the same patterns of methylation were also observed in matched adjacent non-neoplastic pancreatic tissues, suggesting that CpG promoter methylation of these genes does not play a major role in the biology of this tumor. Conversely, PCDH10, that resulted unmethylated in adjacent non-neoplastic pancreatic tissues showed a variable degree of methylation ranging from high to low levels in matched PDAC samples. As expected, PCDH10 methylation status correlated with the lack of expression of the corresponding transcript in
PCDH10 fully methylated cancer cell lines and, conversely, with expression of PCDH10 in the unmethylated cell line analyzed. In line with our findings, a previous study [14] found a significant correlation between PCDH10 methylation and loss of PCDH10 mRNA expression in pancreatic, gastric and colorectal cancers tissues.

The variability of PCDH10 methylation among patients led us to investigate the possible correlations between CpG dinucleotide methylation in this gene and PDAC clinical outcome. In this analysis we found, for the first time, an association between PCDH10 promoter

**Table 7** Risk of progression and death associated with the PCDH10 methylation status

| Outcome | PCDH10 methylation status | HR* | 95% CI  | P     |
|---------|---------------------------|-----|---------|-------|
| PFS     | 4.0                       | 1.3–12.3 | 0.016  |
| OS      | 1.8                       | 0.6–4.9   | 0.263  |

*Hazard Ratio of high versus low levels of PCDH10 methylation.
methylation status and PDAC patients outcomes, being the hypermethylation of the gene associated with shorter progression-free survival.

Deaths occurred at high rates in both cohorts of PDAC patients and the percentage tended to be higher among PDAC patients with \( PCDH10^{\text{high}} \) rather than \( PCDH10^{\text{low}} \) tumors (86% versus 75%, respectively). However, possibly because of the high rates of death, the relatively small differences among cohorts and the limited number of patients analyzed, we did not find any correlation between \( PCDH10 \) status and overall survival.

\( PCDH10 \) was already reported to be inactivated by promoter methylation in various types of cancer, including non-small cell lung cancer [23], gastric cancer [24], colorectal cancer [25], nasopharyngeal, esophageal [17], endometrioid endometrial carcinoma [26, 27], bladder cancer [28], cervical cancer [29], suggesting that it plays an oncosuppressor role in those tumors. In support of a role for \( PCDH10 \) as an oncosuppressor gene, re-expression of this gene by transfection in a gastric cancer cell line inhibited the proliferation, migration, invasion ability, as well as its tumor growth in mice [16]. Further evidence that this gene plays an oncosuppressor role derives from the observation that methylation of \( PCDH10 \) was associated with poor prognosis in patients with gastric cancer [16]. In line with this evidence, the genetic deletion of \( PCDH10 \) represents an adverse prognostic marker for the survival of patients with CRC [30]. In pancreatic tumors, however, the potential role of \( PCDH10 \) as oncosuppressor gene in PDAC was investigated only in pancreatic cancer cell lines where this gene was silenced by methylation and its re-expression by transfection inhibited the proliferation, migration, invasion ability and induced apoptosis [31]. The only study which analyzed \( PCDH10 \) methylation in pancreatic tumor samples failed to find any correlation between \( PCDH10 \) methylation status and PDAC staging, which was the pathologic feature analyzed in that study [14]. Also in our study there was no correlation between methylation and tumor staging, but we found that this epigenetic modification was correlated with PFS, which had not been previously analyzed.

**Conclusions**

Promoter methylation has been reported as a promising predictive biomarker in many human cancers. However, a better understanding of the specific epigenetic changes affecting the prognosis of PDAC is necessary.

In our study, we identified for the first time that methylation status of \( PCDH10 \) can predict the patients’ prognosis and may have a significant impact on the outcome in terms of progression-free survival of the patients with PDAC. In particular, high levels of \( PCDH10 \) promoter methylation could be useful to identify patients at high risk of disease progression and early death after surgical treatment, contributing to a more accurate stratification of PDAC patients for personalized clinical management.

**Abbreviations**

- BGS: Bisulfite genomic sequencing
- COBRA: Combined Bisulfite Restriction Analysis
- dHPLC: denaturing high-performance liquid chromatography
- OS: Overall survival
- PDAC: Pancreatic ductal adenocarcinoma
- PFS: Progression-free survival
- ROC: Receiver operating characteristic

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**Availability of data and materials**

The datasets analyzed in the current study are available from the corresponding author upon request.

**Database Linking**

DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov).
The Cancer Genome Atlas (TCGA) provisional database (https://cancer.gov).
International Cancer Genome Consortium (ICGC) database (https://icgc.org).
Queensland Centre for Medical Genomics (QCMG) database (https://qcmg.org).
UTSouthwestern Medical Center (UTSW) database (https://utswmed.org).

Author's contributions
MCC conceived the study, performed methylation analysis and drafted the manuscript. FP performed methylation and bioinformatics analyses and revised the manuscript. RL performed statistical analysis and revised the manuscript. MF and PS interpreted the data and revised the manuscript. AC conceived the study, performed bioinformatics analysis and drafted the manuscript. All authors ensured the integrity of the work, read and approved the final manuscript.

Ethics approval and consent to participate
Patients gave informed written consent and approval from the ethical committee of the "Casa Sollievo della Sofferenza" IRCCS, San Giovanni Rotondo was obtained.

Consent for publications
Not applicable

Competing interests
The authors declare that they have no competing interests.

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