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Original contribution

Protocadherin γ-A7 is down-regulated in colorectal cancer and associated with the prognosis in patients with wild-type *KRAS*☆,☆☆

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Summary Many clustered protocadherin genes (*PCDHs*) within chromosome 5q31 are frequently down-regulated in colorectal cancer (CRC) due to the hypermethylation of this region, and some of them have been identified as tumor suppressors. However, the association between the expression of the clustered *PCDHs* and prognosis of CRC patients is still unclear. Here, we identified multiple *PCDHs* that were significantly down-regulated in CRC by analyzing the RNA-seq data of the Cancer Genome Atlas (TCGA) cohort. Among them, one γ-PCDH subfamily member, *PCDHGA7*, was found to be associated with overall survival in the patients with wild-type *KRAS*. Next, we experimentally validated the decrease of PCDHGA7 mRNA and protein levels in tumor tissues of 20 CRC patients by using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry assay (IHC). To further investigate whether the expression of PCDHGA7 could predict clinical outcomes, an independent cohort of 138 patients, whose tumors carried wild-type *KRAS,* was enrolled. In-house tissue microarrays (TMAs) were developed to facilitate the protein detection, and prognostic significance was analyzed. The result showed low PCDHGA7 expression was associated with advanced TNM stage, high risk of tumor recurrence and short overall survival. In conclusion, this study demonstrates that PCDHGA7 is down-regulated in CRC, and its expression level is correlated with clinical outcomes in patients with wild-type *KRAS*. Our finding indicates PCDHGA7 could serve as a potential novel biomarker to predict prognosis by combining certain tumor genotypes in patients of CRC.

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1. Introduction

Colorectal cancer, a primary malignancy of the intestine, is one of the most common cancers around the world, with higher incidence in males than in females [1]. The morbidity and mortality of colorectal cancer in several areas tend to stabilize or decline, which is due to the detection and removal of precancerous lesions through colorectal cancer screening [2,3]. Unfortunately, approximately 1.2 million patients worldwide are diagnosed with colorectal cancer each year, while more than 600 000 patients die directly or indirectly from this disease [4]. Despite the progress of diagnosis and treatment strategies in recent decades, CRC remains one of the most deadly human cancers: the 5-year survival rate of patients with colorectal cancer (the most invasive form of CRC) is greater than 10% [5]. CRC is not a cancer with a single risk factor. In addition to age and gender, the most important prognostic factors are TNM staging, surgical resection range, postoperative radiotherapy, and adjuvant chemotherapy [6]. However, the abovementioned clinical parameters do not completely explain the observed difference in survival rates. Therefore, it is urgent to discover valuable diagnostic and prognostic biomarkers to stratify patients into different risk categories, subsequently allowing the development and use of more specific treatment agents.

PCDHs are the largest mammalian subgroup of the cadherin superfamily of homophilic cell-adhesion proteins, and are further divided into clustered and nonclustered PCDH families based on their genomic organization [7,8]. In past decades accumulating evidence has revealed that the clustered *PCDHs* regions of chromosome 5q31 are highly methylated in many malignancies [9-11]. It is also demonstrated that silencing of gene expression is concomitant with DNA hypermethylation and repressive histone modifications, which means that *PCDH* gene clusters represent a long-range epigenetic silencing. Moreover, it has been reported that the clustered PCDHs may serve as favorable prognostic indicators for certain cancers, which suggested that PCDHs may be a predictive marker of prognosis in colorectal cancer [12,13].

In this study, we found that *PCDHGA7*, a *PCDH-γ* gene subfamily member, was significantly down-regulated in colorectal cancer by screening the RNA-Seq data of the Cancer Genome Atlas (TCGA) cohort. Then we examined the expression of PCDHGA7 in our own cohort and investigated whether PCDHGA7 is significantly associated with the prognosis of colorectal cancer.

2. Materials and methods

2.1. Patient samples

This study was approved by the Ethics Committee of Tenth People's Hospital of Tongji University. A total of 230 formalin-fixed, paraffin-embedded (FFPE) specimens (one from each case) were collected from the Department of Pathology, Shanghai Tenth People's Hospital, from 2008 to 2013. Additionally, 20 pairs of CRC tumors and matched normal tissues were collected at the time of surgical resection and were immediately snap-frozen in liquid nitrogen before storage at −80 °C. Sample inclusion criteria of all the samples were: (1) diagnosis of primary CRC without a history of other neoplasms; (2) patient having complete clinical data including: age, gender, clinical manifestations, mean tumor size, tumor TNM stage, adjuvant therapy and follow-up; and (3) a sufficient quality of tissue sample for experimental use. According to the TNM staging system, tumors were classified independently by 2 pathologists. A postoperative follow-up of 5 years was performed and the overall survival was counted from date of initial surgery to death.

2.2. RNA extraction and using quantitative real-time polymerase chain reaction

Total RNA from resected tissues was prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The absorbance ratio OD(260)/OD(280) value was used to evaluate concentration and purity. The RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China). *PCDHGA7* and *β*-actin were then quantified by using quantitative real-time polymerase chain reaction (qRT-PCR). The sequences of primers were as follows: *PCDHGA7*, forward: TTCGGTTTCCGTTAAGCGAGG, and reverse: CCGGGTACTTAGTTTCATCGTC; *β*-actin, forward: CATGTACGTTGCTATCCAGGC, and reverse: CTCCTTAATGTCACGCACGAT. The relative expression quantitation of the target gene was determined by using $2^{-\Delta\Delta Ct}$ method. For each sample, the assay was performed in triplicate.

2.3. Immunohistochemistry assay

The 4-μm TMAs or whole sections of FFPE specimens were used for immunohistochemical staining. The slides were deparaffinized in xylene and rehydrated in graded alcohols and distilled water, followed by antigen retrieval. Specimens were then incubated with rabbit anti-human PCDHGA7 (orb1039, Biorbyt) in a dilution of 1:100. All sections were visualized with diaminobenzidine, and counterstained with hematoxylin. The immunostaining intensity was quantitated using Image J software.

2.4. *KRAS* mutational analysis

Genomic DNA was extracted from FFPE tissues using QIAamp DNA FFPE tissue kit. The sequences of primers used to amplify a fragment containing codons 12 and 13 of the *KRAS* gene were as follows: forward, ATTATAAGGCCTGCTGAAAATGACT and reverse, TCGTCAAGGCACTCTTGC. Polymerase chain reaction (PCR) products were purified using the MN NucleoSpin Gel and PCR Clean-up kit. Sanger

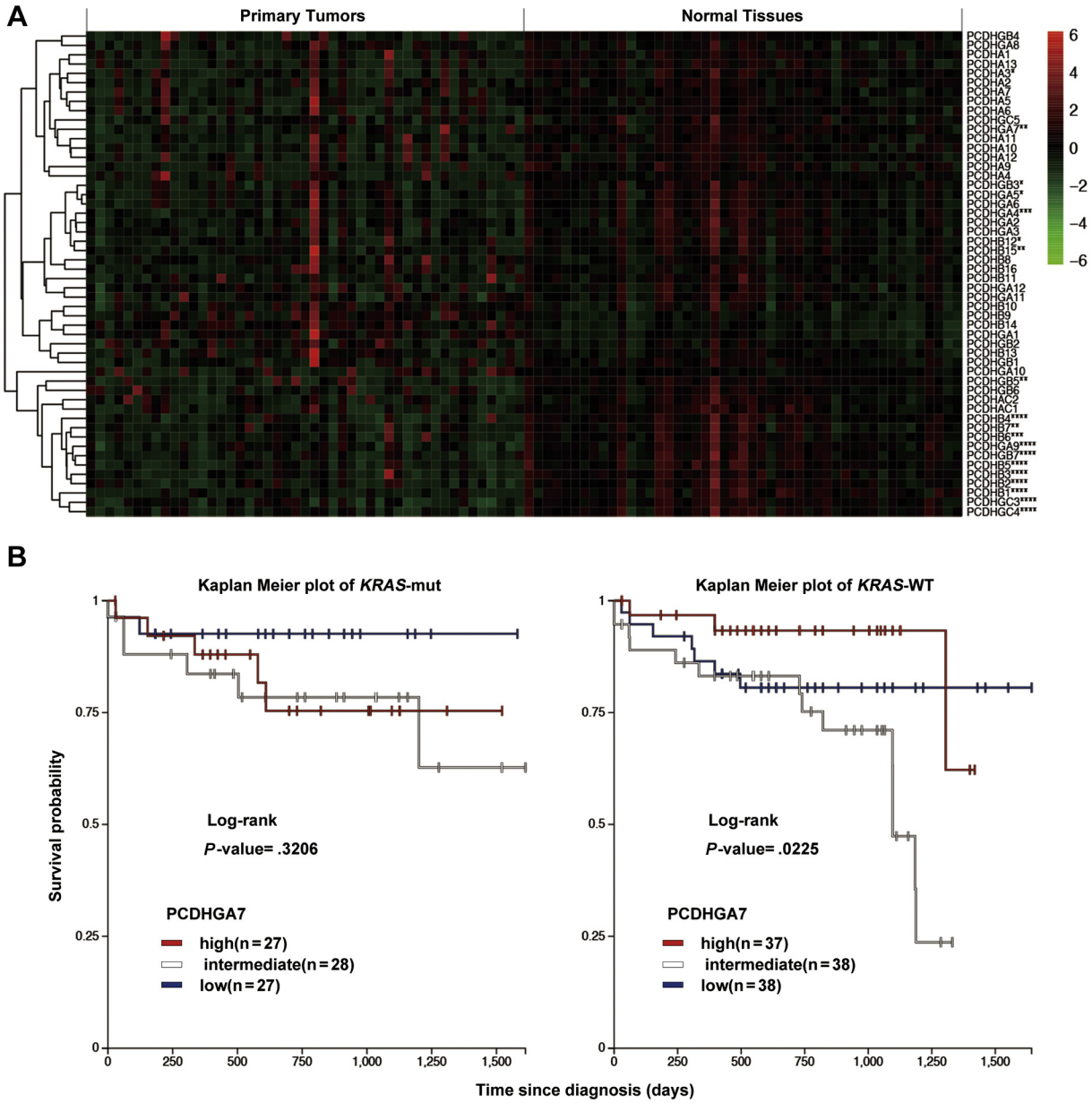

Fig. 1 Gene expression and clinical relevance of *PCDHs* in TCGA cohort of CRC. A, Heat map of clustered protocadherin genes in 47 pairs of CRC tumors and matched normal tissues extracted from TCGA cohort. Significant down-regulation was observed in 19/53 *PCDHs* examined, and most of them were β and γ subfamily members. Data normalized to median, and log values were used to analyze. Bonferroni was used to adjust p-value of all gene, $P < .05$ was considered statistically significant. B, Kaplan–Meier survival analysis showed that one of γ-*PCDHs*, *PCDHGA7*, was significantly correlated with overall survival in patients with wild-type *KRAS*, but not those who patients carry mutated *KRAS*.

sequencing was then performed to identify mutations in codon 12/13 of the *KRAS* gene.

2.5. Statistical analysis

The qRT-PCR data were presented as the mean ± SD. The differences in mRNA or protein levels of PCDHGA7 between colorectal cancer tissues and corresponding normal tissues were analyzed with Student paired *t* test. The correlations between PCDHGA7 expression and clinicopathologic features were analyzed using χ^2 or Fisher's exact test wherever appropriate. Overall survival was calculated by the Kaplan–Meier curve and compared using the log-rank test. All statistical tests were 2-sided, which were performed with Prism (Version 6.0, GraphPad Software, La Jolla, CA). A 2-tailed *P* value less than .05 was considered statistically significant.

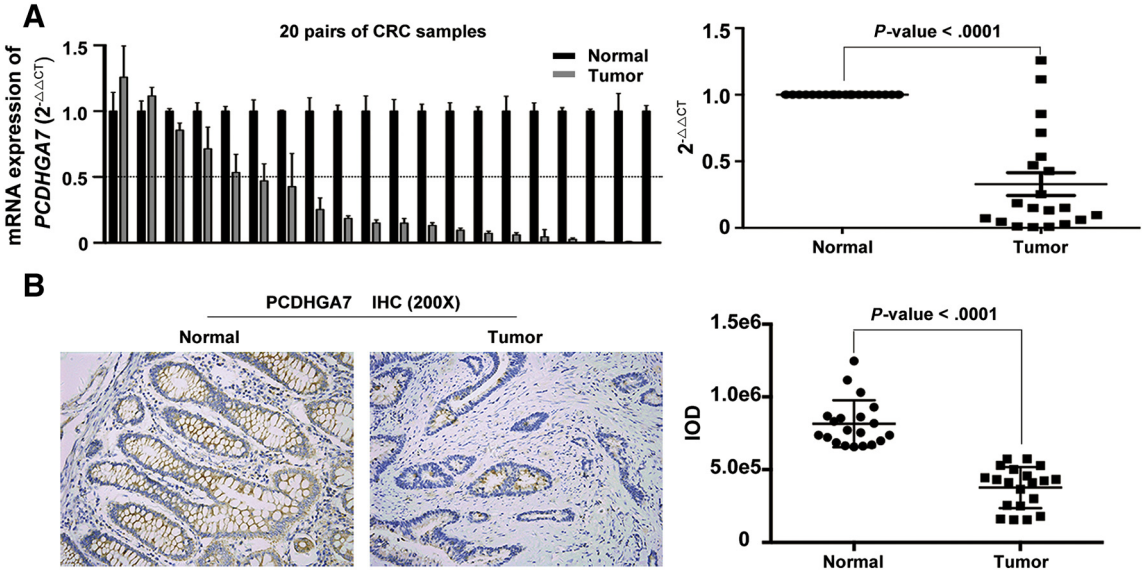

Fig. 2 PCDHGA7 is down-regulated in CRCs. A, mRNA expression of *PCDHGA7* in 20 pairs of tumors and matched normal tissues. Each sample was done in triplicate, and student's paired *t* test was used for statistical analysis. B, PCDHGA7 immunostaining in whole-slide tissue sections of colorectal cancer and adjacent normal. Immunohistochemistry: original magnification ×200. Significance was also evaluated using *t* test, and the result is shown in the right panel.

3. Results

3.1. Expression of multiple clustered protocadherins is down-regulated in CRC in TCGA cohort

The epigenetic silencing of large chromosomal region 5q31 present in CRC has long been intriguing. Previous studies have demonstrated the down-regulation of certain *PCDH* genes in CRC, but the clinical relevance of the 53 individual genes encompassed within this region has been elusive. To screen candidate *PCDHs* for clinical use, we analyzed the RNA-Seq data in 47 pairs of tumors and matching normal tissues retrieved from TCGA cohort of CRC (Supplementary Table S1). As shown in Fig. 1A, the expression of many *PCDHs* spanning the alpha, β and γ clusters was altered in colorectal cancers, with 19 of 53 showing significantly lower than normal tissues, but 3 of 53 showing significantly higher (Supplementary Table S2).

Of note, the most significantly down-regulated genes (Bonferroni adjusted $P < .01$) belong to the β and γ families. This finding led us to interrogate the original FPKM value in TCGA RNA-Seq data set (Supplementary Table S3). As expected, the overall mRNA levels in normal tissues across 15 *PCDHA* genes analyzed in our patient panel showed much lower than β and γ family genes (Supplementary Fig. S1), consistent with the expression profile in kidney and Wilm's tumors [9]. Moreover, our results also were consistent with a previous study in which many of these most down-regulated genes were hypermethylated in CRC tissues [11]. Taken together, our data show that multiple *PCDHs* are down-regulated in CRC tissues by TCGA data analysis.

3.2. Low *PCDHGA7* expression is associated with decreased overall survival in patients with *KRAS*-wild-type CRCs in TCGA cohort

Due to the low mRNA level observed in colorectal carcinomas in TCGA cohort, we further investigated whether these *PCDHs* could serve as prognostic markers. We focused on the *γ-PCDHs* since their aggregate expression showed the highest level (Supplementary Fig. S1). Therefore, we performed survival analyses in TCGA CRC data set for which both overall survival and gene expression were available. After removing duplications and grouping individuals based on *γ-PCDHs* expression, however, the results demonstrated that none of the *γ-PCDHs* was significantly associated with clinical outcome (Supplementary Fig. S2).

We next turned to the role of *KRAS* mutation status. As in patients with CRC that carry mutated *KRAS*, the induced phenotype might 'drown out' others. Therefore, the patients were stratified into 2 groups: *KRAS*-wild-type (*KRAS*-WT) and *KRAS*-Mutant (*KRAS*-Mut). For each group, we performed Kaplan–Meier analyses. As shown in Fig. 1B, one of the *γ-PCDHs*, *PCDHGA7*, was found to be associated with clinical outcome in patients with CRC with wild-type *KRAS* genotype while the expression level was grouped into low, intermediate and high. Collectively, our data indicate *PCDHGA7*, a *PCDH*

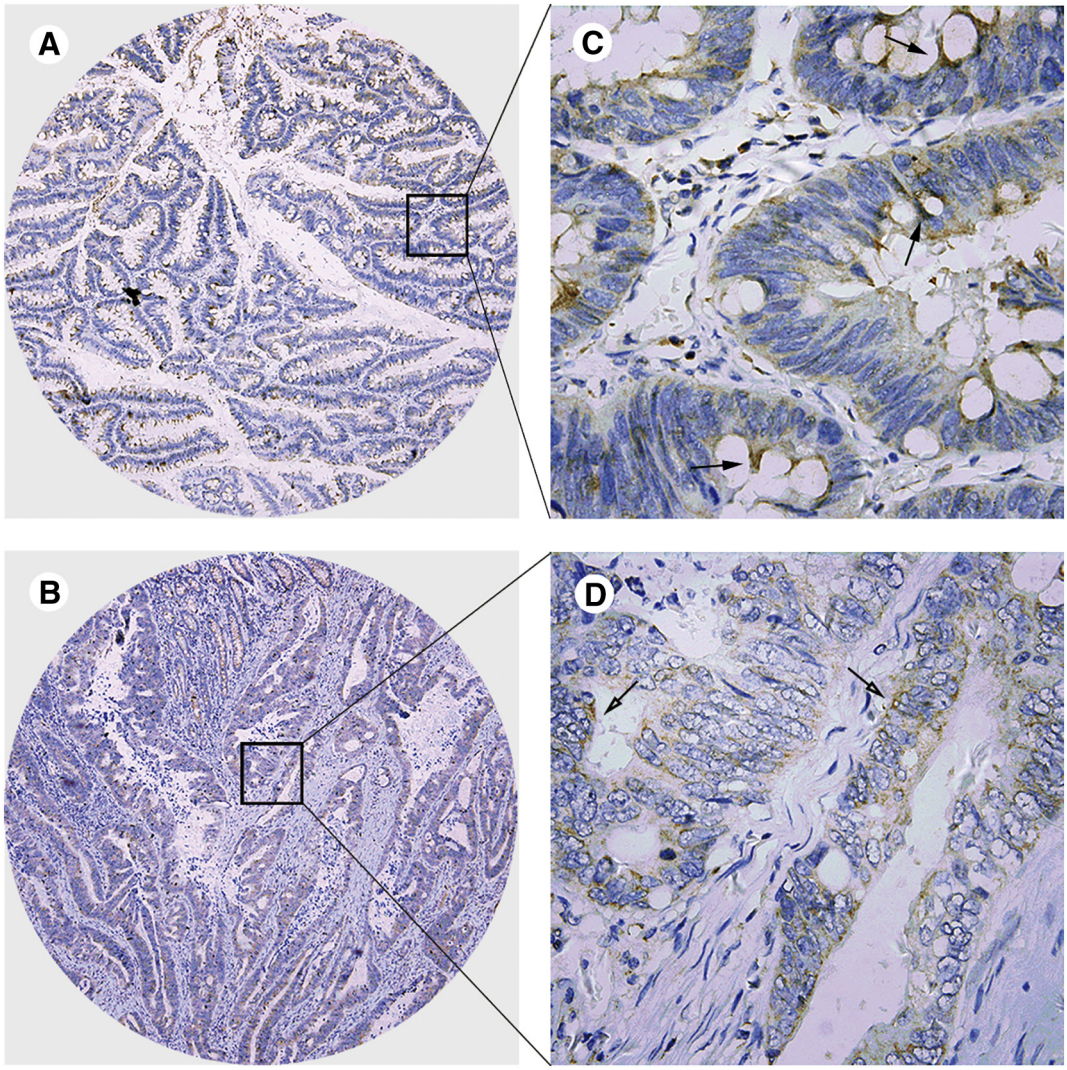

Fig. 3 PCDHGA7 immunostaining in TMAs of CRC with wild-type *KRAS*. Shown are representative sections with high or low protein expression. C and D, Higher-power view of the boxes in panels A and B, respectively. Black arrows indicate the representative spots of positive staining. Immunohistochemistry: original magnification ×40 (A and B), ×400 (C and D).

γ family member, could predict survival in TCGA cohort of CRC.

3.3. PCDHGA7 expression is significantly decreased in CRC

After knowing that *PCDHGA7* was down-regulated in CRC in TCGA cohort and might serve as a prognostic marker, we performed validation in our cohort. We first examined *PCDHGA7* mRNA levels in 20 pairs of colorectal carcinomas and their matched normal tissues (see Supplementary Table S4 for patient information). As shown in Fig. 2A, *PCDHGA7* mRNA expression was significantly lower in tumors compared with normal tissues ($P < .0001$). Next, Immunohistochemistry assay was conducted to determine the protein levels. The result showed PCDHGA7 protein expression was also significantly lower in colorectal tumors compared with normal tissues (Fig. 2B). These data demonstrate that PCDHGA7 is significantly down-regulated in clinical CRC tissues.

3.4. Association between clinicopathologic characteristics and PCDHGA7 protein expression in patients with KRAS-WT

Given the association between *PCDHGA7* expression and overall survival of patients with wild-type *KRAS* in TCGA cohort, we turned to investigating its clinic-pathologic significance in our own CRC cohort. A total of 230 patients were selected as an independent cohort followed by Sanger sequencing of *KRAS* mutation in tumor tissues. As a result, 138 out of 230 CRCs were identified as *KRAS*-WT

Table Clinicopathologic correlation of PCDHGA7 expression in KRAS-WT CRC patients

Clinicopathological variables	PCDHGA7 expression		*P*
	High	Low	
Age (n = 138)			
Young (≤median,71)	37	35	.865 [b]
Old (>median, 71)	32	34	
Gender (n = 138)			
Female	42	37	.390 [b]
Male	27	32	
TNM stage (n = 138)			
Early stage (I-II)	52	40	.030 [a,b]
Late stage (III-IV)	17	29	
Differentiation status (n = 129)			
Moderately/well	58	59	.442 [c,e]
Poorly	4	8	
Tumor size (n = 134)			
Small (≤5 cm)	41	48	.300 [b,c]
Large (>5 cm)	25	20	
Recurrence (n = 138)			
No	57	47	.048 [a,b]
Yes	12	22	
Venous infiltration (n = 138)			
Absent	68	66	.620 [d]
Present	1	3	

[a] Significant difference.
[b] χ^2 Test.
[c] Total number <138 due to missing data.
[d] Fisher's exact test.
[e] χ^2 Test with Yates' correction.

(Supplementary Fig. S3). We next determined the PCDHGA7 protein levels across all the *KRAS*-WT colorectal tumors in tissue microarrays using immunohistochemistry assay (Fig. 3A and B). The correlation between PCDHGA7 expression and the clinic-pathologic features was summarized in the Table. By grouping individuals based on PCDHGA7 expression (with high and low groups reflecting expression above or below the median, respectively), we found that patients whose tumors had high PCDHGA7 expression had a significantly higher risk of tumor recurrence after surgery ($P = .048$, χ^2 test) and they presented more frequently at advanced tumor stages ($P = .030$, χ^2 test). Consistently, patients with low PCDHGA7 expression in their tumors had significantly shorter overall survival than those with high PCDHGA7 expression (Fig. 4). Taken together, our study demonstrates PCDHGA7 down-regulation in *KRAS*-wild-type CRC is correlated with worse clinical outcomes.

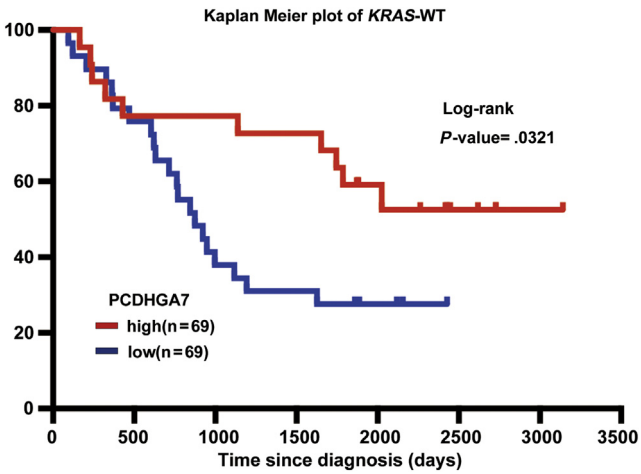

Fig. 4 Low PCDHGA7 expression is associated with shorter overall survival in *KRAS* wild-type CRC. Overall survival curve using Kaplan–Meier estimation in our CRC cohort with wild-type *KRAS*. For analysis, log-rank (Mantel-Cox) test was used to determine the statistical difference between stratified groups. $P < .05$ was considered statistically significant.

4. Discussion

Colorectal cancer, with global morbidity and mortality ranked third and fourth, respectively, is characterized by easy-to-develop metastasis and poor prognosis [14–17]. Consequently, it is imperative to develop new diagnostic and prognostic biomarkers for the management of patients with this deadly malignancy. Frequently long-range epigenetic silencing of chromosome 5q31 protocadherins involved in colorectal tumorigenesis have long been intriguing, but the molecular mechanism and clinical significance have been elusive [18-22].

Although certain PCDHs have been found to have tumor suppressor function in many malignancies [23-27], little is known about the function of the clustered *PCDHs* across this region in the prognosis of colorectal cancer. In the present study, we demonstrated that the mRNA level of multiple clustered *PCDHs* was significantly down-regulated in colorectal cancer tissues as compared to the adjacent normal tissues using TCGA data. Of note, the overall expression of α-*PCDHs* in adjacent normal tissues was very low while the aggregate γ-*PCDHs* showed robust expression, being the highest level among the 3 subfamilies. These results were consistent with previous studies [9,11], therefore leading us to investigate the potential prognostic role of γ subfamily members. However, by combining the RNA expression and clinical information of patients in TCGA cohort, no γ-*PCDHs* were found to be correlated with the patients' overall survival. We further turned to investigate the *KRAS* status in tumor tissues as the prior study have indicated gene signature for survival prediction seems to be associated with *KRAS* genotype [28]. Interestingly, one of the γ-*PCDHs*, *PCDHGA7* was found to be significantly correlated with the overall survival in patients with CRC with wild-type *KRAS*. As depicted in Fig. 1B, the survival curves showed that patients with high expression level of *PCDHGA7* in *KRAS*-wildtype CRCs had longer survival. By contrast, those in *KRAS*-mut group had shorter survival as compared to the patients with low *PCDHGA7*, although the *p* value did not reach statistical significance.

To validate the aberrant expression of PCDHGA7 in CRC observed in TCGA cohort, qRT-PCR and IHC was performed in our cohort. Analysis of an additional 20 pairs of clinical samples confirmed the significant down-regulation of PCDHGA7 in colorectal cancer tissues as compared to the adjacent normals. To further confirm its potential in predicting prognosis, we collected a total of 138 CRC tumors which harbored non-mutated *KRAS* as an independent cohort. The prognosis of patients with colorectal cancer could be affected by many factors, like tumor size, differentiation status, TNM stage, venous infiltration and recurrence and other factors. Our analyses revealed that TNM stage and tumor recurrence were significantly correlated with PCDHGA7 expression levels, exhibited prognostic values of PCDHGA7 in colorectal cancer. Although *KRAS* mutation by itself did not have prognostic significance in both TCGA cohort and our cohort, our results showed PCDHGA7 was effective for predicting survival in patients with CRC with wild-type *KRAS*. This phenomenon [28] indicating a potential subordinate role of PCDHGA7 in predicting prognosis under *KRAS* genotypic status. However, the precise mechanism requires further elucidation.

Several members of the clustered protocadherin superfamily have been shown to play a tumor-suppressing or oncogenic role in certain tumor types, such as PCDHGA3 in B-cell lymphoma 2-positive and negative follicular lymphoma (FL) [26] and PCDHGC3 in Wilms' tumor and colorectal cancer [9,11]. However, to date, the correlation between their expression and clinical outcomes remains elusive. Our findings demonstrate a new member of γ-PCDHs could serve as a potential biomarker for patients' prognosis. The findings would complement and expand the previous notion that the PCDH long-range epigenetic silencing may have great utility in providing biomarkers for many cancers. Taken together, our study uncovered that compared with normal tissues, the expression of PCDHGA7 in colorectal cancer tissues is lower, which was significantly associated with the prognosis of colorectal cancer patients with wild-type *KRAS*. The study also indicated the down-regulation of PCDHGA7 corresponded to advanced mortality. In conclusion, down-regulation of PCDHGA7 expression is involved in the pathogenesis of colorectal cancer, which can be used as a potential biomarker for colorectal cancer prognosis.

Ethical approval

Research on humans was conducted with the understanding and consent of human subjects and has been approved by the Institutional Review Committee of the Shanghai Tenth People's Hospital.

Author contributions

Y.D.L., K.S.P. and H.Q.Y. contributed to specimen preparation. C.M.C and Q.W designed the research study. J.G. and R.W. contributed essential reagents, tools and materials. Y.D.L., K.S.P. and C.M.C analyzed the data. Y.D.L., C.M.C and Q.W. wrote the paper. All authors have read and approved the final version of the manuscript.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.08.007.

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