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High-resolution melting analysis of PCDH10 methylation levels in gastric, colorectal and pancreatic cancers

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Protocadherins are cell-adhesion molecules with 6 or 7 cadherin motifs in their extracellular domain and various cyotoplasmic domains. PCDH10 was characterized a novel tumor suppressive gene in and was epigenetically silenced in multiple haematologic malignancies as well as some solid tumors such as gastric cancer, nasopharyngeal carcinoma and esophageal carcinoma. High-resolution melting (HRM) analysis has been used as a novel tool for analysis of promoter methylation. In our study, we used HRM analysis to detect the methylation levels of PCDH10 gene in 100 gastric cancers, 100 colorectal cancers, 70 pancreatic cancers and equal number of adjacent normal tissues. The frequency of PCDH10 methylation in all three types of cancers was significantly higher than that in normal tissues. Consistent with previous reports, expression levels of PCDH10 were inversely correlated with methylation levels. But we didn't find significant association between PCDH10 methylation status and TNM staging in all three types of cancers. In summary, application of HRM analysis to large amount of clinical samples proves to be a fast and high-throughput way to investigate the epigenetic status of PCDH10. And this is the first study to evaluate the prevalence of PCDH10 methylation based on large amount of tumor samples, showing that epigenetic regulation of PCDH10 was associated with carcinogenesis.

Key words: PCDH10, methylation, HRM, gastric cancer, colorectal cancer, pancreatic cancer.

Protocadherins are cell-adhesion molecules with 6 or 7 cadherin motifs in their extracellular domain and various cyotoplasmic domains [1]. The protocadherin family in the mammalian genome consists of about 50 members. Protocadherin-a (CNR) genes, protocadherin-b (protocadherin 3) genes, and protocadherin-g (protocadherin 2) genes constitute gene clusters on a single chromosome [2], whereas others are scattered over different chromosomes [1]. Most of these protocadherins are expressed in the nervous system, and at least some are localized at synapses [3, 4]. protocadherin 10 (PCDH10/OL-PCDH/KIAA1400) is a nonclustered-type protocadherin with a unique cytoplasmic domain [5]. It is reported that there is a correlation between the expression pattern of PCDH10 and functional systems of the brain such as the visual and limbic systems [6–8]. Unlike other protocadherin family members, PCDH10 was discovered to have widespread expression [9]. Recently, PCDH10 has been characterized as a novel tumor suppressive gene (TSG) [10].

Promoter hyper-methylation is one of the hallmarks of carcinogenesis associated with transcriptional silencing of genes encoding for diverse cellular pathways, and is considered to be an important epigenetic mechanism implicated in the regulation of normal gene expression. Such changes often affect 5 regulatory CpG genomic regions and can be associated with aberrant expression of certain genes in cancer [11]. Studies on mouse models of acute lymphocytic/myeloid leukaemia demonstrated that PCDH10 is a frequently methylated gene involved in leukaemia transformation [12, 13]. Methylation of PCDH10 was also reported to be associated with multiple haematologic malignancies as well as some solid tumors such as gastric cancer, nasopharyngeal carcinoma and esophageal carcinoma [10, 14, 15].

High-resolution melting (HRM) analysis is a novel tool for analysis of promoter methylation [16]. The new approach is based on the "melting" properties of DNA in solution [17], and was originally developed for SNP genotyping [18]. The princi-

ple of this method is that bisulfite-treated DNA templates with different contents of methyl-cytosine can be resolved by melting analysis due to differences in melting temperatures [19]. HRM relies on the precise monitoring of the change of fluorescence as a DNA duplex melts. This technique requires the use of standard PCR reagents and double stranded DNA-binding dyes that can be used at saturating concentrations without inhibiting PCR amplification [18]. The melting analysis does not allow detailed information about the methylation of single cytosines within the sequence of interest, but can distinguish fully and partially methylated samples. The semi-quantitative measurement of methylation is important because low levels of methylation may not be biologically important [20, 21]. Also, quantification of promoter methylation may enable early detection of cancer and early metastatic spread [22].

In our study, we used HRM analysis to detect the methylation levels of PCDH10 gene in 100 gastric cancers, 100 colorectal cancers, 70 pancreatic cancers and equal number of adjacent normal tissues. Methylation levels in all three types of cancers were significantly higher than that in normal tissues. This is the first study to estimate the prevalence of PCDH10 methylation based on large amount of tumor samples, showing that epigenetic regulation of PCDH10 was associated with carcinogenesis.

Materials and methods

*Controls and patient samples***.** CpGenome Universal Methylated and unmethylated DNA (Chemicon, Millipore Billerica, MA, USA) were used as 100% and 0% methylated control DNA, respectively. Methylation standards were constructed by diluting 100% methylated bisulfite-modified control DNA in a pool of unmethylated bisulfite-modified DNA at ratios of 50%, 10% and 1%. These standards were included in each experimental run.

Surgically resected tumor tissues and adjacent normal tissues were collected from 100 primary gastric cancer patients, 100 primary colorectal cancer patients and 70 primary pancreatic cancer patients. Staging was assessed after pathological examination of formalin fixed surgical specimens based on the 2002 TNM classification (6th edition of the staging criteria of the UICC and AJCC). The study was approved by the ethical committee of the Shenzhen Hospital, Peking University. The individuals gave their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

Extraction of genomic DNA and sodium bisulfite modification. Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innogent, Shenzhen, China) according to the manufacturer's instruction. One μg of genomic DNA was subjected to bisulfite conversion with the EZ DNA methylation kit (Zymo research, USA). The eluted DNA (40 μl volume) was used for the HRM analysis.

HRM Analysis. PCR amplification and HRM were performed on the ABI7500 (Applied Biosystems) as adapted from the published protocol (16). The primers were designed as outlined (16). The sequences of the primers for PCDH10 are as follows: forward- GGATTTGCGGGGTTGTTTT and reverse- AATCGCCCAAAAACACCAC (156 bp). PCR was performed in a 20 μl volume containing: 1x buffer, 2 U Hotstart Taq DNA polymerase (Takara), 250 nM of each primer, 2.5 mM SYTO-9, and 10 ng bisulfite treated DNA template, with 3 mM final $MgCl_2$. Each reaction was performed in triplicate. The cycling conditions were as follows: 1 cycle of 95°C for 10 minutes, 60 cycles of 95°C for 10 seconds, 61°C for 10 seconds, and 72°C for 10 seconds; followed by an HRM step of 95°C for 1 minute, 40°C for 1 minute, 65°C for 15 seconds, and continuous acquisition to 95°C at 1 acquisition per 0.3°C. A standard curve with known methylation ratios was included in each assay and was used to deduce the methylation ratio of each tumor and normal sample. HRM data were analyzed using the High Resolution Melting Software (Applied Biosystems). Output plots are in the form of normalized melting curves and difference plots. Statistical analysis was performed using chi-square test through the SPSS 10.0 software for Windows. *P* values less than 0.05 were considered statistically significant.

Quantitative RT-PCR. Total RNA was isolated from tissues by using AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen) according to the manufacturer's instruction. First strand cDNA was synthesized with RevertAidTM First Stand cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. The relative abundance of PCDH10 mRNA level was calculated by using the comparative C(T) method [23, 24] with GAPDH as the internal control. Logarithmic transformation (Log_2 [1+X]) was used to transform primary data to normal distribution. Data from three independent experiments were analyzed by student t-test and $p<0.05$ was considered statistically significant. The primers for PCDH10 are: forward: 5' CCAAGACCGACCTGATGTTTC and reverse: 5' CAGCCTTACCTCGTTGGACAA. The primers for GAPDH are: forward: 5'cagcctcaagatcatcagca and reverse: 5'tgtggtcatgagtccttcca.

Results

The sensitivity of HRM analysis for PCDH10. The sensitivity of the PCDH10 HRM analysis was tested by using dilutions of fully methylated DNA into unmethylated DNA. The HRM standard melting curve was derived from five samples with the following ratios of methylated DNA: 0%, 1%, 10%, 50% and 100% methylation. The inclusion of CpGs in the primer sequence makes it possible to direct the PCR bias towards the methylated templates by manipulating the annealing temperature of PCR amplification. At the annealing temperature of 61^0C, methylation level as low as 1% can be easily detected (Fig. 1). The normalized melting profiles of the PCR product amplified from the same template were consistent between replicates and between different runs, and the shapes of normalized melting profiles were amplification independent as samples with different starting amount of template displayed very similar profiles. (data not shown).

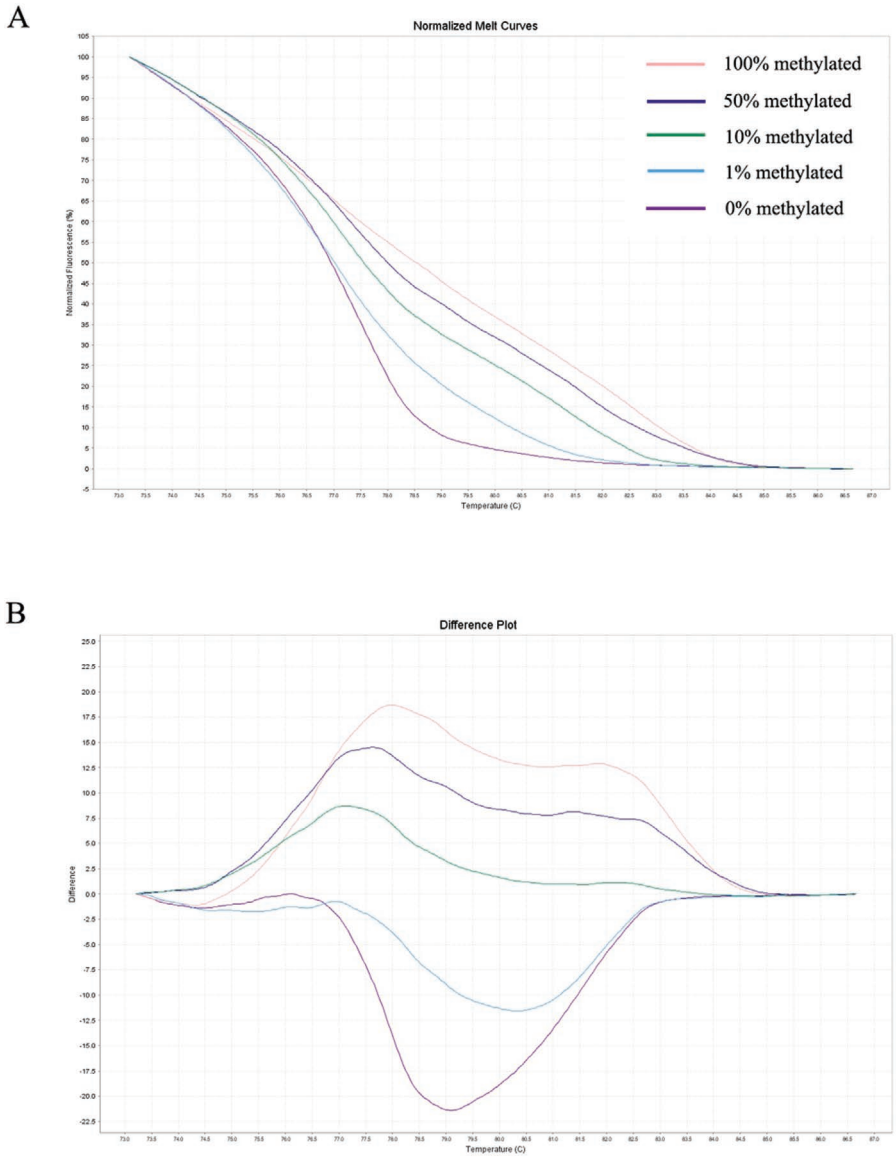

Figure 1. Normalized HRM standard curves and difference plot of PCDH10 gene.

Templates with different ratios (as indicated) of methylated DNA were amplified at the annealing temperature of 61^{0}C, and subjected to HRM analysis. A, normalized melting curves; B, difference plot.

Methylation levels of PCDH10 in tumor samples and normal tissues. Table 1 shows the methylation levels of PCDH10 in 100 gastric, 100 colorectal and 70 pancreatic cancer samples and equal number of adjacent normal tissues. In all three types of cancers, methylation levels of PCDH10 in cancer samples were significantly higher than that in normal tissues.

Correlation between methylation and expression of PCDH10. In order to confirm that methylation of PCDH10 was indeed correlate with the downregulation of its gene expression, we then used real-time PCR to detect the mRNA levels of PCDH10 in the cancer samples. Since low levels of methylation may not be biologically important (20, 21), the cancer samples were sub classified into to groups: <1% methylation and >1% methylation. The numbers of samples in the two groups are 53 and 47 (gastric cancer), 57 and 43 (colorectal cancer), and 46 and 24 (pancreatic cancer), respectively (Table 2). As expected, in all three types of cancers, expression levels of PCDH10 were significantly lower in the groups of >1% methylation (Fig. 2), which is consistent with previous reports.

Correlation between methylation of PCDH10 and staging of cancers. According to the 6th edition of the staging criteria of the UICC and AJCC, the cancer samples used in our study

Table 1. Methylation levels of PCDH10 in cancer samples and normal tissues

□	Total No.	0% methylation	0-1% methylation	1-10% methylation	10-50% methylation	50-100% methylation	*P*
Gastric cancer							
Cancer samples	100	36	17	22	19	6	2.5E-11
Normal tissues	100	79	18	3	0	0	
Colorectal cancer							
Cancer samples	100	33	24	31	9	3	4.9E-14
Normal tissues	100	87	12	1	0	0	
Pancreatic cancer							
Cancer samples	70	31	15	17	3	4	4.8E-06
Normal tissues	70	58	11	1	0	0	□

Table 2. Methylation levels of PCDH10 in different stages of cancers

□	No. of samples	< 1% methylation	> 1% methylation	*P*
Gastric cancer				
Stage I cancers	15	9 (60%)	6 (40%)	0.517
Stage II cancers	27	16 (59%)	11(41%)	
Stage III cancers	37	16 (43%)	21 (57%)	
Stage IV cancers	21	12 (57%)	9 (43%)	
Total	100	53 (53%)	47 (47%)	
Colorectal cancer				
Stage I cancers	11	5 (45%)	6 (55%)	0.820
Stage II cancers	23	13 (57%)	10 (43%)	
Stage III cancers	32	18 (56%)	14 (44%)	
Stage IV cancers	34	21 (62%)	13 (38%)	
Total	100	57 (57%)	43 (43%)	
Pancreatic cancer				
Stage I cancers	12	9 (75%)	3 (25%)	0.498
Stage II cancers	21	12 (57%)	9 (43%)	
Stage III cancers	26	19 (73%)	7 (27%)	
Stage IV cancers	11	6 (55%)	5 (45%)	
Total	70	46 (66%)	24 (34%)	□

were assessed after pathological examination based on the 2002 TNM classification. The number of samples in each stage was summarized in Table 2. There was no significant association between PCDH10 methylation status and TNM staging in all three types of cancers.

Discussion

Several methods have been developed for the analysis of methylation, each with their characteristic strengths and weaknesses. The most widely used method is methylation-specific PCR (MSP) that uses primers specific for methylated, bisulphite-modified DNA [25]. MSP is very sensitive but is not quantitative, thus can lead to the classification of gene methylation when only a small number of cells are positive. Genomic sequencing can be considered the gold standard [26, 27]. It provides the most detailed information but is relatively insensitive, and its expensive cost makes it generally unsuitable for screening. Pyrosequencing was recently introduced with higher sensitive, but is dependent on the availability of the proprietary instrumentation [28]. High-resolution melting (HRM) analysis becomes a novel tool for analysis of promoter methylation [16]. The applications of sequencing and HRM in methylation studies utilize methylation-independent PCR (MIP) where the primers are designed to amplify the bisulphite-modified sequence regardless of its methylation status. However, MIP primers do not always lead to the proportional amplification of methylated and unmethylated sequences [29, 30]. So, inclusion of some CpGs seems necessary in the primer sequence to avoid the underestimate of the degree of methylation [30]. At lower annealing temperatures, the primers bind both methylated and unmethylated templates and PCR bias will favour the amplification of unmethylated sequences. At higher annealing temperatures, primer binding will favour methylated sequences. Thus the optimal annealing temperature is important for the effective amplification of templates independent of methylation status. HRM has several advantages over the other methods. It is high-throughput and relatively cheap. More importantly, it can be used to estimate the proportion or extent of methylation when run with standards. This is especially useful when assessing clinical cancer samples for predictive markers such as PCDH10 where discrimination between different levels of methylation may have diagnostic and prognostic value. In our study, we have shown that HRM is applicable for the very sensitive detection of PCDH10 methylation in an unmethylated background. With HRM, we were able to detect the methylation level of PCDH10 as low as 1%.

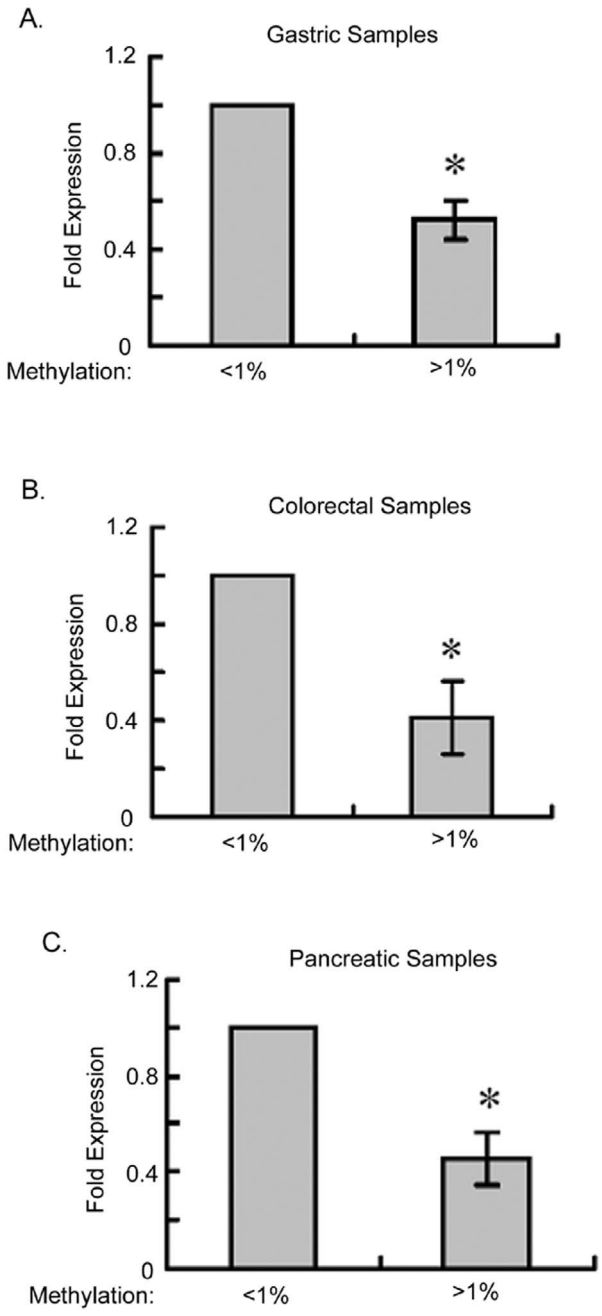

Figure 2. PCDH10 expression in different types of cancer samples.

Total RNA from cancer samples were extracted and subjected to real-time PCR analysis. The relative abundance of PCDH10 mRNA level was calculated by using the comparative C(T) method after logarithmic transformation. Asterisk: $p<0.05$.

PCDH10 has been characterized as a novel tumor suppressive gene, and is found to be epigenetically silenced in multiple haematologic malignancies as well as solid tumors [10, 12, 14, 15]. But only limited number of primary tumor samples was recruited in previous studies. This makes further evaluation necessary to better estimate the prevalence of PCDH10. Our study for the first time investigated PCDH10 methylation in large amount of primary cancer samples. The high prevalence of hypermethylation of PCDH10 in the gastric, colorectal and pancreatic cancers (47%, 43% and 24%, respectively) suggests that transcriptional silencing of PCDH10 by methylation is common and may be involved in the pathogenesis of many types of cancers. On the other hand, methylation levels among different cancer types are not consistent. In our cases, gastric cancers have the highest percentage of methylation. This indicates that contribution of PCDH10 methylation to carcinogenesis relies on the unique property of different cancer types. Furthermore, a certain incidence of PCDH10 methylation was also detected in adjacent normal tissues, although the methylation levels in these normal tissues remain lower than 10% (Table 1). Aberrant hypermethylation could be caused by various factors in normal tissues such as diets or aging [31] and this might result in the low methylation frequencies in normal tissues. Another possibility is the contamination of trace amount of tumor tissues during the surgical resection process, since our normal tissues are resected from adjacent area of the same patient. In the future, application of microdissection would be very helpful to distinguish tumors from normal tissues.

A significant correlation between PCDH10 methylation and loss of expression of PCDH10 was observed in all three types of cancers by real-time PCR. This is not unexpected and is consistent with many previous studies. We also analyzed the staging of the cancers in relation to the methylation of PCDH10. To our surprise, no association was found between them. This can be explained by the fact that PCDH10 methylation is supposed to be the cause, rather than the outcome, of carcinogenesis. Thus, even in the stage I of cancers, PCDH10 gene is already in methylated state, as observed in our study. This suggests that PCDH10 methylation cannot be used as an auxiliary criterion for the staging of cancers, but rather, can be used as a marker for the diagnosis of early stage cancers.

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