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Long-range epigenetic silencing of chromosome 5q31 protocadherins is involved in early and late stages of colorectal tumorigenesis through modulation of oncogenic pathways

A R Dallosso[1,5], **B Øster**[2,5], **A Greenhough**[3], **K Thorsen**[2], **T J Curry**[1], **C Owen**[1], **A L Hancock**[1], **M Szemes**[1], **C Paraskeva**[3], **M Frank**[4], **C L Andersen**[2], and **K Malik**[1]

[1]Cancer Epigenetics Laboratory, School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK

[2]Department of Molecular Medicine, Aarhus University Hospital, Skejby, Denmark

[3]Colorectal Cancer Laboratory, School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK

[4]Developmental Biology, Institute of Biology 1, University of Freiburg, Freiburg, Germany

Abstract

Loss of tumour suppressor gene function can occur as a result of epigenetic silencing of large chromosomal regions, referred to as long-range epigenetic silencing (LRES), and genome-wide analyses have revealed that LRES is present in many cancer types. Here we utilize Illumina Beadchip methylation array analysis to identify LRES across 800 kb of chromosome 5q31 in colorectal adenomas and carcinomas (n=34) relative to normal colonic epithelial DNA (n=6). This region encompasses 53 individual protocadherin (PCDH) genes divided among three gene clusters. Hypermethylation within these gene clusters is asynchronous; while most PCDH hypermethylation occurs early, and is apparent in adenomas, *PCDHGC3* promoter methylation occurs later in the adenoma–carcinoma transition. *PCDHGC3* was hypermethylated in 17/28 carcinomas (60.7%) according to methylation array analysis. Quantitative real-time reverse transcription–polymerase chain reaction showed that *PCDHGC3* is the highest expressed PCDH in normal colonic epithelium, and that there was a strong reciprocal relationship between *PCDHGC3* methylation and expression in carcinomas (R=−0.84). PCDH LRES patterns are reflected in colorectal tumour cell lines; adenoma cell lines are not methylated at *PCDHGC3* and show abundant expression at the mRNA and protein level, while the expression is suppressed in hypermethylated carcinoma cell lines (R=−0.73). Short-interfering RNA-mediated reduction of PCDHGC3 led to a decrease of apoptosis in RG/C2 adenoma cells, and overexpression of PCDHGC3 in HCT116 cells resulted in the reduction of colony formation, consistent with tumour suppressor capabilities for PCDHGC3. Further functional analysis showed that PCDHGC3 can suppress Wnt and mammalian target of rapamycin signalling in colorectal cancer cell lines. Taken together, our data suggest that the PCDH LRES is an important tumour suppressor locus in colorectal cancer, and that PCDHGC3 may be a strong marker and driver for the adenoma–carcinoma transition.

Correspondence: Dr CL Andersen, Department of Molecular Medicine, Aarhus University Hospital, Skejby, DK 8200, Denmark. cla@ki.au.dk; Dr K Malik, Cancer Epigenetics Laboratory, School of Cellular and Molecular Medicine, University of Bristol, Bristol BS8 1TD, UK. k.t.a.malik@bristol.ac.uk.
[5]These authors contributed equally to this work.

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Introduction

The development of colorectal cancers is characterized by the accumulation of multiple genetic and epigenetic alterations (Cho and Vogelstein, 1992). In approximately 60–80% of sporadic colorectal cancers (CRCs), tumorigenesis is initiated by mutations of the adenomatous polyposis coli (*APC*) gene (Miyoshi et al., 1992; Powell et al., 1992). As APC is an essential component of the destruction complex that regulates the turnover of β-catenin, a transcriptional co-activator of the WNT signalling pathway (Klaus and Birchmeier, 2008), this highlights constitutive activation of the Wnt/β-catenin signalling pathway as a prerequisite for colorectal tumorigenesis. Subsequent somatic mutations that are frequently identified in colorectal cancers (CRCs) are considered 'drivers' necessary for tumorigenesis (Wood et al., 2007), and mutations in the *BRAF, KRAS, PIK3CA, PTEN* and *TP53* genes reflect the importance of the RAS-MAPK, PI(3)K and p53 pathways (Fearon, 2011). The majority (~80%) of sporadic CRCs exhibit chromosomal instability characterized by aneuploidy (Pino and Chung, 2010), whereas a smaller group (~15%) with defective mismatch repair exhibit microsatellite instability (MSI). MSI is also associated with an increased frequency of DNA hypermethylation at multiple gene loci and such tumours have been defined as having a CpG island methylator phenotype (CIMP) (Ahuja et al., 1997; Weisenberger et al., 2006).

Although tumour-specific promoter hypermethylation is prevalent in many cancers, including colon cancer, the stage specificity and consequences of diverse epigenetic alterations are less well defined than genetic defects (Jones and Baylin, 2007). Genome-wide DNA hypomethylation is ubiquitous even in early benign tumours and is linked to chromosomal instability (Ji et al., 1997; Matsuzaki et al., 2005), oncogene activation (Baylin and Ohm, 2006) and deregulation of imprinted loci (Cui et al., 2003). Hypermethylation of the SFRP Wnt antagonists (Suzuki et al., 2004) and the tumour suppressor *RASSF1* (Greenspan et al., 2006) is observed in aberrant crypt foci; although most instances of hypermethylation occur early in colorectal tumorigenesis, a small subset of 'late' silenced genes, including *MLH1*, are carcinoma specific and may be associated with malignant progression (Ibrahim et al., 2011; Oster et al., 2011).

Taken together with gene-specific silencing events, epigenetic suppression can also involve transcriptional lock-down spanning large chromosomal domains, resulting in the coordinate silencing of numerous genes. Such long-range epigenetic silencing (LRES) has been described in colon cancer at chromosome 2q14.2, at the *MLH1* locus on 3p22 and, most recently, the Ikaros locus on 7p12 (Frigola et al., 2006; Hitchins et al., 2007; Javierre et al., 2011). LRES and CIMP are suggested to share a common aetiology (Wong et al., 2011). LRES has also been identified at additional loci and in different adult cancer types, including lung (Rauch et al., 2007), breast (Novak et al., 2008) and prostate cancer (Coolen et al., 2010), and also in Wilms' tumour-a paediatric kidney cancer (Dallosso et al., 2009). A common 800 kbp LRES region identified in breast cancer and Wilms' tumour resides on chromosome 5q31; this region contains 53 genes of the protocadherin (PCDH) superfamily in three multi-gene clusters (α-, β- and γ-PCDHs; Human Genome Organization nomenclature *PCDHA@, PCDHB@* and *PCDHG@*, respectively).

PCDH functions have predominantly been studied in neuronal cells, although PCDH expression is also observed in other cells including gut, lung and kidney (Bass et al., 2007;

Dallosso et al., 2009). PCDHs have extracellular cadherin domains involved in cell adhesion (Obata et al., 1995; Fernandez-Monreal et al., 2009; Schreiner and Weiner, 2010) and intracellular domains, which can translocate to the nucleus and regulate gene expression (Haas et al., 2005; Hambsch et al., 2005). γ-Protocadherins are capable of binding and negatively regulating members of the focal adhesion kinase (FAK) family, including *PYK2* (Wang et al., 2009), and also regulate neuronal survival through cytoplasmic domain interactions with programmed cell death 10 (PDCD10) (Lin et al., 2010). Protocadherins also interact with, and are phosphorylated by, the receptor tyrosine kinase encoded by the *RET* proto-oncogene (Schalm et al., 2010), and also exhibit intracellular retention, which can influence intracellular membrane shaping via their variable cytoplasmic domains (Fernandez-Monreal et al., 2009; Hanson et al., 2010; O'Leary et al., 2011).

Although little is known about protocadherin molecular functions in relation to tumour biology, the diverse and complex cellular activities they mediate likely impinge on key cellular pathways involved in cell death and proliferation. Indeed, our studies of selected γ-PCDHs, PCDHGA2, PCDHGA6 or PCDHGA12, in Wilms' tumour demonstrated their ability to suppress Wnt pathway activity in vitro (Dallosso et al., 2009); this was supported by proteomic analysis of the γ-PCDH-interacting proteins, which showed interactions with β-catenin (Han et al., 2010), together with several other proteins implicated in carcinogenesis.

We previously detected hypermethylation of some 5q31 PCDH genes in the colorectal cancer cell line HCT116, prompting us to investigate whether PCDH LRES might also occur in primary colorectal cancers. Here we show that 5q31 LRES is also frequent in colorectal adenoma (CRA) and carcinoma. We establish that, unlike the majority of silencing events within the LRES region, protocadherin *PCDHGC3* is largely unmethylated in normal and adenoma tissues, but frequently epigenetically silenced in carcinomas. As this is also the highest expressed PCDH in normal colonic epithelium, the role of *PCDHGC3* was examined in CRA and carcinoma models. PCDHGC3 is capable of negatively regulating Wnt and mammalian target of rapamycin (mTOR) signalling consistent with tumour suppressor function in colorectal cancer.

Results

Epigenetic alterations of multiple 5q31 protocadherins in colorectal tumorigenesis and carcinoma-associated silencing of *PCDHGC3*

We examined the DNA methylation profile of genes at 5q31 (Figure 1a) in normal colonic epithelia from 6 non-cancer individuals, 6 CRAs (all microsatellite stable (MSS)) and 28 CRCs (five MSI and 23 MSS) using methylation arrays. The majority of PCDHs from PCDHA@, PCDHB@ and PCDHG@ are hypermethylated in either adenomas, carcinomas or both, relative to normal tissue (18 of 22 PCDHs examined by the arrays, 82%) (Figure 1b), and all adenomas and carcinomas displayed hypermethylation of genes spanning the three PCDH clusters. Methylation at non-PCDH genes flanking the gene cluster is stable compared with the widespread changes observed in all three of the PCDH clusters (Figure 1c). None of the genes located within 500 kbp (7 centromeric genes and 11 telomeric) of the PCDH locus show any evidence of hypermethylation relative to normal controls. Thus, methylation array analysis identifies LRES at the PCDH locus on chromosome 5q31 in colorectal cancer.

PCDH hypermethylation is found in both MSI and MSS tumours, suggesting that increased methylation is not merely a consequence of CIMP. For 17/18 hypermethylated PCDHs, increased methylation is found in both adenomas and carcinomas with respect to normal colon (Supplementary Figure 1; $P<0.03$ and <0.04, respectively, Mann–Whitney U-test).

Late hypermethylation, that is in carcinomas rather than adenomas is only observed at the *PCDHGC3* gene (Figures 1b and c). *PCDHGC3* has low methylation levels in all normal and adenoma samples and is hypermethylated in about half of the examined carcinomas (15/28, 53.6%) according to array analysis.

In our study of Wilms' tumour, we observed that LRES encompassed many genes which are not expressed in corresponding normal tissue (Dallosso et al., 2009). To identify gene expression variations pertinent to colon cancer, we interrogated our Affymetrix exon microarray data set (Oster et al., 2011). Analysis of normal colon epithelium gene expression shows that the abundance of individual PCDH transcripts is overall very low (Supplementary Table 1). Many transcripts are not detectable above background (14/47 PCDHs examined) and median PCDH expression is in the 10th percentile of mean colonic epithelium gene expression (n=6). However, robust expression of aggregate *PCDHG@* was detectable via analysis of their downstream constant-region exons 2–4 (Figure 1a). By comparison, it was not possible to identify aggregate *PCDHA@* expression due to a paucity of probe sets with signals detectable above background. Of the individual PCDHs readily detectable by multiple probe sets in colon tissue, *PCDHGC3* exhibits robust expression and importantly also appears to show a reciprocal relationship with Infinium DNA methylation levels (Supplementary Figure 2).

To validate the expression microarray data, we used quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis of PCDH expression. Analysis of two normal colonic epithelium tissue samples showed an aggregate *PCDHG@* expression, which was more than 40-fold higher than aggregate *PCDHA@* expression (Figure 1d). Real-time quantification of individual PCDH transcripts confirms their relatively low abundance. However, among our quantitative analysis of 19 individual PCDHs, PCDHGC3 expression was significantly higher than other PCDHs analysed (36- to 938-fold), approximately equivalent to the housekeeping gene TBP. This pattern of expression is consistent with our previous work and that of others, describing contrasting expression profiles for the *PCDHA@* and *PCDHG@* gene clusters, with strictly neuronal-specific expression of *PCDHA@*, but widespread *PCDHG@* expression in embryonic and adult tissues (Bass et al., 2007; Novak et al., 2008; Dallosso et al., 2009). We endeavoured to characterize cellular PCDHGC3 protein expression in colorectal cancer tissue microarrays, but unfortunately our PCDHGC3 antibodies were unsuitable for immunohistochemistry.

This analysis shows that PCDH LRES is frequently associated with the early stages of colorectal tumorigenesis. Conversely, and uniquely within the locus, the array data suggested that hypermethylation of the protocadherin *PCDHGC3* is strictly associated with the adenoma–carcinoma transition. As *PCDHGC3* is the highest expressed PCDH at 5q31, our data suggest that its epigenetic deregulation may be important in colorectal cancer progression. For these reasons, we focused our subsequent investigations on *PCDHGC3*.

Epigenetic silencing of *PCDHGC3* in colorectal cancer

For rigorous validation of our array data, we performed quantitative analysis of *PCDHGC3* methylation and expression in a subset of our normal, adenoma and carcinoma sample groups using bisulphite pyrosequencing and real-time RT–PCR, respectively (Figure 2). Pyrosequencing of six adjacent CpG dinucleotides located between +15 and +47 relative to the *PCDHGC3* transcriptional start site (Figure 2a) confirmed that methylation levels alter between sample groups ($P<0.0005$, Kruskal–Wallis test). *PCDHGC3* is virtually unmethylated in normal tissues (mean 5.6%, standard deviation (s.d.) 2.8%, n=10). Average methylation in adenomas was higher (mean 14.2%, s.d.=15.3%, n=10). However, this increase was attributable to those adenomas exhibiting high-grade dysplasia (mean 24.7%,

s.d.=15.9%, n=5) and adenomas with low-grade dysplasia were not hypermethylated (mean 3.8%, s.d.=2.5%, n=5). This contrasts with carcinomas, the majority (81%) of which are hypermethylated (mean 43.7%, s.d.=28.0%, n=16). Thus, the analysis of multiple CpGs by pyrosequencing reveals a higher frequency of carcinoma hypermethylation than indicated using methylation arrays, the latter technique being discrepant for three carcinomas in our analysis (Supplementary Table 2).

As expected, *PCDHGC3* methylation and gene expression were inversely correlated (Spearman's rank correlation coefficient=−0.78, $P=2.58 \times 10^{-5}$), suggesting that methylation is functionally linked to gene silencing (Figure 2b). Gene expression is unchanged between normal and adenoma or unmethylated carcinomas, but *PCDHGC3* expression in methylated carcinomas is significantly reduced (P<0.01, Mann–Whitney U-test) (Figure 2c). The PCDHGC3 hypermethylated subset of tumours includes three MSI cancers and MSI is closely linked to CIMP. However, although these tumours showed significantly lower PCDHGC3 expression than the rest of the methylated cancers (P<0.02, MSS-methylated versus MSI-methylated, Mann–Whitney U-test), PCDHGC3 methylation levels were not significantly higher (P=0.37, Mann–Whitney U-test). These studies strongly support PCDHGC3 methylation as a marker of CRA–carcinoma transition, and a potentially critical epigenetic lesion in colon cancer progression.

PCDH LRES in colorectal cancer cell lines

Because cancer cell lines often faithfully reflect the epigenetic defects of their tumour of origin, they have proven invaluable in the functional characterization of important cancer pathways (Paz et al., 2003). We assessed LRES in cell lines derived from CRAs and carcinomas by examining the DNA methylation of selected 5q31 gene promoters located across the locus. As shown above for primary tumour samples, PCDH hypermethylation is widespread in genes from the *PCDHA@, PCDHB@* and *PCDHG@* gene clusters (Figure 3a; Supplementary Table 2). Methylation was observed in each PCDH examined (between 42.9% (3/7) and 100% (7/7) of cell lines examined for each assay). We examined an additional five PCDH genes in HCT116 cells and all were found to be highly methylated (Supplementary Table 2). Importantly, this analysis also shows that the boundaries of the hypermethylated region are maintained in cultured cells as methylation was not found in flanking genes (*DNAJC1, WDR55* and *DIAPH1*). The HCT116 cell line, reported as CIMP-high (Yagi et al., 2010), has the highest frequency of methylated PCDHs (8/8). Significantly, methylation of the *PCDHGC3* gene is low or absent in the adenoma cell lines, and the gene is fully or partially methylated in the majority of carcinoma lines (Figures 3a and b). Quantitative analysis of expression and DNA methylation identified a strong reciprocal relationship (R=−0.73, P=0.048, Spearman's rank correlation), as was shown above for primary samples (Figure 3c).

Quantitative real-time RT-PCR analysis of the 'DKO' cell line, a DNMT1/DNMT3b double knockout line derived from HCT116 (Rhee et al., 2002), shows derepression of *PCDHGC3* relative to the parental line. Treatment with the DNA methyltransferase inhibitor 5-azacytidine was also able to restore expression, but to a lesser extent (465- and 62-fold expression over parental HCT116 cells, respectively) (Figure 3d). Derepression was also verified at the protein level by immunoblotting (Figure 3d, inset). As expected, the expression of non-PCDH unmethylated genes flanking the LRES region was unaffected by epigenetic unmasking experiments. The expression of several other PCDHs were either unaffected or showed much-reduced fold-change increases in gene expression in these experiments, possibly suggesting additional non-epigenetic rate-limiting factors in these cells (Supplementary Table 3).

The PCDHGC3 protein expression pattern among adenoma and carcinoma cell lines was also assessed by immunoblotting (Figure 3e). Whereas five adenoma-derived cell lines all showed easily detectable PCDHGC3 protein, 6/8 carcinoma-derived cell-lines showed little or no PCDHGC3. Of the two other carcinoma lines, SW480 cells also showed reduced PCDHGC3; thus only one of eight carcinoma lines (HCA7) retain PCDHGC3 protein levels similar to adenoma lines. These analyses confirm PCDHGC3 protein expression changes at the adenoma–carcinoma transition, and also demonstrate that colorectal cell lines are suitable surrogates for *in vitro* analysis of PCDH function.

PCDHGC3 influence on apoptosis and Wnt signalling

The data above imply that *PCDHGC3* silencing is critically associated with the adenoma–carcinoma transition in colorectal cancer. We sought to investigate the functional implications of *PCDHGC3* loss at this critical period during carcinogenesis using an in vitro adenoma model that we have characterized previously (Greenhough et al., 2010). This involved short interfering RNA (siRNA)-mediated knockdown of *PCDHGC3* in a non-tumorigenic premalignant CRA cell line, RG/C2 (Paraskeva et al., 1989), which we have shown to express PCDHGC3 protein (Figure 3e). Under normal conditions, this cell line undergoes relatively high basal apoptosis and thus represents a model system for investigating survival-modulating factors. After *PCDHGC3* knockdown, the proportion of floating (apoptotic) cells was reduced (71%; P=0.020, Student's t-test) compared with control siRNA transfections (Figure 4a). This reduced floating cell count was associated with a clear reduction in cleaved poly-(ADP-ribose) polymerase (PARP) (from 55.5 to 36.4% cleaved PARP by image densitometry), a substrate of caspase-3, thus demonstrating a reduction in apoptosis in the cells with reduced *PCDHGC3* (Figure 4a).

Our previous work has shown that PCDHGA2, PCDHGA6 and PCDHGA12 can regulate Wnt signalling and growth properties in normal and cancer cells (Dallosso et al., 2009). PCDHGC3 expression also significantly inhibited colony growth (66% suppression of colony formation) in HCT116 cells (Figure 4b). We then examined the effect of PCDHGC3 overexpression on β-catenin/T-cell factor-mediated transcription. In these experiments, we used both SW480 and HCT116 cells as they are known to have different Wnt pathway mutations (a truncating APC mutation and an activating β-catenin mutation in SW480 and HCT116, respectively). In both cell lines, PCDHGC3 overexpression was able to markedly suppress reporter activity (Figure 4c). In SW480 cells, the reduction in reporter activity was associated with a reduction in endogenous levels of 'active' (non-phosphorylated) β-catenin (Figure 4c, lower panel).

Taken together, these experiments show that PCDHGC3 is pro-apoptotic in an adenoma context, suppresses the growth of carcinoma cells and is able to repress Wnt/β-catenin/T-cell factor transcriptional activity as we have previously observed with several other PCDH paralogues (PCDHGA2, PCDHGA6, PCDHGA12) (Dallosso et al., 2009). Thus, the biological activities of *PCDHGC3* are consistent with tumour suppressor gene function.

PCDHGC3 and other oncogenic pathways

Given that γ-PCDHs have been shown to repress FAKs (Wang et al., 2009), and that *FAK* has been shown to be required downstream of Wnt/c-myc signalling in intestinal tumorigenesis (Ashton et al., 2010), we investigated the effect of PCDHGC3 knockdown in RG/C2 cells on FAK and PYK2 levels (Figure 5a). Immunoblotting revealed no changes in total FAK, phospho-Tyr402 PYK2 or total PYK2. We also analysed mTOR, as Wnt signalling has been shown to exert β-catenin-independent post-transcriptional influence via the mTOR pathway (Inoki et al., 2006) and inhibition of mTOR can inhibit colorectal tumorigenesis (Gulhati et al., 2009). Both phospho-Ser2448 mTOR and total mTOR were

increased following *PCDHGC3* knockdown in RG/C2 adenoma cells, as was phosphorylated 4EBP1, an important factor in enhanced protein translation downstream of mTOR (Hsieh et al., 2010). We confirmed the PCDHGC3 knockdown effect on mTOR was not cell specific or due to off-target effects by knocking down PCDHGC3 with a second siRNA in HCA7 cells (Supplementary Figure 3a). Overexpression of full-length *PCDHGC3* in SW480 carcinoma cells, on the other hand, led to markedly decreased mTOR and phosphorylated 4EBP1 (Figure 5b); transfection of the C-terminal domain of PCDHGC3 alone had no effect on mTOR levels (Supplementary Figure 3b). We also examined whether PCDHGC3 modulated phospho-AKT, which is known to be upstream of both Wnt and mTOR pathways, but no changes resulting from PCDHGC3 modulation were observed (data not shown). As mTOR is an important regulator of autophagy (Jung et al., 2010), and protocadherins have been shown to colocalize with the autophagy marker LC3 (Hanson et al., 2010), we also analysed LC3 proteins in our adenoma and carcinoma cells, but no shift in LC3-I/LC3-II, or change in p62 levels, which are markers of autophagy (Klionsky et al., 2008) was apparent by immunoblotting (Figure 5).

These studies further emphasize the regulatory capacities of PCDHGC3 in oncogenic pathways, and suggest that loss of PCDHGC3 can have downstream effects not only on transcriptional activities mediated by Wnt/β-catenin, but also on protein translation through mTOR (Hsieh et al., 2010).

Discussion

In this study, we have characterized a new region of LRES at chromosome 5q31 in colorectal cancers. Furthermore, we show that DNA hypermethylation of multiple genes within an LRES region does not occur in a synchronized manner; one gene in particular, *PCDHGC3*, is only silenced at the adenoma–carcinoma transition. Our functional studies demonstrate that PCDHGC3 protein has growth-inhibitory capacity and is able to suppress key oncogenic pathways in colorectal cancer cell lines, consistent with tumour suppressor function.

Previously, we demonstrated PCDH LRES in Wilms' tumour (Dallosso et al., 2009), and a similar LRES was demonstrated in breast cancer (Novak et al., 2008). More recently, PCDH hypermethylation was also apparent in a genome-wide analysis of prostate cancer (Kobayashi et al., 2011). Taken together with this study, this suggests that the chromosome 5q31 clustered PCDHs are tumour suppressors in several different cancers. In addition to the PCDH locus on chromosome 5q31, LRES loci have now been identified in tumours originating in breast (Novak et al., 2008), head and neck (Smith et al., 2006), lung (Rauch et al., 2007), prostate (Coolen et al., 2010) and colon (Frigola et al., 2006; Hitchins et al., 2007; Javierre et al., 2011), suggesting that events leading to LRES are pervasive during tumorigenesis; in fact, a recent comprehensive study in prostate cancer integrated genome-wide histone modification, DNA methylation and transcript data to characterize 47 novel LRES regions in prostate cancer, and showed these were significantly associated with both tumour suppressor and gene cluster loci (Coolen et al., 2010).

In addition to some LRES regions being specific to cancer types, a degree of tissue specificity of individual PCDH genes undergoing epigenetic silencing is also apparent; in Wilms' tumours, *PCDHGC3* was never found to be methylated, indicating that PCDHGC3 protein is indispensable for renal cells. PCDHGC3 also appears to escape methylation in prostate cancer (Kobayashi et al., 2011). In breast cancer, PCDHGC3 does undergo hypermethylation, and cellular models mimicking breast cancer progression demonstrated that PCDHGC3, unlike other PCDHs, acquired methylation only in cancer cell lines, as opposed to post-stasis or immortalized cell lines (Novak et al., 2009). Therefore, similar to

colorectal cancer, loss of PCDHGC3 function may be integral to cancer progression in breast cancer, although no functional studies of PCDHs in breast cancer has been carried out to date. It has recently been shown that DNA methyltransferase 3B overexpression is associated with sequential methylation in colorectal cancer progression (Ibrahim et al., 2011) and upregulation of DNA methyltransferases is most pronounced at adenoma–carcinoma transition (Schmidt et al., 2007). Such changes highlight the importance of epigenetic alterations in colorectal cancer progression, with *PCDHGC3* as a possible key target.

Several members of the protocadherin superfamily have been shown to have tumour-specific hypermethylation, including *PCDH10* in colorectal cancer (Ying et al., 2006). However, to date, relatively little is known about the functional consequences of altered PCDH expression on oncogenic mechanisms. Taken together with our previous study on Wilms' tumour, this study suggests that PCDHs can affect Wnt signalling in different cellular contexts and PCDH epigenetic silencing can therefore be added to other Wnt inhibitory molecules that are suppressed during carcinogenesis (Baylin and Ohm, 2006). Although the Wnt pathway is activated early by *APC* mutation in colon cancer, a two-step model for APC-driven cancer initiation and progression, requiring additional lesions/oncogenic activities has been proposed (Phelps et al., 2009). We suggest that epigenetic events such as *PCDHGC3* silencing can prompt cancer progression by further elevation of Wnt signalling, as well as other oncogenic activities such as the mTOR pathway, which our studies have shown to be inhibited by PCDHGC3 (Figure 6). Although the mechanisms by which PCDHGC3 influences mTOR remain unclear, they appear to require full-length protein, as the C-terminal domain did not affect mTOR levels when transfected into SW480 cells. Given the documented nuclear transcriptional functions ascribed to protocadherins (Haas et al., 2005; Hambsch et al., 2005), together with non-conventional intracellular roles (Fernandez-Monreal et al., 2009; Hanson et al., 2010; O'Leary et al., 2011) and extensive proteomic interactions of protocadherins with cellular signalling factors (Han et al., 2010), it will be of great interest to perform integrated transcriptomic and proteomic analyses of tumour cells with differing PCDH epigenetic profiles to delineate the complex mechanisms by which protocadherins influence tumour cell biology.

Finally, we propose that the PCDH LRES may have great utility in providing biomarkers for many cancers. The high density of epigenetically deregulated genes within a relatively small locus may be especially useful compared with the use of biomarker panels consisting of genes from several genetic loci (Lind et al., 2011), as genetic heterozygosity could be easily verified and an epigenetic 'barcode' for the region would provide markers for both early and late events in tumorigenesis.

Materials and methods

Patient samples

DNA samples were obtained from patients diagnosed with CRA or adenocarcinoma, no cancer other than colorectal cancer, no indication of heredity disease and no radio- or chemotherapy before resection. Informed consent was obtained from all patients and protocols approved by the Central Denmark Region Committees on Biomedical Research Ethics. Sample details are given in Supplementary Table 4.

Cell culture, transfections and reporter assays

CRA and carcinoma cell lines used have been described previously (Paraskeva et al., 1989; Elder et al., 1996). All cell lines were propagated using standard methods in Dulbecco's modified Eagle's medium with 2 mm l-glutamine, 50 U/ml penicillin/streptomycin and 10%

fetal bovine serum, unless stated otherwise. Growth media were identical for the AA/C1, AA/C1/SB10 and RG/C2 lines with the addition of 20% fetal bovine serum. All cell lines were propagated using standard protocols at 37 °C and 5% CO2.

For RNA interference, siRNAs for PCDHGC3 (5′-AGGAAAGAGAGAAGGGTTTTT-3′; Eurogentec, Liège, Belgium) and Silencer Select siRNA s200447 (Ambion Inc, Austin, TX, USA) were used together with appropriate negative controls. Cells were reverse (suspension) transfected with siRNA sequences targeted to human PCDHGC3, or a validated non-targeting negative control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, for transfection in suspension, RG/C2 cells were seeded overnight in Opti-MEM (Invitrogen) at a density of 1.6 × 105/cm2 in T12.5 flasks in the presence of 20 nm siRNA/Lipofectamine complexes, as described previously (Greenhough et al., 2010). Following transfection of PCDHGC3 siRNA, RG/C2 cells were placed in 10% fetal bovine serum containing Dulbecco's modified Eagle's medium for 96 h. Floating cells (those cells that had detached from the flask and were present in the culture media) were collected, and the attached cells were harvested by trypsinization with trypsin-EDTA. The attached and floating cells were counted separately using a Neubauer counting chamber. We have previously determined that the majority of floating cells in the RG/C2 cell line are apoptotic, and therefore the proportion of floating cells as a percentage of the total cell population (attached plus floating cells) represents a good surrogate for the percentage of apoptosis in the treated culture (Elder et al., 1996). Assays were repeated at least three times.

Wnt/β-catenin reporter assays, colony formation experiments and plasmid subcloning were performed as described previously (Dallosso et al., 2009).

Immunoblotting

Analysis of proteins was as described previously (Dallosso et al., 2009). The antibodies used were as follows: tubulin, GAPDH and LC3 (Sigma-Aldrich, St Louis, MO, USA), β-actin (Abcam, Cambridge, UK), phospho-Ser2448 mTOR, total mTOR, phospho-Thr37/46 4EBP1, phospho-Tyr402 PYK2, total PYK2, total FAK, phospho-Ser473 AKT (all from Cell Signalling Technology, Danvers, MA, USA), active β-catenin (Millipore, Billerica, MA, USA), poly-(ADP-ribose) polymerase (Alexis, Lausen, Switzerland) and p62 (BD Biosciences, Franklin Lakes, NJ, USA). PCDHGC3 antibodies have been reported previously (Haas et al., 2005).

DNA methylation analysis

DNA was extracted from tissue samples using Puregene DNA purification kit (Gentra systems, Plymouth, MN, USA). Bisulphite treatment was carried out using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. PCR amplification was carried out using Jumpstart Red Taq Polymerase (Sigma) using: 95 °C/2 min, then 45 cycles of 94 °C/30 s, and annealing/30 s, 72 °C/30 s. Quantitative pyrosequencing of bisulphite PCR products was carried out using the Pyromark Q96 MD platform using the Pyromark Gold system and analysed using the Pyro Q-CpG analysis software (Qiagen, Hilden, Germany). Primer sets and restriction enzymes used for combined bisulphite and restriction assay (COBRA) are given in Supplementary Table 6, together with locations of COBRA, Illumina and pyrosequencing assays for PCDHGC3.

Bisulphite-converted genomic DNA was analysed using Illumina's Infinium Human Methylation27 Beadchip Kit (WG-311-1202). Chip processing and data analysis were performed using the recommended reagents and protocol. Chips were image-processed in Illumina's iScan scanner and data were extracted using the BeadStudio v.3.0 software (Illumina, San Diego, CA, USA). Methylation values for each CpG locus are expressed as a

β-value, representing a continuous measurement from 0 (completely unmethylated) to 1 (completely methylated). This value is based on the following definition and calculation:

β-value=(signal intensity of methylation-detection probe) / (signal intensity of methylation-detection probe+signal intensity of non-methylation-detection probe).

Statistical analysis

Methylation comparisons between groups were carried out using the Mann–Whitney U-test after trimming of extremes and filtering for genes with median methylation value between groups of >0.1. All statistical analyses were carried out using R scripts (cran.r-project.org).

RNA expression analysis

Microarray analysis of gene expression was carried out using the Affymetrix GeneChip Human Exon 1.0 ST Array platform as described previously (Oster et al., 2011). Because the transcripts encoded by the PCDHA@ and PCDHG@ clusters contain perfectly homologous sequences via their shared downstream exons, only probes located in the unique regions (exon 1) were used in the analysis. Probes were used for comparative analysis if detected above background in at least 3 of 6 normal tissue samples and all transcripts with at least two valid probe sets meeting these criteria were analysed.

Comparative real-time RT–PCR was performed as described previously (Dallosso et al., 2009). Primer sequences are available in Supplementary Table 5. All assays were performed in at least duplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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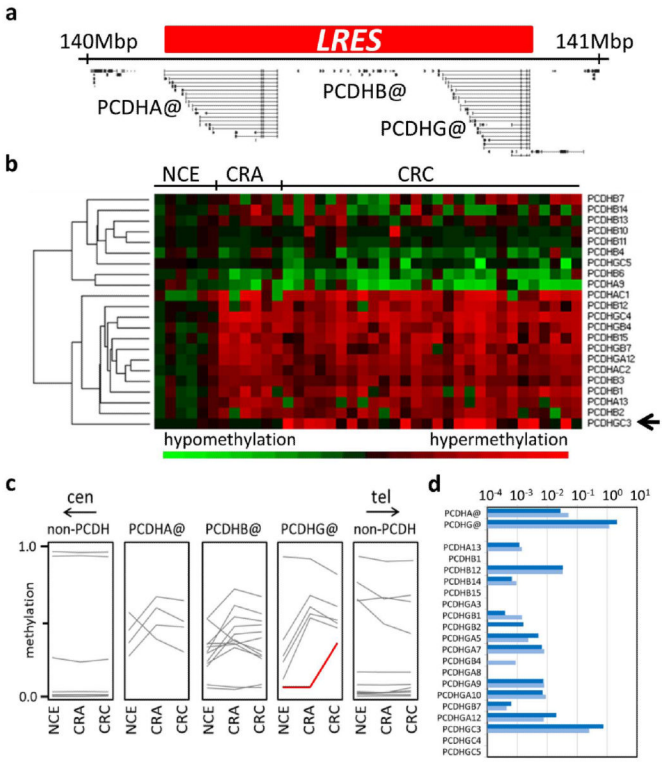

Figure 1.

Hypermethylation of multiple protocadherin genes at 5q31 in colorectal tumours. (a) Organization of the protocadherin cluster located on chromosome 5q31. The 800 kbp locus contains a tandem arrangement of 53 paralogous protocadherin-encoding genes belonging to the α, β and γ subclusters (*PCDHA@, PCDHB@, PCDHG@*). The breadth of the previously identified LRES region is shown by the red box. (b) Hierarchical clustering of data from Illumina Infinium Human Methylation27 methylation arrays of normal colonic epithelia(NCE) from 6 non-cancer individuals, 6 CRAs (all MSS) and 28 CRCs (5 MSI and 23 MSS). Data normalized relative to mean NCE. PCDH hypermethylation is observed in 18/22 PCDHs examined and is prevalent in both CRA and CRC. PCDHGC3 is an exception (arrowed). (c) The 5q31 DNA methylation changes are specific to the PCDH gene clusters. Each box groups genes into those belonging to the α, β and γ PCDH clusters or non-PCDH genes located within 500 kbp upstream (cen) or downstream (tel) of the PCDH gene cluster. Mean Illumina Infinium methylation levels for each tissue group are plotted, showing that, despite widespread epigenetic PCDH alterations in both CRA and CRC, promoter methylation is largely unchanged outside the LRES. Atypical ‘late’ hypermethylation of *PCDHGC3* is highlighted in red. (d) Real-time RT–PCR of two normal colon samples (blue and light blue) relative to the housekeeping gene TBP.

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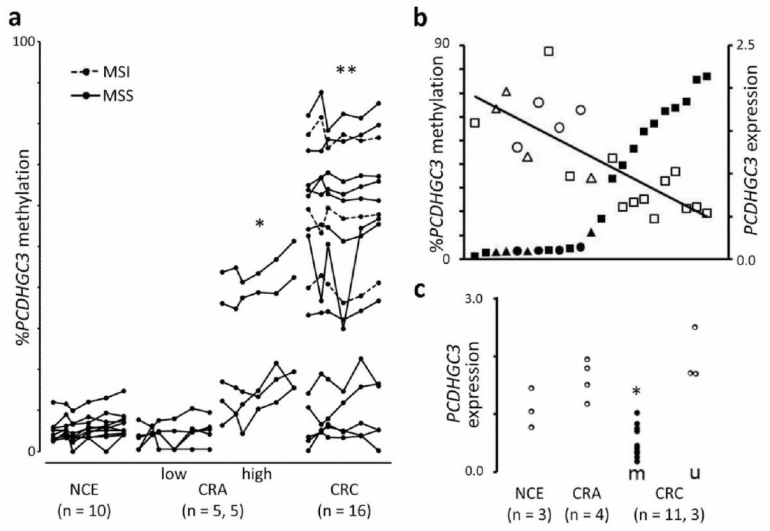

Figure 2.

Quantitative methylation and gene expression analyses of PCDHGC3. (a) Pyrosequencing of six CpG dinucleotides adjacent to the *PCDHGC3* transcriptional start site (+15 to +47). For adenomas: low, low-grade dysplasia; high, high-grade dysplasia. One asterisk indicates the significant change ($P<0.03$) between normal colonic epithelium and high-grade dysplasia adenomas, and two asterisks indicate the significant differences between carcinomas and normal ($P<0.02$) and low-grade dysplasia adenomas ($P<0.05$) (Kruskal–Wallis one-way analysis of variance with Bonferroni correction for multiple testing). (b) Reciprocal relationship between *PCDHGC3* mean pyrosequencing methylation levels (black markers) and PCDHGC3 real-time RT–PCR gene expression (open markers) in patient samples (circular, triangular and square markers represent normal samples, adenomas and carcinoma, respectively). (c) *PCDHGC3* expression analysed by real-time RT–PCR is lower in methylated (M) carcinomas than normal epithelium (NCE), colorectal adenoma (CRA) and unmethylated (U) carcinoma (CRC). *$P<0.01$, t-test.

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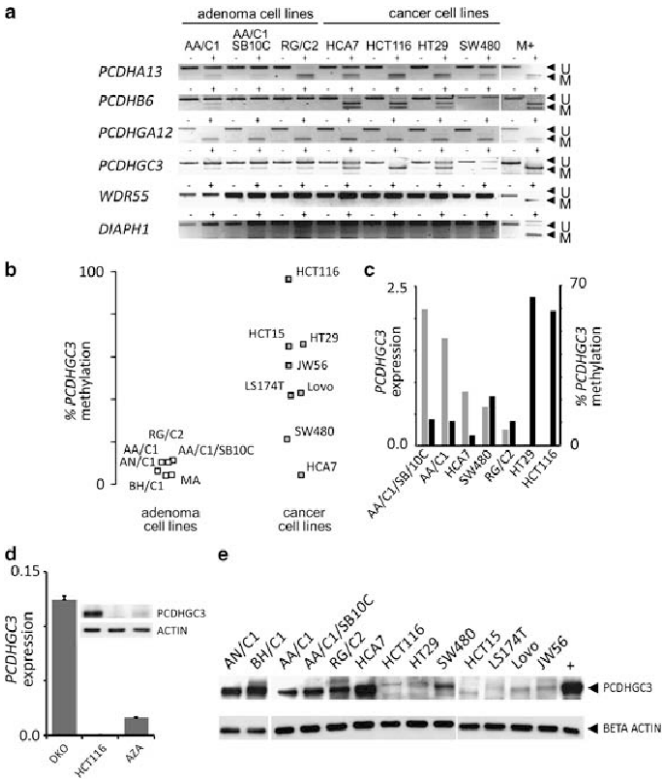

Figure 3.

Epigenetic silencing of *PCDHGC3* in colorectal cancer cell lines. (a) DNA methylation of PCDH genes in colorectal adenoma and carcinoma cell lines. Combined bisulphite and restriction analysis of PCDHs and flanking non-PCDH genes shows that the cultured cells recapitulate the 5q31 methylation pattern observed in patient tumours. −/+, mock/digested samples, respectively. U, unmethylated COBRA signal; M, methylated signal. Cell-line analysis of additional PCDHs is given in Supplementary Table 2. (b) Pyrosequencing of PCDHGC3 in colorectal tumour cell lines confirms carcinoma-specific hypermethylation. Adenoma lines are shown by open boxes, and carcinoma lines by grey boxes. (c) Reciprocal relationship between *PCDHGC3* promoter methylation (black bars) and expression (grey bars) in tumour cell lines ascertained by pyrosequencing and real-time RT–PCR, respectively. Gene expression levels are shown relative to the housekeeping gene TBP. (d) Pharmacological and genetic reactivation of *PCDHGC3* transcription. Quantitative real-time RT–PCR for *PCDHGC3* expression in HCT116 DNMT1 and DNMT3B double knockout cells (DKO), compared with parental HCT116 cells and HCT116 cells treated with the demethylating agent 5-aza-2-deoxycytidine (AZA). Inset is an immunoblot demonstrating reactivation of PCDHGC3 protein. (e) Immunoblotting of CRA and CRC cell lines for PCDHGC3 protein shows higher expression in unmethylated adenoma and carcinoma lines and decreased protein in methylated carcinomas.

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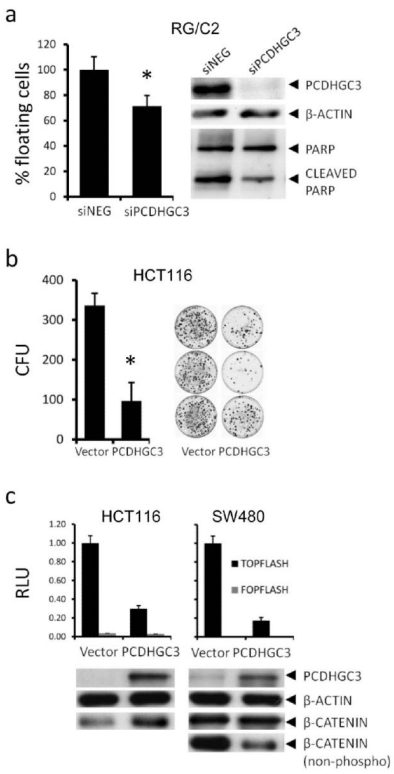

Figure 4.

PCDHGC3 effects on apoptosis, cell growth and Wnt signalling. (a) PCDHGC3 suppresses apoptosis in an adenoma cell-line model. Targeted siRNA knockdown of PCDHGC3 in RG/C2 colorectal adenoma cells results in a reduction in floating cells (*P<0.05 t-test of triplicate assays). Immunoblotting (below) confirms effective PCDHGC3 knockdown and housekeeping control β-actin levels. A lower proportion of cleaved poly-(ADP-ribose) polymerase in knockdown samples indicates that the observed cell counts are associated with a reduction in apoptosis in transfected cells. (b) Suppression of HCT116 colony formation following PCDHGC3 cDNA transfection. Following selection and staining, colonies were counted. Mean colony counts and representative plates are shown. *P<0.01 t-test of triplicate assays; CFU, colony-forming units. (c) Suppression of β-catenin/T-cell factor reporter activity accompanying PCDHGC3 expression. HCT116 and SW480 cells were co-transfected with a PCDHGC3 cDNA or empty vector plasmid along with Super8XTOPFLASH (TOPFLASH) or Super8XFOPFLASH (FOPFLASH) plasmids. Immunoblotting (below) shows expression levels of heterologous protein and loading control β-actin and the levels of endogenous total and non-phosphorylated β-catenin. As observed previously, non-phospho-β-catenin is not detectable in HCT116 cells. RLU, relative light units.

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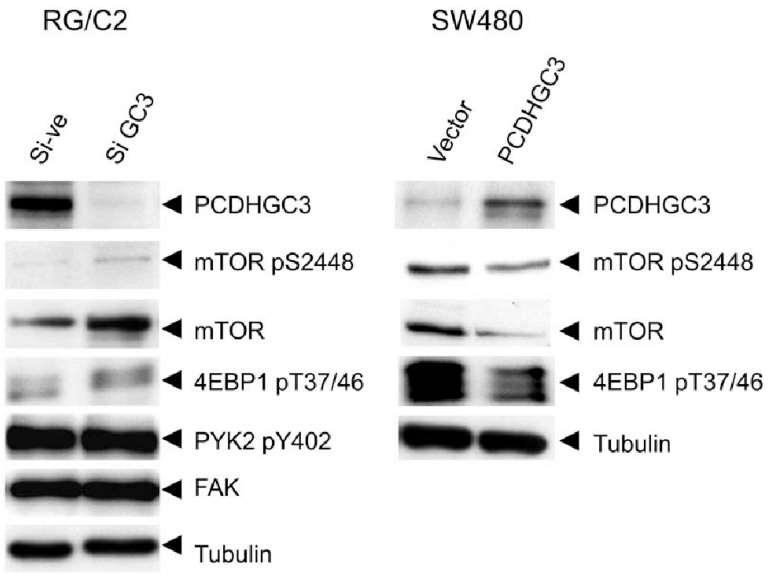

Figure 5.
Modulation of PCDHGC3 protein levels affects the mTOR pathway. (a) RG/C2 cells were transfected with either scrambled control siRNA (si-ve) or *PCDHGC3* siRNA (siGC3), and proteins were analysed by immunoblotting, (b) SW480 carcinoma cells were transfected with empty vector (Vector) or PCDHGC3 overexpression plasmid, and proteins were analysed by immunoblotting.

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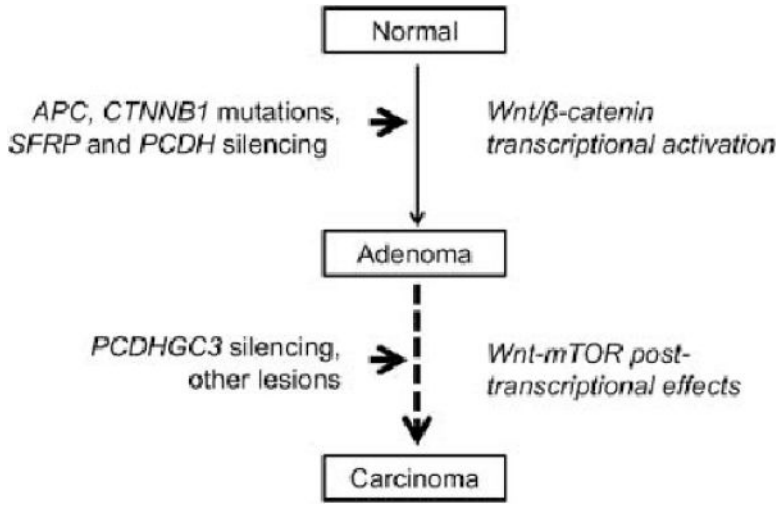

Figure 6.

Model for PCDH effects in colorectal tumorigenesis. Early lesions at the PCDH LRES region may contribute to transcriptional deregulation through the Wnt/β-catenin pathway, and disruption of membrane and intracellular activities; the adenoma–carcinoma transition requires further epigenetic silencing of genes such as PCDHGC3, which also contributes to mTOR-dependent post-transcriptional deregulation.